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N-Myristoylation-Dependent c-SRC Interactions

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List of Abbreviations

AFM – Atomic Force Microscopy
 AKAP – A-Kinase Anchoring Protein
 BASP – Brain Acid Soluble Protein
 CHAPS – 3-((3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate
 CSP – Chemical Shift Perturbation
 DHPC – 1,2-dihexanoyl-sn-glycero-3-phosphocholine
 DMPC – 1,2-dimyristoyl-sn-glycero-3-phosphocholine
 DMPE – 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine
 DMPG – 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
 DOPC – 1,2-dioleoyl-sn-glycero-3-phosphocholine
 DOPG – 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
 DPPC – 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
 DPPG – 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
 EDTA – EthyleneDiamineTetraacetic Acid
 FWHM – Full Width at Half Maximum
 GFP – Green Fluorescent Protein
 IDD – Intrinsically Disordered Domain
 IDP – Intrinsically Disordered Protein
 LaurCoA – Laurylated Coenzyme A
 LaurUSH3 – Laurylated Unique and SH3 domains of c-Src
 LC/MS – Liquid Chromatography/Mass Spectrometry
 LSA – Lipid Sedimentation Assay
 LUV – Large Unilamellar Vesicles
 MARCKS – Myristoylated Alanine-rich Kinase Substrate
 MIP – Maximum Insertion Pressure
 MLV – MultiLamellar Vesicles
 MyrCoA – Myristoylated Coenzyme A
 MyrSH4 – Myristoylated SH4 domain of c-Src
 MyrUGFP – Myristoylated Unique and SH3 domains of c-Src
 MyrUSrc – Myristoylated Unique domain of c-Src
 MyrUSH3 – Myristoylated Unique and SH3 domains of c-Src

NaP – Sodium Phosphate
NMR – Nuclear Magnetic Resonance
NMT – N-Myristoyl Tansferase
PAT – Palmitoyl AcylTransferase
PB – Persistently Bound
PCD – Positively Charged Domain
PI(3,4,5)P3 – Phosphatidylinositol-3,4,5-TriPhosphate
PKA – Protein Kinase A
PTM – Post-Translational Modification
RI – Refractive Index
SDS – Sodium Dodecyl Sulfate
SDS-PAGE – Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SEC – Size Exclusion Chromatography
SFK – Src Family Tyrosine Kinase
SH – Src Homology
SLB – Supported Lipid Bilayer
SLiMs – Short Linear Motifs
SOFAS-HMQC – Band-Selective Optimized-Flip-Angle Short-Transient Heteronuclear Multiple Quantum Correlation
SPP – Surface Plasmon Polariton
SPR – Surface plasmon Resonance
SMP – Single Molecule Photobleaching
TIR – Total Internal Reflexion
ULBR – Unique Lipid Binding Region
VILIP – VIsinin-Like Protein
WT – Wild Type

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Chapter 1

Introduction

1.1. Membrane anchored proteins

1.1.1. Membranes

To the origin of life certainly contributed the spontaneous self association of amphiphilic lipid molecules, that formed a physical boundary permitting the segregation of specific chemical reactions. Simple organisms have evolved to increasingly complex prokaryotes and eukaryotes. About 2,5 billions of years ago, eukaryote life appeared, concomitantly with an increase of oxygen in the composition of atmosphere. Eukaryote cells acquired the ability of sub-segregating chemical reactions in their different organelles. Membranes of eukaryote cells and organelles are mainly composed of glycerophospholipids, sphingolipids and cholesterol (which appearance also corresponds with the increase in atmospheric oxygen). An extremely wide variety of lipids composes these families and is present in membranes (more than 100 different lipids for bacteria but more than 1000 for eukaryotes). The lipid composition of membranes is highly variable in different organisms, but also in different cells of an organism, and in different organelles in a cell. In addition to phospholipids, membranes contain carbohydrates, and also a huge amount of proteins (depending on the membrane type, it can vary from 20 % up to 75 %) ([Sonmino and Prinetti, 2009](#)). Membrane asymmetry results from the difference in composition of lipids, carbohydrates and proteins in the inner and outer leaflets. Lipid polymorphism makes lipids able to accommodate for the presence of proteins (by “filling the holes”) ([Simons and Sampaio, 2011](#)) and is also source of membrane plasticity. Cell and organelles

surfaces are not flat, two-dimensional entities, but adopt a wide variety of shapes (local concave or convex deformations). Membranes are maintained in a fluid state, enabling lateral diffusion of the embedded molecules (both lipids and proteins) (Sonnino and Prinetti, 2013).

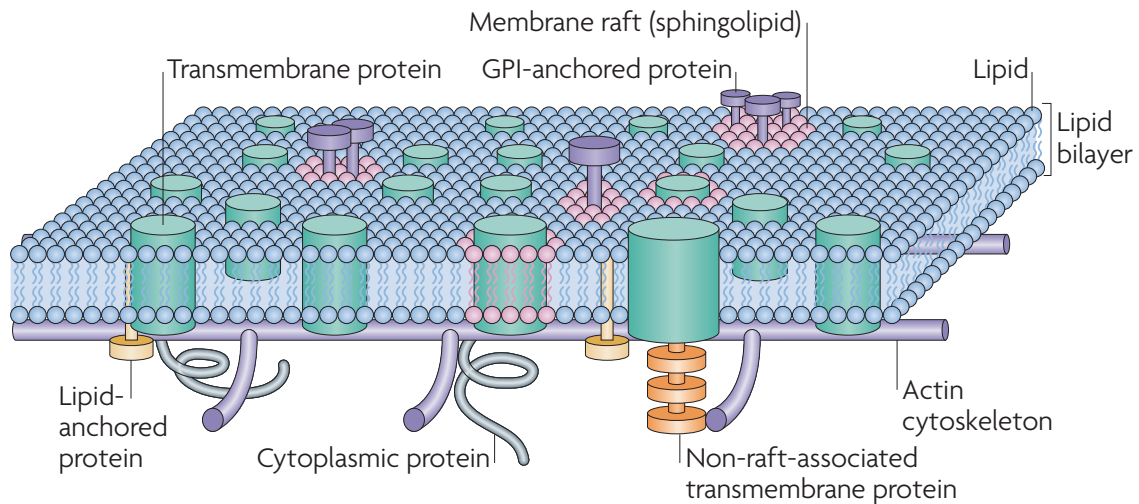


Figure 1.1: *The plasma membrane is composed of lipids, proteins and carbohydrates that are associated through non-covalent interactions. The illustration accounts for membrane asymmetry, for the presence of lipid rafts and that of protein-lipid shells. Figure from Viola and Gupta (2007).*

Nevertheless, nanodomains enriched in sphingolipids, cholesterol, and specific lipids (such as phosphoinositides) or proteins (often possessing long saturated carbon chains) have been observed in the recent years by different techniques (AFM, fluorescence microscopy) as transient lateral clusters with reduced disorder (McIntosh, 2007). These “liquid-ordered phase” domains dynamically assemble and disassemble among the “liquid disordered phase” environment. These so-called lipid rafts constitute an additional two-dimensional segregation of chemical reactions, since they have the capacity of including or excluding biomolecules: rafts are entities of physical and chemical specificity (Sonnino and Prinetti, 2013). Lipid rafts are described as signaling nanoplateforms, from which signal transduction can be initiated by complex protein-protein interactions. Yet, the lipid raft theory probably does not fully describe the membrane complexity; the amount of membrane proteins is still often underestimated, and membranes should be viewed as lipid-protein composites instead of entities in which proteins are diluted in a lipid sol-

vent (Ken Jacobson et al., 2007). In addition, interactions of the membrane constituents with the cytoskeleton probably play an important role in membrane segregation. Additional theories are emerging, such as the “shell model”. It accounts for the observation of non-raft clustering phenomena. In this model, shelled proteins recruit specific lipids and exist alone or in nanoclusters. This hypothesis supports the observation of “known” raft proteins that are diffusing at different speeds, and in particular at a rate compatible with that of monomers (Ken Jacobson et al., 2007). One of the hypothesis linking rafts and signalization describes small lipid rafts and/or protein shells as inactive entities, until they merge into larger active rafts, placing the proteins in a new and specific environment, from which protein signaling can start, protected from outside competing reactions (for instance kinases protected from the role of phosphatases) (Simons and Toomre, 2000) (Ken Jacobson et al., 2007). A schematic view of a membrane is represented in Figure 1.1 (Viola and Gupta, 2007).

Figure 1.2 contains most of the configurations found for proteins residing at the cell membrane environment; integral membranes proteins and anchored proteins.

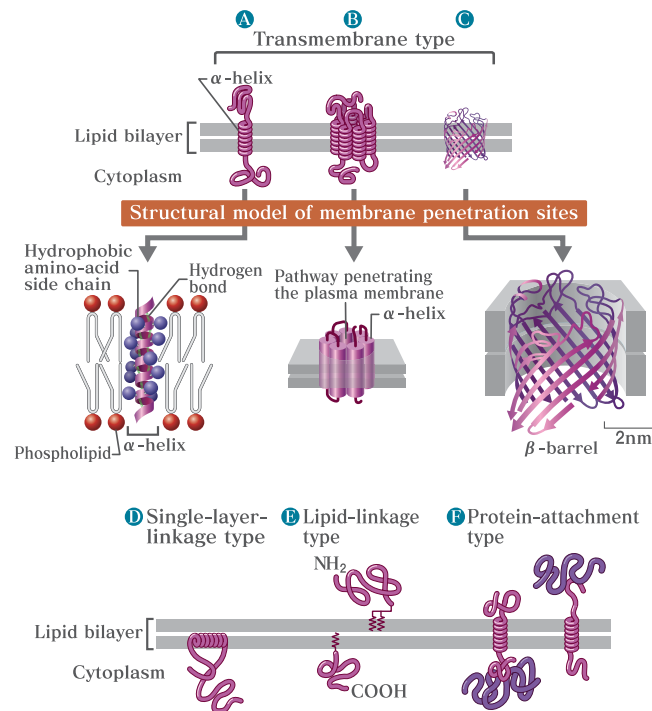


Figure 1.2: *Types of attachment for proteins residing at the cell membrane. Figure from Makoto Asashima et al. (2010)*

Among them, lipid anchored proteins constitute a peculiar family, by acting as soluble protein with a two-dimensional restricted movement. Protein modified by the addition of one or several acyl chains acquire an amphiphilic character, which affects in a subtle manner their transport, location and function in the cell, through the interplay of hydrophobic and electrostatic forces. More than 1000 proteins undergo covalent attachment of a lipophilic group (Resh, 2013), in the form of fatty acid, isoprenoid, sterol, phospholipid and GPI anchor. Only a few fatty acids have been selected during evolution as candidates for such modification. The primary role of protein acylation is to target these molecules to membranes. Nevertheless, acylated proteins acquire unique features in protein-lipid and protein-protein interactions.

1.1.2. Lipid Modifications

Figure 1.3 illustrates some of the lipid chains involved in protein modifications (Resh, 2006). Proteins may undergo many kinds of modification, but we will focus in three main lipidation mechanisms, namely myristoylation, palmitoylation and farnesylation.

Myristoylation is a co-translational modification occurring at the N-terminal glycine on the nascent polypeptide, after removal of the initiator methionine residue (Kasaharaa et al., 2007) (Sandilands et al., 2004) (Martin et al., 2011). The reaction is catalyzed by N-Myristoyl Transferase (NMT) and results in the covalent attachment of the myristoyl chain at the N-terminal side of the protein. Myristoylation is a very stable modification and only rare cases of demyristoylation have been reported (the Myristoylated Alanine-rich C Kinase Substrate (MARCKS) protein being one of the cases observed (Brauna et al., 2000) (Resh, 1996)). The amino-terminal glycine residue is absolutely required for myristoylation – substitution of this residue to any other abrogates

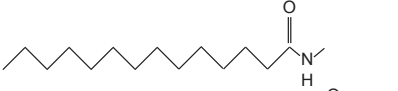
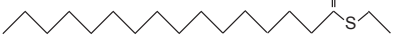
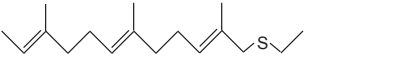
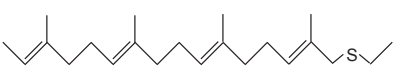
Modifying Group	Chemical Structure
Myristate	
Palmitate	
Farnesyl	
Geranylgeranyl	

Figure 1.3: *Myristoyl, Palmitoyl and Farnesyl are three common lipids co- or post-translationally added to proteins.*

myristoylation (Martin et al., 2011). But an additional "consensus peptide" is needed for protein recognition by NMT, typically consisting in a Met-Gly-X-X-X-Ser/Thr sequence. Despite of the low abundance of myristic acid in cells, about 1% (Resh, 1996), this modification is very specific; myristoyl constitutes the shortest lipid motif capable of stably (but not irreversibly) anchoring proteins to membranes (Peitzsch and McLaughlin, 1993) (Resh, 1996). Although myristate is irreversibly bound to proteins it allows the acylated protein to reversibly sample membranes because of its weak hydrophobic nature (Martin et al., 2011). A common regulation mechanism of reversible binding to membranes occurs in proteins having a hydrophobic pocket in which the acyl chain can burry. If myristoyl is hidden in the pocket, the protein is cytoplasmic. When a triggering signal is received and the chain is released from the pocket, binding of the protein to membrane is allowed. This is called the myristoyl switch. The transition can be induced by different mechanisms (Resh, 1999): ligand-dependent conformational change (Ames et al., 1996), electrostatic switch (McLaughlin and Aderem, 1995), proteolytic switch (Hermida-Matsumoto and Resh, 1999), and entropic switch (Tang et al., 2004). Myristic acid possesses the minimum chain length able to trigger micelle formation – at non physiological high protein concentration (Pool and Thompson, 1998) – and is involved in protein protein interactions (Taniguchia, 1999). An example is the transition of the Matrix-Associated (MA) viral protein, through a myristoyl-switch mechanism, from a monomeric cytoplasmic state to a membrane bound trimer configuration (Resh, 2004). Although the mechanism remains unclear, myristoylated proteins are often preferentially targeted to the plasma membrane (Resh, 1999). They can be found both within or out of rafts, which can affect their activity (Seong et al., 2009). Myristoyl is generally added in its saturated form, but alternative chains have been found (Bereta and Palczewski, 2011), namely C14:1 or C14:2, which can influence the localization of the protein (Liang et al., 2001).

Palmitoylation consists in S-acylation of a 16 or 18 carbon chain, usually palmitoyl, in which the acyl chain is reversibly attached to cysteine residues of a proteins (there is no other known sequence requirements). It is a post-translational modification that requires first membrane targeting, since the enzyme catalyzing the reaction is a membrane protein, Palmitoyl AcylTransferase (PAT) (Bijlmakers and Marsh, 2003). Palmitoylation serves primarily as a strong lipid anchor, which makes the substrate specificity for palmitic

not as strict as for myristoyl (Liang et al., 2001). Since it acts as a second membrane anchoring point, palmitoylation confers a stable protein anchoring to membrane, but in a reversible way, through palmitoylation and depalmitoylation cycles (via palmitoyl protein thioesterases (PTE) mechanisms) (Drisdel et al., 2006) (Goodwin et al., 2005). Palmitoylation also plays a role in membrane targeting and usually directs proteins to lipid rafts (Mumby, 1997). A class of palmitoylated proteins, transmembrane proteins, are also directed to lipid rafts by palmitoylation (Resh, 1999).

Prenylation is a posttranslational reaction that occurs in the cytosol. A 15-carbon (farnesyl) or 20-carbon (geranylgeranyl) isoprenoid is linked through a thioether bond to one or more cysteine residues at or near the C terminus of the protein. Many prenylated proteins contain a C-terminal “CAAX box” (Cysaliphatic- aliphatic-X). The X amino acid determines whether the cysteine within the CAAX box is farnesylated by farnesyltransferase or geranylgeranylated by geranylgeranyltransferase I. The three C-terminal amino acids (AAX) are then cleaved, and the C-terminal prenylated cysteine residue is carboxymethylated in the endoplasmic reticulum (Sorek et al., 2009). Like myristoyl, farnesyl is not enough to stably anchor a protein to the membrane, but geranylgeranyl is longer and provides a much stronger binding (Resh, 2013).

1.1.3. Two-signal membrane anchoring

Often, such proteins are modified by more than one lipid chain, which modulates in a cooperative manner its binding to membranes (Resh, 1999). Many cases of myristoylated protein that are subsequently palmitoylated have been found (Resh, 1996). Dual myristoylation and palmitoylation greatly enhances the protein affinity towards membranes. Indeed, the binding energy corresponding to myristoyl insertion is very low, with an apparent affinity of 10^{-4} M^{-1} and additional palmitoylation increases this affinity with a 10-fold (Taniguchia, 1999) (Resh, 1996). The half-life of a membrane bound myristoylated peptide is in the order of minutes in comparison to hours for palmitoylated or dually acylated peptides (Martin et al., 2011).

In some instances, the second membrane binding signal is not a lipid chain, but a highly positively charged peptide in the protein sequence, that we will call Positively Charged Domain, PCD (Tao et al., 2006). A PCD can interact with the negatively charged acidic lipids and stabilize in a cooperative way the protein attachment. On their

own, neither the acyl chain nor the basic peptide would bind lipids with strong affinity, but the cooperative action is more than additive, and the resulting total affinity is greatly enhanced (Ladokhin and White, 2001). Location of the PCD can vary within the protein sequence; c-Src possesses a PCD just after the myristoyl tail (Sigal et al., 1994), while the PCD of MARCKS is located further away from the N-terminal (Arbuzova et al., 1997). A farnesyl tail can also be found instead of myristoyl: K-Ras4B is farnesylated and contains a PCD (Resh, 2013). PCD binding to membranes can be electrostatically modulated, in particular by phosphorylation of amino acids in the proximity of the PCD sequence. Serine will become negatively charged under phosphorylation, which will diminish the overall charge around the PCD (Tournaviti et al., 2009) and consequently the electrostatic interaction. Variation of the lipid composition can also modulate such electrostatic interaction, since the presence of negative charges and their distribution can be tuned by the types of lipids present in the interacting membrane area. In some proteins, modulation of the electrostatic interaction is the triggering signal involved in the myristoyl switch (called in this case the myristoyl electrostatic switch). As an example, phosphorylation events in the PCD of MARCKS release the peptide from the membrane and lower the protein affinity towards the membrane. In the released soluble form of MARCKS, the acyl chain hides back in its hydrophobic pocket (Arbuzova et al., 1998).

Other dual-signal mechanisms exist, for instance H-Ras and N-Ras are prenylated and palmitoylated (Weise et al., 2013). Some α -subunits of G protein possess a hydrophobic peptide interacting with lipids, and are also palmitoylated (Veit et al., 1994). This demonstrates that myristoylation is not essential for palmitoylation (Resh, 1996); membrane association rather than myristoylation is important for palmitoylation (Degtyarev et al., 1994).

Figure 1.4 (Resh, 1999) summarizes the main configurations of dual-signal lipid binding involving a myristoyl group.

More than two interactions are also found among lipidated proteins. Table 1.1 lists examples of such proteins. A myristoylated protein comprising a PCD may also be stabilized once bound to membranes by the hydrophobic interaction of another domain in the amino acid chain; the MARCKS protein contains 5 phenylalanine residues inserting in the lipid bilayer (Arbuzova et al., 1997) upon membrane association. Additional binding to a membrane protein may also occur (Liu et al., 2014). Thus the role of the myristoyl

can be quite diverse according to the other coexisting binding motifs. In some cases, the specificity of the acyl chain is crucial for protein function; replacing the myristoyl of Arf6 by a prenyl group directly hampers its function (Boutin, 1997). In other cases, it is less clear, and protein function can even seem unaltered in the absence of the acyl chain; no striking effect was observed in absence of the myristoyl tail of calcineurin (Kennedy et al., 1996). The role of the acyl chain might be more subtle; the effect of the lack of myristoylation is more subtle for c-Src than for Lck proteins (Abraham and Veillette, 1990), both belonging to the Src family tyrosine kinase.

Another role of the dual signal mechanism is related to protein localization, in which the palmitoyl chain often plays a crucial role. Localization can occur *via* at least two phenomena. First, kinetic trapping can occur when a protein undergoes palmitoylation, in which the localization of LAT will determine that of the protein, since the addition of the palmitic acid produces an almost reversible binding of the protein to the membrane (Cabrer-

Vera et al., 2003). Second, the degree of saturation of the acyl chain may localize the protein in a more local way. As previously mentioned, saturated carbon chains bind preferentially to lipid rafts, so the presence of a palmitoyl usually targets proteins there (Resh, 2006). Addition of a cysteine at position 3 of the c-Src sequence (which is normally not palmitoylated and not specifically directed to rafts) induces c-Src palmitoylation and raft targeting (Alland et al., 1994). Fine tuning of the myristoyl saturation state may also occur: it has been demonstrated that incorporation of an unsaturated myristoyl chain to Fyn (normally targeted to raft via myristoylation and palmytoylation) displaces the protein from lipid rafts (Liang et al., 2001).

In the case of the dual myristoyl-PCD signal, specific targeting can also be achieved because of the presence of the charges. The plasma membrane is indeed more negatively charged than the other membranes in the cell, possibly attracting effectively the PCDs of

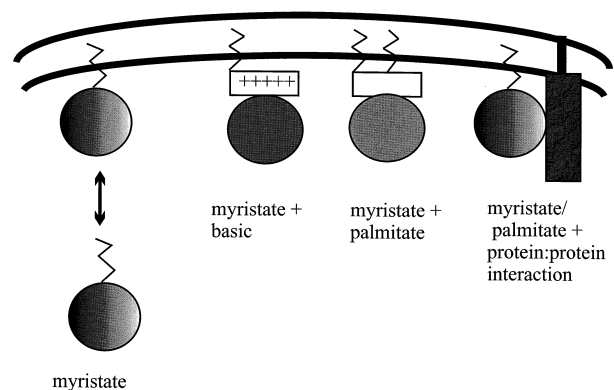


Figure 1.4: *Dual Signal Configuration enabling strong binding of Myristoylated proteins to membranes. Figure from Resh (1999)*

myristoylated proteins. Presence of specific lipids (phosphoinositides) also plays a role in the targeting of PCD-containing proteins to the plasma membrane (Heo et al., 2006). An interesting remark is the similarity of polybasic sequences with that of nuclear localization, suggesting a possible role in which the lipid chain may impede nuclear localization (Li et al., 2014).

Apart from the PCD, other protein domains may have lipid specificity: the Visinin-Like Protein (VILIP) has enhanced affinity for phosphatidylinositol-4-phosphates, which can specifically direct the protein to the correct location (Wang et al., 2014). Arf1 can interact specifically with both a membrane protein and phosphatidylinositol 4,5-bisphosphate (Liu et al., 2014).

Table 1.1: *Examples of lipidated proteins; the second lipid-anchoring signal is indicated*

Protein	Signal 1	Signal 2	Signal 3
c-Src	Myristate	PCD	
SFKs	Myristate	Palmitate	
PKA	Myristate	PCD	
G α_i subunits	Myristate	Palmitate	
G α_q subunit	Palmitate	Palmitate	
Arf1	Myristate	Palmitate	PI45P
MARCKS	Myristate	PCD	alpha helix
Gravin	Myristate	PCD	alpha helix
Rhodopsin	Farnesyl	PCD	
H-Ras	Farnesyl	Palmitate	
H-Ras	Farnesyl	Palmitate	
K-Ras4B	Farnesyl	PCD	
RhoGTPase	Geranylgeranyl		
Hedgehog	Palmitate	Cholesterol	

1.2. Disorder in myristoylated proteins

1.2.1. Intrinsically Disordered Proteins

The paradigm linking the three-dimensional structure of proteins with their function has been reshaped in the recent years. All these domains that usually correspond to a lack of electron density in the protein structure derived from X-ray crystallography raised increasing interest in the recent years. It is now well accepted that many functional proteins or domains lack a three dimensional structure. They are called Intrinsically Disordered Proteins or Domains, IDPs or IDD – among all the name they have been attributed. They are biologically active but do not have a well defined equilibrium structure and form an heterogeneous ensemble of conformations, resulting from a relatively flat free energy surface (Dunker and Obradovic, 2014). These highly dynamics structures interconvert on a number of time scales. By assuming that the lack of structure must be encoded in the sequence, the characteristics of “coded disorder” have been studied. There is a clear balance between hydrophobicity and charges: IDPs usually contain many charged residues and have a very high net charge, with a relatively low hydrophobicity (Uversky et al., 2000); they contain disorder promoting residues (A and G) and also have a low sequence complexity (Radivojac, 2007). In a Charged-Hydropathy plot, IDPs occupy a quite confined region, while well-structured domains stand in another, more dispersed, area (Habchi et al., 2014). Identification of the properties of IDPs enabled to develop predictive algorithms based on protein sequences. These predictions point out the underestimated abundance of IDPs in the proteome. In fact, the number of discovered functional IDPs is still increasing very fast. Some algorithms forecast that 25 % of proteins in Swiss-Prot have predicted IDDs longer than 40 consecutive residues, and that at least 11 % of the residues among the proteome are likely to be disordered (Radivojac, 2007). Higher IDP abundance is found in eukaryotes, certainly because of the gain of regulation they provide in the cell function. IDPs are evolutionary persistent (Dunker and Obradovic, 2014), which means that disorder is conserved. But they are also subject to fast evolution: domains that were gained during evolution by the extension of existing exons contain the highest degree of disordered regions (Lee et al., 2014). They are involved in a wide variety of functions which have been classified in many different ways.

IDPs often have an important role within signal control pathways. Because of their

high plasticity, their activity must be under tight control (Uversky et al., 2014). For instance some IDD probably adopt a “misfolded conformation”, in which intramolecular interactions with adjacent folded domains prevent non native interactions (Habchi et al., 2014). Their deregulation can lead to dramatic consequences and is often related to serious diseases such as cancers (Uversky et al., 2014). Thus, IDPs are also seen as potential new drug targets (Dunker et al., 2008). For instance, allosteric modulation of Bcr-Abl involving the myristate binding site of Abl can be used to design drug targeted to this domain and directly influence the enzymatic activity of the oncogene protein (Zhang et al., 2010).

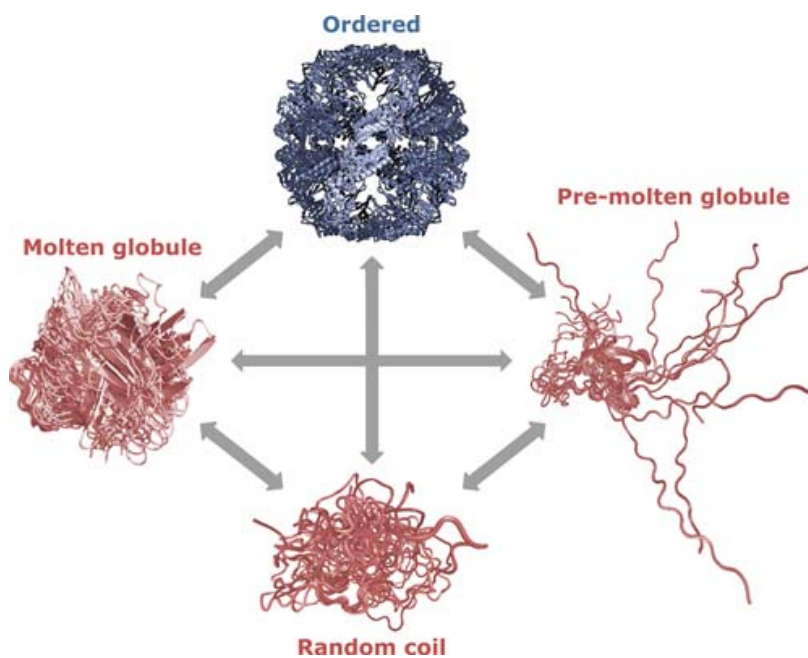


Figure 1.5: *Different “configurations” of IDDs: disordered, premolten, molten globule and folded domain. Figure from Dunker et al. (2008).*

The lack of 3D-structure is often needed for protein function, but the “disordered state” is not as random as it was initially thought. Among the different IDPs, and even within a same IDP, compactness can vary significantly. Different states corresponding to the degree of disorder have been described, from totally disordered to premolten, molten globule and folded domain, as illustrated in Figure 1.5 (Dunker et al., 2008). The net charge is usually related to the compactness (Habchi et al., 2014), but as said, IDPs can interchange between different states. These properties confer them the possibility of interacting with a multitude of partners. IDPs can bind their ligands with both specificity

and low affinity, leading to specific interactions that can be easily dispersed, which is an important feature for regulation ([Dunker and Obradovic, 2001](#)).

Some small regions contain a higher degree of compactness ([Radivojac, 2007](#)), indicative for short-structure binding motifs, which are the ones that can undergo unfolded to folded transitions. These transitions can be triggered by passive factors (e.g. variations in the pH, the temperature, the redox conditions, or response to light) or active factors (e.g. membrane induced changes, ligand binding, protein-protein interactions). These common functional modules within IDPs are composed of short stretches of adjacent amino acids, known as short linear motifs (SLiMs). Depending on the partner proteins that recognize them, these sites can facilitate a diverse set of functions including targeting a protein to a specific subcellular location, determining its modification state, controlling its stability, and regulating its context-dependent activity ([Van Roey et al., 2014](#)). Highly flexible domains containing SLiMs have an important role in allosteric regulations and autoinhibition processes ([Tomba, 2014](#)). Probably due to their exposed character, they are also often subject to degradation and PTMs ([Jakob et al., 2014](#)); one common PTM is phosphorylation which takes place very often in disordered regions ([Iakouchava and other, 2004](#)). More than 300 different PTMs exist, through which the targeted protein will gain new functions. As we saw, myristoylation induces in many cases membrane binding; frequently, myristoylated proteins undergo other PTMs (phosphorylation, palmitoylation) that provide them with additional regulation capabilities.

1.2.2. Myristoylation and disorder

Myristoylation usually happens cotranslationally before protein folding, thus it is tempting to classify the N-terminal NMT recognition peptide as a SLiM. This peptide sometimes does not participate in the 3D-structure of the protein, or can even be part of a longer N-terminal disordered region. In fact, N-terminal disordered domains are found in many myristoylated proteins, and the total abundance of such proteins may be higher than we think. Combination of disorder and myristoylation increases the possibility of fine tuning protein functions at the membrane interface, often together with other PTMs. We will look into more detail into the gain of function provided to a protein possessing an N-terminal myristoylated disordered domain.

Facilitate membrane binding N-terminal disordered segments can enhance membrane binding. Indeed, steric hindrance has been found to decrease the binding partitioning of a protein lacking a flexible N-terminal (for instance in bovine pancreatic trypsin inhibitor derived with a myristoyl chain) when compared with proteins featured with a flexible peptide segment (such as c-Src or MARCKS) (Pool and Thompson, 1998).

Favor PTMs

- Myristoylated proteins acquire a membrane binding capacity which in turn permits S-acylation (Resh, 1999), often catalyzed by a membrane-embedded palmitoyltransferase. This second PTM is probably favored in case of an exposed cysteine among a disordered region. The subsequent membrane binding regulation by palmitoylation / depalmitoylation cycles may be facilitated at this disordered region. Among the not so many examples of dually myristoylated and palmitoylated proteins stand almost all members of the Src family kinase, some α -subunits of G proteins, some AKAPs proteins like AKAP15 (Gray et al., 1998), and eNOS. The first two have a N-terminal intrinsically disordered region (Ingley, 2008) (Cabrera-Vera et al., 2003), AKAP15 also is probably disordered at its N-terminus (Few et al., 2007).
- The myristoylated proteins possessing a PCD are plenty, and membrane binding is very often modulated by phosphorylation; addition of phosphate groups decreases the peptide charge and therefore its binding to membranes (Tournaviti et al., 2009). One can think that phosphorylation is facilitated if PCDs are disordered and more accessible. Among proteins possessing a PCD, many are regulated by an electrostatic switch triggered through phosphorylation events, probably facilitated by an easier access to disordered segments, like in the case of MARCKS (McLaughlin and Aderem, 1995).

Favor protein protein interactions The HIV-Matrix protein also possesses a myristoyl switch, and it has been shown that it can form trimers upon membrane binding. The N-terminal residues involved in the myristoyl switch appears to be critical determinants of the myr(in)/myr(out) equilibrium and have higher than average intrinsic conformational mobility (Tang et al., 2004). In this example, protein-protein interactions are favored by

the flexibility of the N-terminal region capable of adopting diverse conformations ([Resh, 2004](#))

Favor protein ligand interactions Modulation of membrane binding can also be achieved by a ligand binding mechanism. MARCKS and AKAP12 (gravin) have several PCDs at their N-terminal disordered domain. These domains are also capable of binding Calmodulin/ Ca^{2+} . By this mechanism, the protein is released from the membrane since the electrostatic interaction from the PCDs to the membrane is diminished ([Malbon et al., 2004](#)) ([Tao et al., 2006](#)). By interacting with several partners (lipid or ligand), the IDD favors membrane binding modulation. Some myristoylated proteins also shows specific interactions with lipids (for instance PI(45)P2 which is a lipid often involved in signaling pathways), like the HIV-matrix protein, which also regulates the myristoyl-switch mechanism ([Saad et al., 2006](#)). Once again, a disordered segment is capable of interacting with multiple partners.

Play a role in protein targeting PCDs often have a disordered character, and their electrostatic interactions with acidic lipids may be quite unspecific; a myristoylated protein possessing a PCD may have the capacity of spanning unspecifically the membrane until finding a proper localization – e.g. getting palmitoylated by a palmytoyl acyl transferase ([Li et al., 2014](#)).

IDPs are subjected to degradation It has been found that MARCKS can be cleaved between amino acids 6 and 7 in certain extracts, and that this cleavage is myristoylation-dependent ([Brauna et al., 2000](#)). The disordered character of the N-terminal probably facilitates this cleavage – that could hypothetically be a possible mechanism to detach myristoylated proteins from membranes.

Disorder to order: alpha helix formation Myristoyl is capable of stabilizing an intrinsically disordered N-terminal domain to an alpha helix. In the case of Protein Kinase A (PKA), the first amino acids of the protein are disordered in absence of the myristoyl chain, and myristoylation promotes helix formation, which in turn promotes membrane binding. Nevertheless, this helix can be destabilized under phosphorylation of serine 10, increasing its disordered state, and probably decreasing the membrane affinity ([Tholey](#)

et al., 2001). Fine tuning of membrane binding is achieved through the plasticity of the disordered domain. In the case of Nef, the unfolding to folding transition seems to happen when encountering the membrane. After myristoyl insertion and electrostatic attraction, formation of an alpha helix occurs and subsequently inserts into the bilayer, magnifying membrane binding (Gerlach et al., 2010).

Allostery at the interface c-Abl has a structure very close to that of c-Src kinase, but possesses a myristoyl switch features; non-myristoylated forms of c-Abl have much higher activity levels. The myristoylated disordered domain is directly involved in the myristoyl switch, which in turn is related to kinase activation (Hantschel et al., 2003). This example shows the crucial role of the flexible linker undergoing conformational changes and involved in intramolecular interactions, which ultimately results in kinase activation or inactivation.

Table 1.2 lists several myristoylated proteins among which the disordered regions are pointed out.

Myristoylation only occurs in eukaryote organisms. Two myristoylated proteins were identified in 1982, PKA and calcineurin. The subsequent discovery of more than 100 fatty acid modified proteins occurred rapidly (Resh, 1996) (Taniguchia, 1999). The extent of the “myristoylome” is still under study, since the techniques to assess protein myristoylation *in-vivo* are not straightforward (but under improvement (Traverso et al., 2013)). As previously described, specific combinations of amino acids have been identified for N-myristoylation of the candidate protein. Removal of initiator methionine is a common modification of proteins, occurring in up to 80 % of total proteins. The reaction is catalyzed by methionine aminopeptidase and is even more efficient when the amino acid residue following the initiator methionine is a glycine residue, yet only selected proteins are myristoylated due to the selectivity of NMT. Several myristoylation prediction models have been developed (Martin et al., 2011); the 17 N-terminal residues following the initial methionine were actually demonstrated to be required for the prediction of the myristoylation status of the protein. There are several exceptions to the basic rules, for instance the Fyn anchor (Maurer-Stroh et al., 2002b). Another type of myristoylation, which occurs posttranslationally, is known to happen as a result of caspase cleavage during cell apopto-

Table 1.2: *List of some myristoylated proteins; the second lipid-anchoring signal is indicated; the presence of a short or a long disordered region at the N-terminal is also pointed out*

Protein	Lipid 1	Signal 2	Comment	N-terminal
Protein kinases and phosphatases				
c-Src	Myristate	PCD		Disordered
SFKs	Myristate	Palmitate		Disordered
c-Abl	Myristate	PCD	Hydrophobic Cleft	Disordered
PKA	Myristate	PCD/ α helix	Hydrophobic Cleft	Short disordered
AKAP15	Myristate	Palmitate		
AKAP12 (gravin)	Myristate	PCD		Disordered
Calcineurin B	Myristate	PCD		
Guanidine nucleotide binding proteins				
G α_i 1 subunit	Myristate	PCD		Disordered
G α_i subunits	Myristate	Palmitate		Short Disordered
Arf1	Myristate	Palmitate	PI45P	
Arf6	Myristate	PCD		
Calcium binding proteins				
Recoverin	Myristate	PCD	Hydrophobic Cleft	
Neurocalcin	Myristate	PI45P	Hydrophobic Cleft	
VILIP	Myristate	PI4P	Hydrophobic Cleft	
Membrane bound structural proteins				
MARCKS	Myristate	PCD/ α helix		Disordered
Viral proteins				
MA	Myristate			Short disordered
Nef	Myristate			Disordered
Miscellaneous				
eNOS	Myristate	Palmitate		
BASP	Myristate	PCD/ α helix		Disordered

sis. This pool could significantly increase the total abundance of myristoylated proteins. As said, myristoyl is a rare fatty acid in cells ([Boutin, 1997](#)), but myristoylated proteins constitute 0,5 to 2 % of the proteome in human ([Traverso et al., 2013](#)) (or 0.5 to 3 % of the mammalian and plant proteomes ([Martin et al., 2011](#))), and it might be larger ([Traverso et al., 2013](#)). Appearance of PTMs (and in particular myristoylation) and of IDPs are features brought by evolution in higher order organisms, and they are present in proteins playing crucial roles in signaling cascades. Here we showed that by acting in synergy, they provide important gain of function to proteins and improve their regulation mechanisms. Though they are difficult to detect and study, computational predictions nowadays highlight their underestimated abundance ([Boisson et al., 2003](#)) ([Uverskya, 2011](#)) and importance. Consequently, the abundance of myristoylated IDDs could be more important than is currently thought and have critical functions in signaling pathways.

1.3. c-Src and the Src family

1.3.1. c-Src family

The Src tyrosine kinase family (SFK) takes its name from its leading member, the c-Src protein, and is composed of at least 9 other members: Blk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Yes and Yrk. Some members are ubiquitously expressed, like c-Src, and some are found in specific cells (Thomas and Brugge, 1997). SFKs are enzymes catalyzing the phosphorylation of a tyrosine, readily transferring a phosphate group from ATP to the hydroxyl group of tyrosine residues of their substrate (Parsons and Parsons, 2004). Tyrosine phosphorylation is considered to be one of the key steps in signal transduction and regulation of enzymatic activity. SFKs are indeed involved in many signaling pathways and their deregulation, in particular that of c-Src, affects cell migration, proliferation and survival (Parsons and Parsons, 2004) (Thomas and Brugge, 1997). Redundancy has been observed in many instances among the family, but c-Src is one of the members having a broad range of functions. c-Src itself was the first discovered oncogene and takes its name from Sarcoma. Peyton Rous observed in 1911 that a virus was capable of inducing tumors in chicken. Src viral counterpart v-Src was identified in the viral genome in the 1970s. Its capacity of transforming cells and inducing tumors made it the first discovered viral oncogene. Subsequently, the human form of the gene was evidenced to be a proto-oncogene that has a key signaling function in cells, and is potentially oncogenic only upon deregulated activity (Steven Martin, 2001). As a cytoplasmic membrane anchored protein, c-Src has a critical role in mediating signal transduction via interactions with multiple proteins and protein complexes (Kim et al., 2009). Depending on its cellular localization, c-Src phosphorylates different substrates in the cytosol or at the inner face of the plasma membrane, at cell-matrix or cell-cell adhesions. These signaling complexes initiate pathways that regulate protein synthesis, gene expression, cytoskeletal assembly and many other aspects of cell function such as adhesion, invasion, motility or survival (Thomas and Brugge, 1997) (Yeatman, 2004) (Kefalas et al., 1995). Its activity is under tight control (Sen and Johnson, 2010) via phosphorylation/dephosphorylation cycles (Roskoski, 2005), and acts as a cancer promoter when deregulated (Irby, 2000). Overexpression of the c-Src protein and an increase in its specific activity have been observed in numerous cancer types (in colorectal cancer, several other gastrointestinal malignancies, as well as in

breast, ovarian and lung cancers); increased c-Src kinase activity is often associated with advanced-stage tumors that readily metastasize and is thought to have an important role in the increased metastatic potential of these tumors. Therefore, it is since many years an important therapeutic target (Kim et al., 2009).

c-Src was the fifth protein in which incorporation of a N-terminal myristoyl was discovered (in 1984) (Buss and Sefton, 1985). All the SFK members actually possess a myristoyl chain (Kefalas et al., 1995), except Frk, and are usually also palmitoylated. They are lipid anchored kinases acting at the membrane interface. c-Src does not use S-acylation, but its membrane association is enhanced by the presence of a PCD (Buser et al., 1994). Table 1.3 shows the consensus sequences of the SFK members, enabling myristoylation and/or palmitoylation of their N-terminal domain (Resh, 1999).

Table 1.3: *Sequence alignment of the N-terminal domains of the SFK members. Myristoylation occurs at glycine 2 while palmitoylation at cysteine 3 when present.*

SFK	Amino acid sequence															
Src	M	G	S	N	K	S	K	P	K	D	P	S	Q	R	R	R
Yes	M	G	<u>C</u>	I	K	S	K	E	D	K	G	P	A	M	K	Y
Fyn	M	G	<u>C</u>	V	Q	C	K	D	K	E	A	T	L	K	T	E
Lyn	M	G	<u>C</u>	I	K	S	K	R	K	D	N	L	N	D	D	E
Lck	M	G	<u>C</u>	V	C	S	S	N	P	E	D	D	W	M	E	N
Hck	M	G	<u>C</u>	M	K	S	K	F	L	Q	V	G	G	N	T	G
Fgr	M	G	<u>C</u>	V	C	F	K	K	L	E	P	V	A	T	A	K
Yrk	M	G	<u>C</u>	V	H	C	K	E	K	I	S	G	K	G	Q	G
Blk	M	G	L	L	S	S	K	R	Q	V	S	E	K	G	K	G

The association of c-Src with the plasma membrane is considered as an essential feature for its function. In absence of myristoylation, v-Src is not able to transform cells (Kamps et al., 1985), and c-Src stability and kinase activity is also affected (Patwardhan and Resh, 2010). The N-terminal domain of all SFKs is intrinsically disordered and constitutes a poorly conserved region among the family; therefore, it was named the Unique Domain (Ingley, 2008).

c-Src is composed of 536 amino acids (Maffei, 2014). Its first X-ray structure was obtained in 1997 (Sicheri and Kuriyan, 1997). The structures determined by x-ray lack the Unique Domain because of its disordered character. Apart for the N-terminal region,

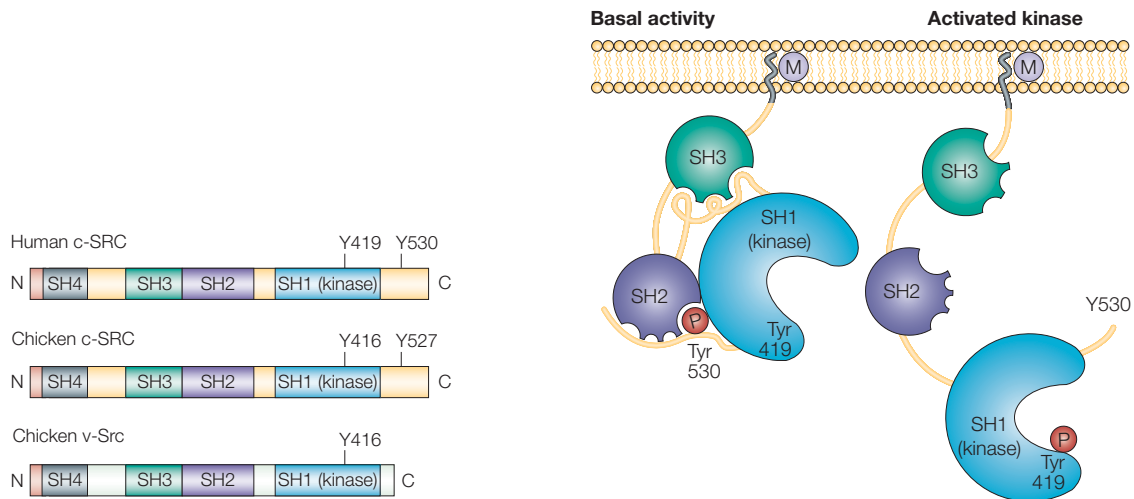


Figure 1.6: *Structure and activation of c-Src protein. Figure from Yeatman (2004).*

SFKs share a similar structure conserved among the members. Three Src Homology domains are connected to the Unique Domain, namely the SH3, the SH2 and the SH1 (or kinase) domains. The SH2 and SH1 are connected by another partially disordered loop, containing a proline rich motif. After the SH1 domain follows a C-terminal tail. These kinases have a mechanism for autoinhibition and activation driven by two key tyrosine residues, one negative-regulatory in the C-tail (Y527), and the other positive-regulatory in the so-called A-loop of the SH1 domain (Y416). Mutation of Y527 results in constitutive activation of c-Src, therefore loss of Y527 phosphorylation leads to activation of c-Src catalytic activity. v-Src lacks the C-terminal regulatory tail, which makes it always activated in the viral form (Thomas and Brugge, 1997). When phosphorylated, the kinase is locked in an inactive form through two intramolecular interactions: the SH2 domains binds the phosphorylated tyrosine Y527 while the SH3 domain binds the proline motif of the region connecting the SH2 and SH1. Upon dephosphorylation, the kinase adopts an open conformation and becomes kinetically active (Yeatman, 2004). Autophosphorylation of Y416 results in an additional activation step in which the enzyme reaches maximal activity. The activation mechanism is illustrated in Figure 1.6 (Yeatman, 2004).

Y527 is phosphorylated by the cytoplasmic tyrosine kinase Csk (Irby, 2000), thereby inactivating c-Src. Among the ligands capable of regulating c-Src activity stand the phosphatases and the receptor tyrosine kinases (RTKs). The mechanism of c-Src autoinhibition and activation relies on intramolecular interactions between the different domains, but the latter also participate in protein-protein interactions, thus playing an additional role in

the regulation of the enzyme. SH3 domains bind short contiguous amino acid sequences rich in proline residues and its ligands contain a core consensus sequence of P-X-X-P. SH2 domains bind to short contiguous amino acid sequences containing phosphotyrosine. The SH2 and SH3 domains play a central role in regulating c-Src catalytic activity. In addition to intramolecular interactions, the SH3 domain is important for intermolecular interactions, that regulate c-Src catalytic activity, localization, and recruitment of substrates (Ingley, 2008), the SH2 for regulation of the localization of c-Src or its binding proteins (Thomas and Brugge, 1997).

In vivo, c-Src molecules are present mainly in a inactive dormant form; in about 90-95 % of c-Src proteins, Y527 is phosphorylated in basal conditions (Roskoski, 2005). If c-Src is not phosphorylated at Y416, its activity is low (Superti-Furga, 1995). Autophosphorylation increases the catalytic activity and modifies substrate specificity (Sun et al., 2002). Mutation of Y413 (equivalent to Y416 in c-Src) diminishes the transforming potential of v-Src (Thomas and Brugge, 1997) and Y416 is constitutively phosphorylated in activated oncogenic c-Src mutants. Autophosphorylation has also been found to override the negative regulation process in some instances (Ingley, 2008). At high concentration and activation levels, v-Src displays a high transformation potential. The requirement for a trans-autophosphorylation of the A-loop for full activation suggests that SFKs are activated in clusters of at least two identical or related molecules, that may implicate adaptor molecules having a crucial regulatory role in this process (Ingley, 2008). Very tight regulation of c-Src is needed to avoid aberrant kinase activity.

Myristoylation targets c-Src proteins to membranes, which dramatically affects its cellular localization (Buss et al., 1986) (Pellman et al., 1985). In one study, non myristoylated c-Src has been found in the cytoplasm but also in the nucleus, which suggests that a possible role for myristoylation might be to prevent unregulated nuclear transport of proteins (David-Pfeuty et al., 1993). This phenomenon could be explained by the presence of a PCD sequence of c-Src (that may resemble a nuclear targeting domain (Li et al., 2014)), or by that of FG repeats in the Unique Lipid Binding Region – also found to promote nuclear localization in Hough et al. (2015). It is not clear whether non myristoylated forms of c-Src can exist in cells – David-Pfeuty et al. (1993) specify that they have been experimentally observed in some studies (Buss and Sefton, 1985). Several studies established that v-Src in its wild type form was present at both the plasma membrane (Courtneidge,

1980) and the perinuclear region of transformed cells (Kim et al., 2009). v-Src was found to be inactive in the perinuclear region, but it was in the active state at the cell periphery (Welham and Wyke, 1988). This activity-localization relationship was also observed for c-Src: studies on the subcellular localization of c-Src revealed that it was associated with the plasma, perinuclear, and endosomal membranes. The inactive form of c-Src has juxtanuclear localization (Yeatman, 2004). The actin cytoskeleton is needed for catalytic activation (Sandilands et al., 2004): upon activation by phosphorylation, c-Src SH3 domain associates with actin filaments, which then drives the translocation of c-Src to cell-cell and cell-matrix adhesion sites, where c-Src can interact with plasma membrane-bound molecular partners (Sen and Johnson, 2010). It is not really known how the kinase is spatially regulated. Endosomal trafficking is involved in the delivery of c-Src to the plasma membrane, and also in the recycling and degradation pathways (Sandilands and Frame, 2008). A soluble pool of c-Src proteins has also been observed (Buss et al., 1994) (David-Pfeuty et al., 1993) (Donepudi and Resh, 2008). Unlike the other members of the SFK, c-Src does not possess a palmitoyl chain, which is known to target proteins to rafts. c-Src is found to be localized in both raft and non-raft regions, with a different activity at the different compartments (Seong et al., 2009). In addition, c-Src can localize to late endosomes / lysosomes, which is specific to non palmitoylated SFKs, and is involved in lysosome distribution. An altered distribution of this distribution is often found in cancer cells (Kasahara et al., 2008).

1.3.2. The Unique and SH3 Domains of c-Src

Figure 1.7 displays the domain structure and sequence of the first N-terminal domains of c-Src, namely the SH4 and Unique disordered domains (USrc), and the SH3 folded domain. Figure 1.8-A is a scheme of c-Src bound to membranes, and Figure 1.8-B shows the scheme of the domains we will focus on during this work, namely the Myristoylated Unique and SH3 domains.

As already mentioned, c-Src does not undergo S-acetylation but possesses a PCD, called the SH4 domain, situated just after the glycine 2 residue. The SH4 domain of c-Src is enriched in three lysines and three arginine among its first 17 amino acids. c-Src binding to membranes via the myristoylated SH4 domain (MyrSH4) is one of the most studied case of cooperative binding between a myristoyl group and a PCD (Sigal et al., 1994).

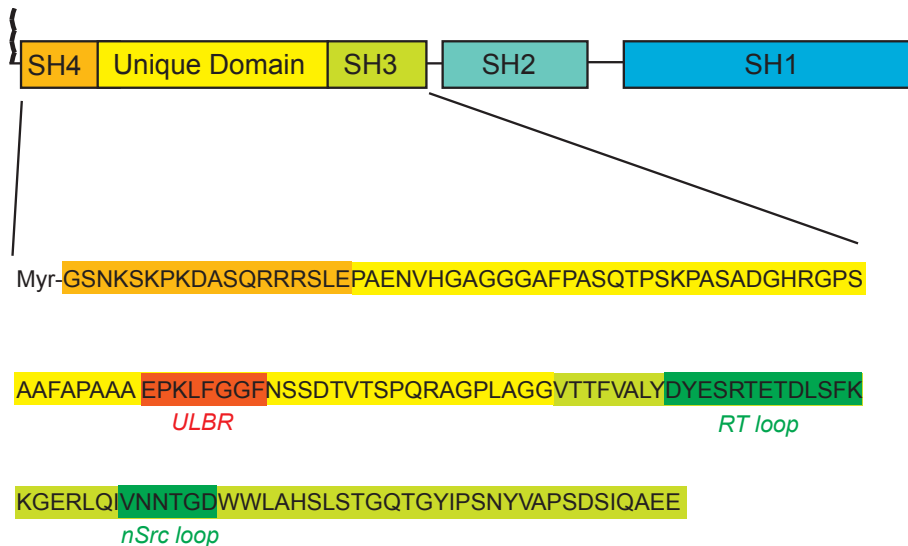


Figure 1.7: Domain structure and amino acid sequence of the SH4, Unique and SH3 domains of c-Src. The main functional domains (ULBR, RT and nSrc loops) are highlighted.

Binding to lipid vesicles (made of charged or neutral lipids) of variants of MyrSH4 showed that the electrostatic interactions increased the peptide partition to membranes in a non-additive manner (Buser et al., 1994). The partition coefficient derived were compatible with that of c-Src alone (Murray et al., 1998), confirming the essential role of MyrSH4 in c-Src anchoring to membranes. The three arginines seem to have a dominant role in the electrostatic interaction processes – as seen in NMR experiments of USrc binding to charged bicelles (Pérez et al., 2013). Myristoyl chain insertion has been studied by solid state NMR and showed that the acyl chain insertion depth varied according to the lipid composition; the depth was influenced by the degree of saturation, the chain length and the charge of the lipids (Scheidt and Huster, 2009). In the molecular model computed for MyrSH4 binding to a bilayer, the SH4 domain does not penetrate the bilayer but stands on top of it through electrostatic interactions (Murray et al., 1997) (Murray et al., 1998). Experimentally, non myristoylated SH4 binding to a lipid monolayer was not found to insert into it (Sigal et al., 1994). MyrSH4 can be phosphorylated in S17 (Amata et al., 2013), which decreases the total charge of the SH4 domain and therefore the binding of SH4 to charged lipids (Pérez et al., 2013).

Going towards the C-terminal, after the SH4 domain comes the Unique Domain, and both constitute the intrinsically disordered N-terminal domain of c-Src. This unfolded domain possesses the general characteristics of IDD, (for instance it has a high isoelectric

point), with some peculiarities: it contains more aromatic residues than a usual IDD. Within its sequence, a partially ordered region has been found (Bernadó et al., 2008), that was also shown to have lipid binding capabilities. For this reason, it was called the Unique Lipid Binding Region (ULBR) (Pérez et al., 2013). This secondary, or weak, interaction towards lipids is postulated not to play a role in c-Src anchoring, but in fine tuning of c-Src interactions with membranes and possibly kinase activity regulation. The Unique and SH4 domains are non conserved among the Src family, and thought to provide unique functions to each member Ingley (2008). Though it has raised low interest until now, a few studies highlight its biological importance. Swapping the N-terminal region, including the Unique domain, between c-Src and c-Yes, results in functional exchange (Hoey et al., 2000) (Summy, 2003). Additional phosphorylations to S17 have been found in the Unique Domain, close to the ULBR, at S69 and S75 (Amata et al., 2013), which also abolishes the interaction of ULBR with membranes (Pérez et al., 2013). Other phosphorylation events at serine and threonine residues have been observed in the Unique Domain (Ralston and Bishop, 1985), for instance at S12, T34 and S72, and have been shown to modulate the activity of c-Src (Pan et al., 2011) (Joung et al., 1995) (Gould and Hunter, 1988) (Amata et al., 2014). Mutations in the ULBR of full length c-Src resulted in strong phenotypes in model organisms (Pérez et al., 2013) as well as colorectal cancer cell lines (Maffei, 2014), suggesting that fine tuning of membrane interactions is an important functional element in c-Src regulation. As a consequence, the membrane environment probably plays an important role for c-Src regulation.

After the Unique Domain comes the small folded SH3 Domain. It consists of a beta-sandwich formed by five beta-strands connected by three loops (the RT, nSrc, and distal loops) and a short 3_{10} -helix. The SH3 also contains lipid binding regions, in particular in the RT loop, and also in the nSrc loop (Pérez et al., 2013). Binding of a polyproline on the other side of the SH3 allosterically hampers the RT loop to bind lipids (Amata et al., 2013).

In-vitro studies on the non-myristoylated form of USH3 revealed intramolecular interactions between the SH3 and the Unique domains, highlighting the role of intramolecular interactions at the N-terminal of c-Src. Indeed, these interactions take place in regions already involved in lipid binding. The ULBR has been found to interact with the RT loop. Another interaction between the n-Src loop and the SH4 domain has been observed, and

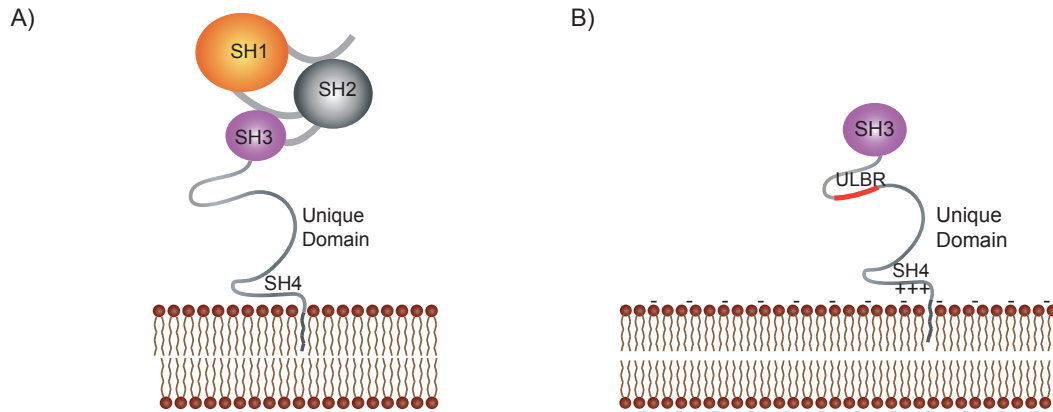


Figure 1.8: *A) Scheme of c-Src bound to membranes B) Scheme of the myristoylated Unique + SH3 domain of c-Src bound to membranes. Additional electrostatic interaction occurs at the PCD SH4.*

revealed that the Unique Domain possibly adopts a restricted conformation through intramolecular interactions. External ligands are also capable of competing with the same sites (for instance ligands containing a polyproline-like motif) ([Maffei et al., 2015](#)). Figure 1.9 represents the different intramolecular interactions taking place in the Unique and SH3 domains of c-Src.

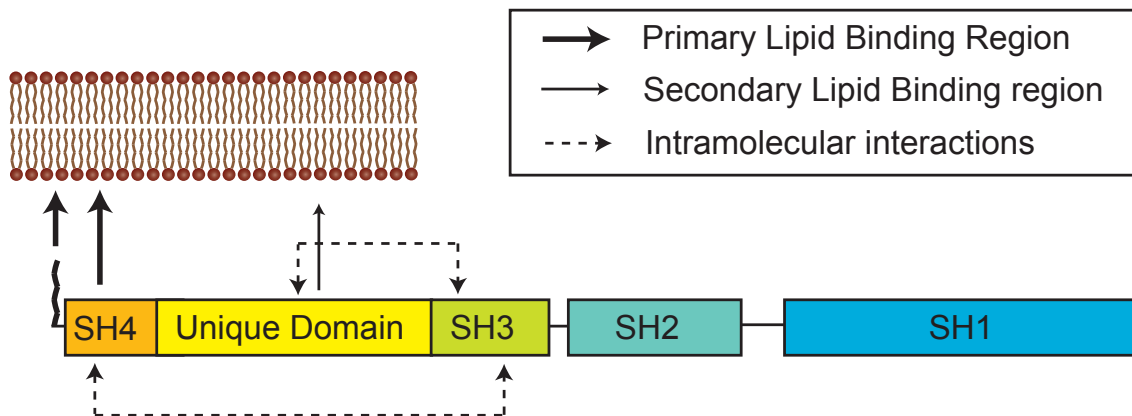


Figure 1.9: *Scheme of USH3 intramolecular interactions, as well as its interactions with lipids*

c-Src constitutes a striking example of an unfolded domain with high functional versatility. It remains disordered, yet is conformationally restricted, interacts with multiple partners with low affinity and acts in synergy with other PTMs such as phosphorylation,

and all this at the membrane boundary via its myristoyl anchoring. This current knowledge sheds light on the existence of the rich repertoire of regulatory interactions at the N-terminal domain of c-Src and highlights the functional role of the disordered N-terminal of c-Src. As said earlier, the Unique and SH3 Domains can influence c-Src function, and are thought to add a regulation layer in c-Src activation mechanism, specific to the protein because of its unique sequence. A detailed understanding of the regulation mechanism would allow this domain to constitute a novel drug target.

However, the in-vitro studies that have been carried out on the N-terminal region of Src in order to characterize its detailed mechanism at the lipid bilayer interface either lack the Unique Domain or the myristoyl chain. A study of the myristoylated Unique and SH3 domains would constitute a better approximation of the natural form of c-Src.

1.4. Lipid membrane models and Protein-Membranes interactions

To study the binding of proteins or peptides to membrane, there is a need to mimic biological membranes in order to conduct biophysical assays. As discussed earlier, membranes are highly complex environments, made of a high number of different lipids and proteins. They are neither flat nor symmetric and even have lateral inhomogeneities (lipid rafts). Fully understanding the mechanisms underlying the wide variety of membrane function is a huge research field, and more research is needed before artificial membranes can perform the full range of roles required of them: reproducing asymmetry, specialized patches and membrane curvatures are still current challenges ([Ken Jacobson et al., 2007](#)). Nevertheless, very simple membrane models can provide very valuable information on the interaction of proteins to lipid layers of certain compositions.

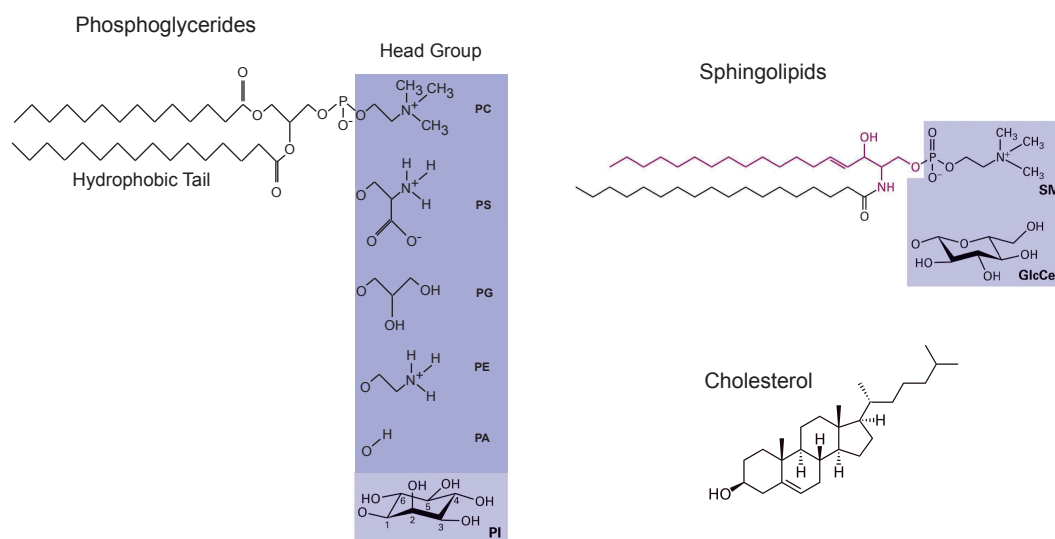


Figure 1.10: *Classes of the most common lipid-composing membranes*

Glycerophospholipids are the main components of membranes and are responsible for its architecture. They are composed of two hydrophobic carbons chains linked to a polar head, as illustrated in Figure 1.10.

The chain can vary in length and saturation, and the head can be of distinct composition. Phosphatidylcholine is the most abundant phospholipid and is zwitterionic. Smaller amounts of negatively (phosphatidylglycerol, phosphatidic acid, phosphatidylserine) or positively (phosphatidylethanolamine) charged are found, in quantities depending on the

membrane location in the organism. Different types of phosphoinositides, considered as signaling lipids with specific functions, are found in very small amounts, especially in rafts. Rafts are also enriched in sphingolipids and cholesterol.

Bilayer formation is a consequence of the dominance of glycerophospholipids in the membrane composition. A bilayer formed of a single lipid can be either in the gel phase or in the liquid phase, depending on the temperature. The temperature at which the transition from the gel to the liquid phase occurs is called the melting temperature (T_m). This temperature depends a lot on the carbon chain length and saturation, and to some extent on the head.

Three classical chain lengths are very useful in the common experimental conditions used. Unsaturated C18-chains ensure that at 4 °C or more, the bilayer is always in a fluid state (for instance DOPC has a T_m of -17 °C). Saturated C14-chains are in the gel phase below room temperature, and are fluid above (for instance DMPC, with a T_m of 23 °C). Saturated C16-chains have a high transition temperature, thus are in the gel phase already at room temperature (DPPC has a T_m of 41 °C).

According to the needs, the proper lipids can be chosen to obtain a gel or fluid phase in the experimental conditions. Indeed, in the fluid phase, lipids and proteins will diffuse laterally, but this diffusion will be significantly slowed down in the gel phase. Often, a lipid mixture is used, and the lipids can be chosen with matching T_m (for instance DMPC and DMPG), or not (to induce phase separation for instance). Cholesterol is also often added as an important component of the phase behavior of the membrane, by having the property of creating “liquid order phases”, which are fluid but less disordered than liquid phases lacking cholesterol (“liquid disordered phases”). The phase state of the bilayer from a complex mixture is represented in a phase diagram (as in Figure 1.11, in which the composition of each lipid

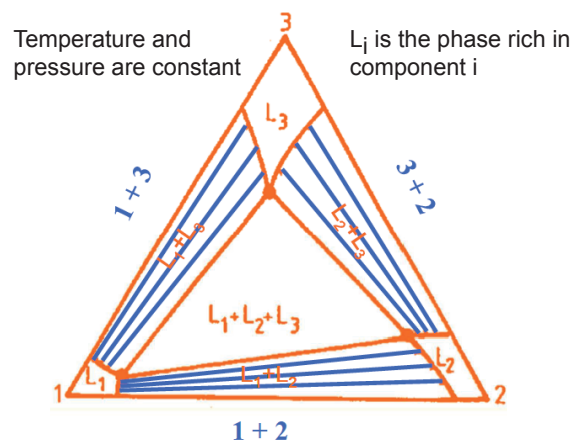


Figure 1.11: *Phase diagram for a bilayer composed of three lipids, showing in which phase it is according to lipid composition i , at constant temperature and pressure*

influences the bilayer phase state.

Table 1.4: *Transition temperature of the most common phospholipids (source from Avanti Polar Lipids webpage)*

Phosphatidylcholine		Phosphatidylglycerol	
12:0 PC (DLPC)	-2	12:0 PG (DLPG)	-3
13:0 PC	14	14:0 PG (DMPG)	23
14:0 PC (DMPC)	23	16:0 PG (DPPG)	41
15:0 PC	35	18:0 PG (DSPG)	55
16:0 PC (DPPC)	41	18:1 PG (DOPG)	-18
17:0 PC	50	16:0-18:1 PG (POPG)	-2
18:0 PC (DSPC)	55	Phosphatidylserine	
19:0 PC	62	14:0 PS (DMPS)	35
20:0 PC	66	16:0 PS (DPPS)	54
21:0 PC	71	18:0 PS (DSPS)	68
22:0 PC	75	18:1 PS (DOPS)	-11
23:0 PC	79.5	16:0-18:1 PS (POPS)	14
24:0 PC	80.3	Phosphatidic Acid	
16:1 PC	-36	12:0 PA (DLPA)	31
18:1c9 PC (DOPC)	-17	14:0 PA (DMPA)	52
18:1t9 PC	12	16:0 PA (DPPA)	65
18:1c6 PC	1	18:0 PA (DSPA)	75
22:1c13 PC	13	18:1 PA (DOPA)	-4
18:2 PC	-57	16:0-18:1 PA (POPA)	28
18:3 PC	-60	Phosphatidylethanolamine	
20:4 PC	-69	12:0 PE (DLPE)	29
14:0-16:0 PC	35	14:0 PE (DMPE)	50
14:0-18:0 PC	40	16:0 PE (DPPE)	63
16:0-14:0 PC	27	18:0 PE (DSPE)	74
16:0-18:0 PC	49	20:0 PE	83
16:0-18:1 PC (POPC)	-2	18:1c9 PE (DOPE)	-16
16:0-22:6 PC	-27	18:1t9 PE	38
18:0-14:0 PC	30	18:2 PE	-40
18:0-16:0 PC	44	16:0-18:1 PE (POPE)	25
18:0-18:1 PC	6		
18:0-18:1 PC	6		
18:1-16:0 PC	-9		
18:1-18:0 PC	9		

A wide range of techniques are available to screen and analyze protein-lipid layer interactions (Cooper, 2004); many of them imply measurements at equilibrium which renders information on the partitioning of the protein towards the lipid. The concept of partitioning is preferred to that of affinity since the interactions usually do not have a specific stoichiometry (Leo et al., 1971). Some techniques also enables to study the kinetics

of the interaction (the velocity at which the protein associate to or dissociate from the lipid layer), as well as its penetration capacity into the layer. Other techniques allow for peptide secondary structure and orientation study at the lipid layer. The factors influencing partitioning, kinetics and penetration can be studied by changing the composition of the lipid layer, as well as its pressure, its curvatures, etc.

Figure 1.12 shows the main membrane mimics in solution, that will be described in the following section.

The simplest mimic consists in a large multilamellar vesicle (MLVs) spontaneously formed upon rehydration of a lipid film (Akashi et al., 1996). The process must be performed above the transition temperature of the lipids in order to obtain a fluid phase. The composition of the enclosed aqueous phase matches that of the phase medium. However, if the external medium is replaced, the composition of the internal phase is unchanged. This can be used, for example, to prepare vesicles loaded with sucrose to increase their density and facilitate phase separation upon centrifugation. MLVs have a large diameter, are very heterogeneous in size (from 1 to 10 μ m) and are multilamellar. Upon centrifugation of the MLVs with the protein, a lipid sedimentation assay can be performed to determine the partitioning of the protein to the lipid phase versus the aqueous phase. Quantification of the amount of protein present in each phase can be done in a simple SDS-page gel, or by more sophisticated methods implying for instance radioactive (Buser et al., 1994) or fluorescence measurements (Ehrlich et al., 2011). Giant Unilamellar vesicles (GUVs) can be formed by electroporation (Riske and Dimova, 2005). At least three techniques permit to obtain smaller and unilamellar vesicles. By extruding MLVs through pores of a definite size, large unilamellar vesicles (LUV) of the size ranging from about 100 nm to 400 nm can be obtained. By sonicating MLVs, small unilamellar vesicles (SUV) are obtained and have a diameter ranging from about 20 nm to 80 nm. These liposomes are very useful in many assays conducted in solution, since they are formed of a single lipid bilayer. Their size or curvature can be controlled (Hamai et al., 2006) (Wu and Baumgart, 2014), and they do not diffract light so much, especially at low concentration. A variation of liposomes are bicelles, in which long chain lipids are mixed with short chain lipids; by hydration of such a lipid film with an aqueous solution, spontaneously form bicelle-shaped structures, the short chain lipids situated at the highest curvature position (P  rez et al., 2013). Studying

the interaction of a protein with liposomes by Isothermal Titration Calorimetry (ITC) renders a partition coefficient of the interaction (Wu and Baumgart, 2014), and also thermodynamics data. Liposomes can be useful in fluorescence assay, giving information of the partitioning of the protein to the bilayer (Ladokhin et al., 1997); if a stop-flow assay is used, kinetics information can be derived (Lorch and Booth, 2004) (Daniel R. Dries and Newton, 2008). Liposomes are also used in NMR experiments (Bensikaddour et al., 2008), and bicelles can be used when there is need for aligning them in the magnetic field. Circular Dicroism (CD) enables to study the conformation of the peptide at the liposome interface, for instance the formation of an alpha helix upon liposome binding (Zhao and Ta, 2003).

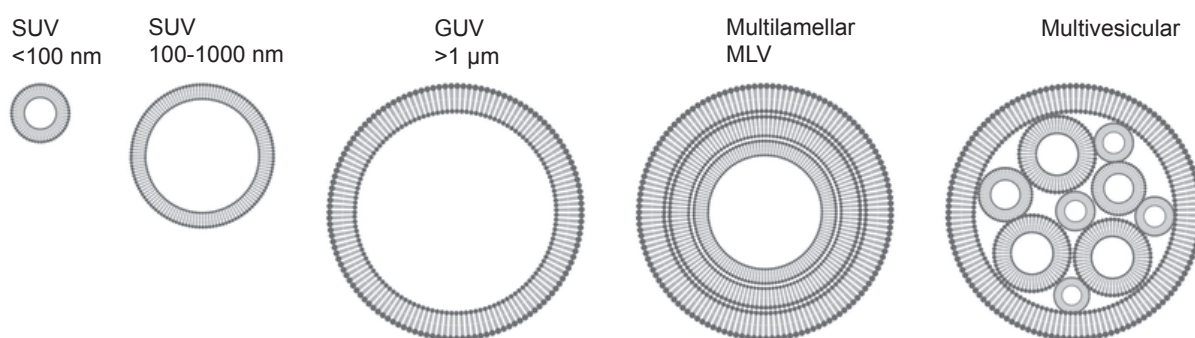


Figure 1.12: *Vesicles (multilamellar and unilamellar) and liposomes (SUV and LUV)*

A different lipid model consists in depositing lipids at an air water interface, using the so-called monolayer technique, as illustrated in Figure 1.13-A. The lipids are dissolved in a solvent, and upon reaching the aqueous surface, spread and organize as a monolayer in which the head is in contact with the water, and the tail with the air. The equivalence between the lateral pressure of this so-called Langmuir monolayer, and lipid bilayers lateral compressibility, has been established (Marsh, 1996). Langmuir monolayers are widely used to study protein interaction with (Wang et al., 2011) and insertion into the monolayer (Calvez et al., 2009). Indeed, the surface pressure can be measured using a Wilhemy plate (Maget-Dan, 1999). By adding a protein in the subphase, protein insertion is recorded by measuring the change in pressure. The monolayer can also be compressed and extended thanks to the use of superficial moving barriers (Figure 1.13-B), rendering for instance the area needed for a peptide to insert in the monolayer (Beschi-schvili and Seelig, 1990) (Sugár et al., 2005) (Desmeules et al., 2007). Combining a

monolayer with another spectroscopic technique, for instance Infrared Reflection Absorption Spectroscopy (IRRAS), can render information about peptide conformation at the interface ([Annette Meister et al., 2007](#)).

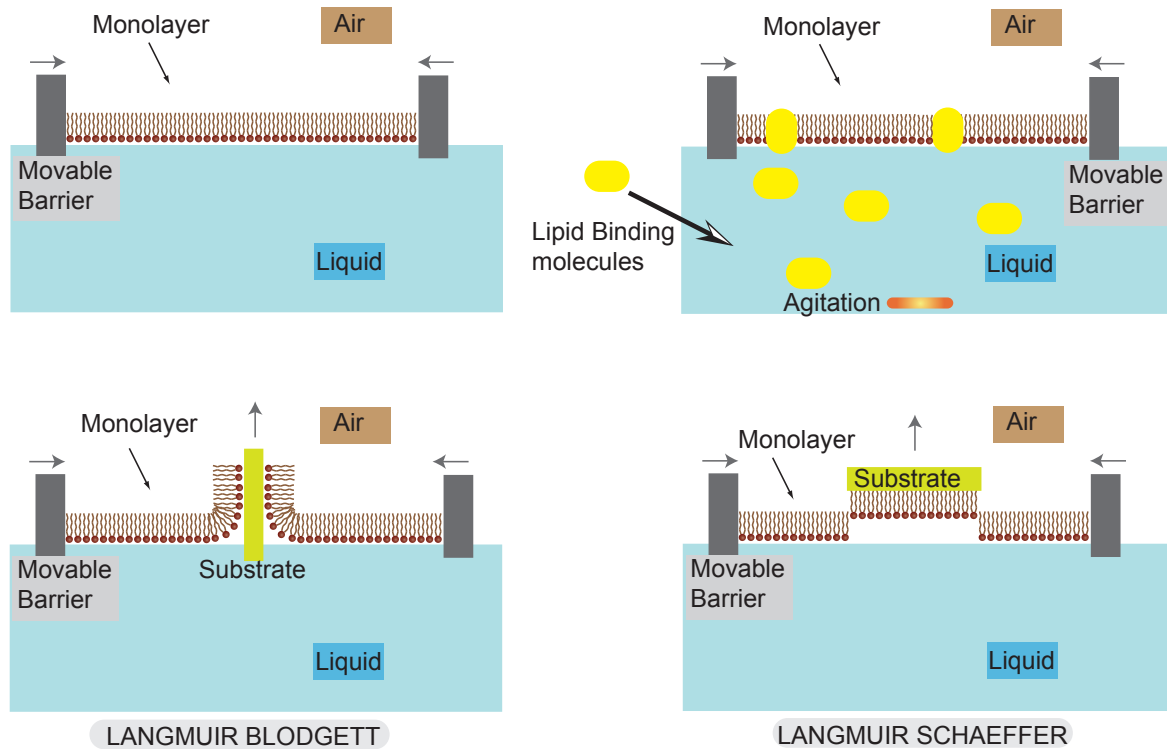


Figure 1.13: *Monolayer set-up*

A vast category of membrane mimics take the form of Supported Lipid Bilayers, SLBs, which is a generic term for mono, bi or multilayers standing over a solid substrate. For instance, Langmuir monolayers can be transmitted to a substrate (glass, mica, etc) by using the Langmuir Blodgett or Langmuir Schaeffer techniques ([Charitat et al., 1999](#)), as illustrated in Figure 1.13-C and -D. SLBs can also be formed simply by depositing liposomes over a variety of substrates. One of the most straightforward technique consists in vesicle fusion over a hydrophilic substrate like glass, mica, silicon, etc ([Buss and Sefton, 1985](#)). The liposomes readily adsorb under certain conditions. Many parameters influence vesicle fusion, such as liposome composition, pH, ionic strength of the buffer ([Johnson et al., 2002](#)), presence of calcium in the buffer, the solid support, etc ([Richter et al., 2006](#)). Functionalized substrates are also used, for example, hydrophobic anchors can be chemically coupled to capture liposomes, or pegylated glass surfaces containing streptavidin molecules can capture liposomes containing biotin. ([Cremer and Boxer, 1999](#)) ([Jass](#)

et al., 2000) (Ehrlich et al., 2011). The liposomes can either spread completely and form a bilayer, or remain intact over the surface; a mixture of the two cases can also be encountered (Cremer and Boxer, 1999). Figure 1.14 shows different spreading events (Richter et al., 2006).

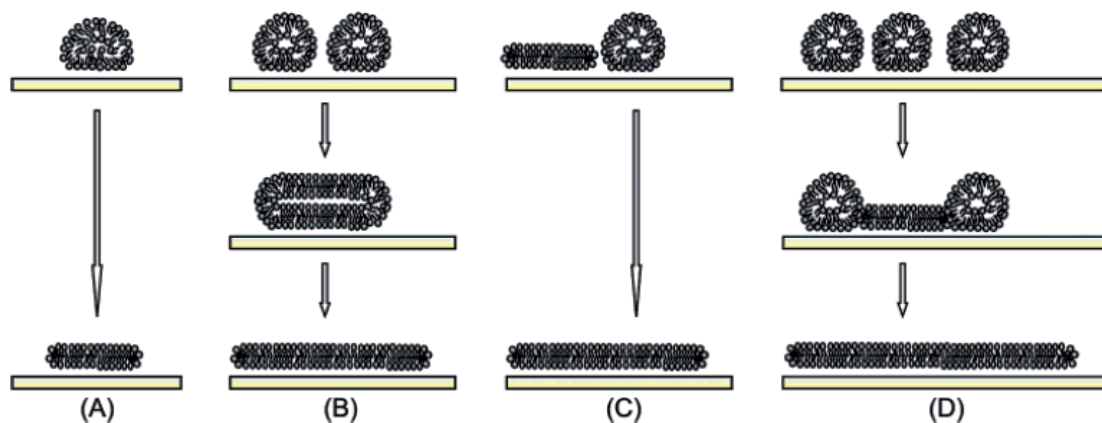


Figure 1.14: *Formation of a Supported Lipid Bilayer by liposome deposition, as described in the text. Figure from Richter et al. (2006)*

These SLBs are used in a variety of techniques. In a Surface Plasmon Resonance (SPR) assay, the kinetics of a protein interacting with a SLB can be followed and the affinity measured (Daniel R. Dries and Newton, 2011). SLB formation has been widely studied by Atomic Force Microscopy (AFM) (Jass et al., 2000) and can be further used to study peptide-SLB interactions (Chaibva et al., 2014). Other spectroscopic methods use SLBs such as fluorescence microscopy, in which the Total Internal ReFlection (TIRF) mode is interesting because it focuses at the interface only. ATF-FTIR can also be conducted to study structural features of the peptide at the SLB interface (Zhao and Ta, 2003) (Bensikaddour et al., 2008). Solid state NMR is also able to give structural information (Lindblom and Quist, 1998).

1.5. Motivations

Recent work demonstrated the biological relevance of the Unique Domain of c-Src in the enzyme function and regulation (Pérez et al., 2013) (Maffei et al., 2015). The biophysical characterization of this disordered domain in absence of the myristoylated tail showed that it was involved in lipid binding, as well as in intramolecular interactions with the SH3 domain. However, since c-Src is anchored to membranes in its natural form, characterization of the myristoylated form is highly relevant, specifically when bound to a lipid bilayer. In this work, we decided to use simple membrane models, and to focus on the complexity of the interactions between the Unique and SH3 domains and lipid layers in myristoylated constructs of c-Src.

Anchoring of c-Src to membranes has been attributed to the MyrSH4 peptide only (Murray et al., 1997). The thermodynamics of MyrSH4 binding to lipid vesicles has been extensively studied (Sigal et al., 1994). The equilibrium constant of c-Src binding to membranes has been found to be of the same order of magnitude. However, the additional lipid binding regions in the Unique and SH3 domains are obviously not present in the MyrSH4 construct. Would the binding of longer myristoylated constructs to lipids differ from that of MyrSH4 because of these additional lipid binding domains? In addition, what is the kinetics behavior of myristoylated forms of c-Src binding to membranes?

Tethering to the membrane surface, even if the connection is a flexible, long, intrinsically disordered domain, creates singular boundary conditions resulting from the transition between 3D (cytoplasm) and 2D (membrane) environments. Cluster formation could be facilitated in this boundary region and has already been observed in the small MyrSH4 peptide (Owen et al., 2010). Can we observe analogous phenomena when constructs longer than MyrSH4 bind to membranes?

Another challenge of our system is to study low affinity interactions in the presence of a high affinity lipid binding region (MyrSH4). In many biophysical techniques, the high affinity interaction is expected to “hide” the weak interactions. The weak interaction would be difficult to study on its own; it would require high protein amounts in the assays, often favoring non specific binding. In addition, the actual affinity would probably be different in the presence or the absence of the high affinity interaction, because of the entropic advantage gained by belonging to the same peptide: the weak interaction is expected to be increased or directed in the presence of the strong interaction. Can we find

ways to study the effect of myristoylation on the lipid binding capacities of the Unique Lipid Binding Region or the RT loop of USH3? Would these domains have enhanced affinity to lipid bilayers when the protein is already anchored to it? Do these domains have selective affinity for specific lipids? Could the Unique Lipid Binding Domain regulate in some way the distance of the folded domains of c-Src from the membrane, by interacting or not with lipids?

Deeper characterization of the SH4-Unique-SH3 ensemble in its myristoylated form, in solution or bound to a lipid bilayer, will increase the understanding of the possible role of these domains in the regulation mechanisms of c-Src kinase.

Chapter 2

Objectives

Objective 1

Establishing the experimental procedures for the efficient production of myristoylated proteins

- I Set up robust methods, as universal as possible, permitting the expression and purification of recombinant myristoylated proteins, folded or unfolded, by coexpressing them with N-myristoyl Transferase in *E.coli*. Propose strategies to eliminate all possible sources of contamination.
- II Scale *in-vitro* myristoylation reactions and identify purification protocols.
- III Produce pure samples at a scale adequate for biophysical studies, in particular obtaining the samples in minimum medium at a concentration suitable for Nuclear Magnetic Resonance studies.

Objective 2

Characterizing the binding of myristoylated constructs containing the Unique Domain of c-Src to lipid bilayers.

- I Study the binding kinetics of MyrUSH3 to lipid bilayers; set up an SPR assay to obtain kinetics and affinity constants of the binding of MyrUSH3 to supported lipid bilayers of different compositions.
- II Explore the possible clustering of c-Src upon membrane binding. Develop a suitable assay at the single molecule level, in which a myristoylated construct containing a fluorescent reporter fused to the Unique Domain is bound to supported lipid bilayers.
- III Examine by NMR the effect of myristoylation at a residue level
 - on the USH3 intramolecular interactions
 - on the binding of “lipid binding regions”, in the USH3, domain to lipid bilayers

Chapter 3

Protein Myristoylation

3.1. Myristoylation

Myristoylation

Myristoylation is found in low and high eukaryotes. The enzyme catalyzing the addition of a myristoyl chain at the N-terminal glycine of a protein was identified in 1986 (Towler and Glaser, 1986) and is called N-Myristoyl Transferase (NMT). It catalyzes the transfer of the acyl chain of Myristoylated Coenzyme A (MyrCoA) to the peptide substrate. Despite the fact that myristic acid is not abundant in cells, NMT usually catalyzes the acyl addition with high specificity for myristoyl. The initiating methionine is removed by methionine amino peptidase during translation and glycine 2 becomes the N-terminal amino acid. The requirement for glycine at the N-terminus is absolute; no other amino acid will substitute. However, not all proteins with an N-terminal glycine are N-myristoylated and the ability to be recognized by NMT depends on the downstream amino acid sequence. The most common N-terminal consensus sequence for NMT substrates is: Met-Gly-X-X-X-Ser/Thr, with generally serine or threonine being preferred at position 6 and lysine or arginine at positions 7 and/or 8 (Resh, 1999), as illustrated in Figure 3.1. Still, the universal rules governing the nature of the following amino acid stretch is yet unclear and variations and exceptions have been described (Boutin, 1997) (Traverso et al., 2013). Up to the first 17 amino acids may play a role in sequence recognition and exhaustive models are investigated in order to be applied in predictive algorithms (Maurer-Stroh

et al., 2002a).

The problem of the specificities of the NMT is interesting, since it concerns both substrate and cosubstrate. Though myristoyl is very specifically coupled to NMT substrates in many cases, examples of heterogeneously acylated proteins have been found, for instance recoverin and the α -subunit of the G protein transducin (Iram and Cronan, 1992) (Boutin, 1997). In these

proteins and others, lauric acid and C14 fatty acids containing a cis double bond between C5-C6 (C14:1) or C5-C6 and C8-C9 (C14:2), have been observed; the reduced hydrophobicity of these acyl chains compared with that of myristoyl may be required for these proteins to properly operate (Sonnenburg and Gordon, 2004). The effect of substituting myristic acid with oxygen incorporated into the fatty acid backbone in place of the position 11 methylene group (which reduces the hydrophobicity of the fatty acid with only a minor effect on chain length) has been studied on different proteins. It resulted in changes and reductions of the in-vitro peptide catalytic efficiency of one peptide with respect to another. For most yeast and mammalian N-myristoyl proteins, it did not alter membrane association, however a few proteins were affected. The 63-kDa N-myristoyl protein in BC3H1 cells and the tyrosine kinase v-Src both underwent dramatic redistribution from membrane to cytosolic fractions (Heuckeroth and Gordon, 1989). More than one hundred different chains were tested as possible substitutes of the natural cosubstrates, in the form of MyrCoA analogues (Boutin, 1997). NMT was able to recognize a wide range of them as cosubstrates for the acylation of peptide or protein. It can usually accommodate shorter acyl chains but not the longer ones, such as palmitic acid (Rudnik et al., 1992) (Heuckeroth and Gordon, 1989) (Towler and Glaser, 1986). NMT is mainly found in the cytosol but also in the reticulum endoplasmic, and usually acts cotranslationally (Deichaite et al., 1988), by modifying the nascent polypeptide chain (Boutin, 1997). Binding of the MyrCoA cofactor to one site of NMT is a prerequisite, since it induces a conformational change in the enzyme allowing for peptide recognition. When the ternary complex NMT:CoA:substrate is formed, the chemical transformation (the nucleophilic

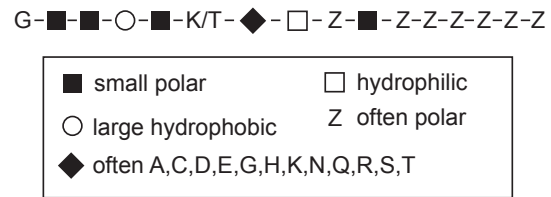


Figure 3.1: *Consensus Sequence for NMT N-terminal peptide recognition*

glycine attacks the thioester carbonyl bond of MyrCoA) is facilitated by NMT. The CoA is released and the Myristoylated peptide as well (Farazi et al., 2001). Figure 3.2 shows a schematic representation of the myristoylation reaction.

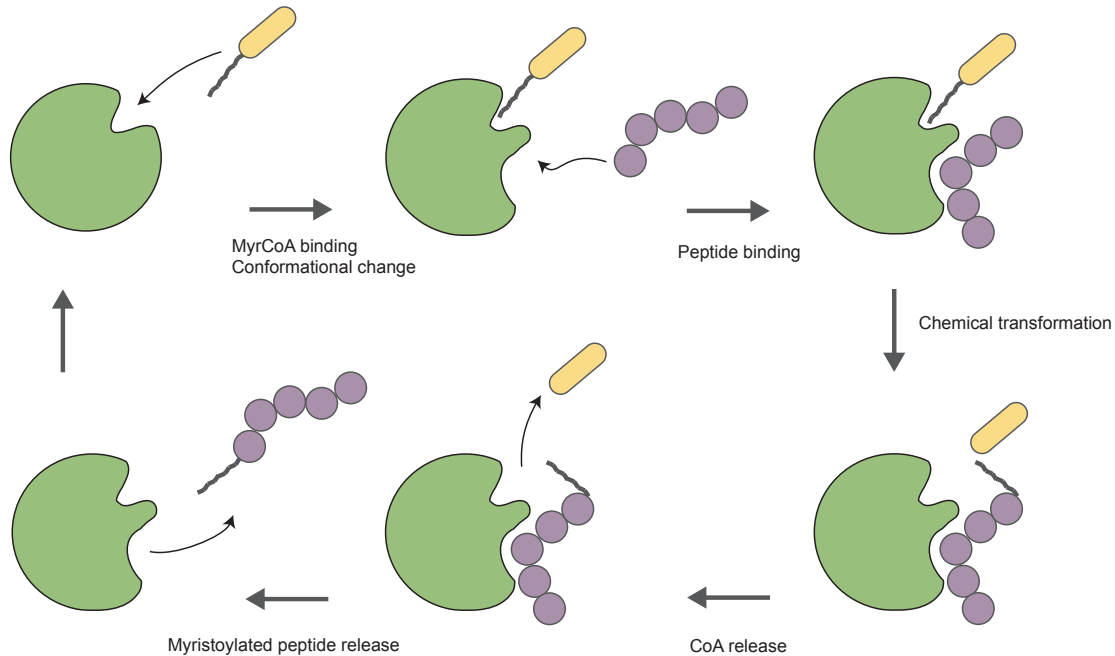


Figure 3.2: *Myristoylation mechanism*

Recombinant proteins are usually expressed in bacterial system, but since bacteria do not express NMT, the protein would not be myristoylated in such a system. This limitation is overcome by coexpressing the protein and the enzyme, since MyrCoA is present in bacteria (used in the β -oxydation pathway of energy production). The standard method initially consisted in a two-plasmid expression system, but in many instances, partial myristoylation was achieved. This lowered the protein yield, and also implied separation of the myristoylated and non-myristoylated proteins (Kroupa et al., 2014) (Ha et al., 2005). Improvement of the myristoylation efficiency was achieved by using a bicistronic vector, in which the plasmid contains the sequence of both the enzyme and the substrate (Gluck et al., 2010). Purification is generally performed in a standard way, by lysing the cells, centrifugating the lysis product and purifying the supernatant, with (Gluck et al., 2010) or without detergents (Morgan et al., 2011) (Kroupa et al., 2014). In some protocols, the protein is extracted from the pellet (Ha et al., 2005).

We used the bicistronic method to express and purify our myristoylated proteins.

When using this strategy, we encountered several unexpected observations:

- First, the myristoylated protein was forming a tight complex with the enzyme upon resolubilization during cell lysis. Rebinding of the myristoylated protein to NMT has also been observed for Nef ([Morgan et al., 2011](#)).
- Second, shorter acyl chain, especially lauryl chains, were also attached to the protein in a non negligible amount, in particular when cells were grown in minimum medium. This phenomenon has also been observed upon myristoylation of Arf1 ([Yizhou Liu et al., 2009](#)). As explained previously, the length of the acyl chain can influence the binding properties of the protein to membranes, so this issue should be addressed in order to obtain pure samples of myristoylated proteins.
- Third, additional protein degradation occurred upon myristoylation. Thus expression and purification protocols were optimized in order to obtain pure myristoylated proteins.

We used the coexpression system to produce several constructs of myristoylated forms of Src. These proteins were expressed in rich medium for most of the assays, and also in minimum media containing ^{15}N labelled NH_4 for further use in NMR experiments. MyrUSH3 was expressed and used for expression and purification optimization. In order to conduct fluorescence experiments, the SH3 domain was replaced with a fluorescent domain, namely the enhanced GFP.

Alternatively, myristoylation can be performed *in-vitro*, by mixing the NMT enzyme, the MyrCoA cofactor and the protein substrate ([Padovani et al., 2013](#)) ([Gaffarogullari et al., 2011](#)). The different components have to be separately produced before being mixed. CoA can be chemically myristoylated ([Gaffarogullari et al., 2011](#)), and the protein and NMT can be expressed and purified separately. When the reaction has finished, the mixture has to be purified from the NMT, the unreacted MyrCoA and the non myristoylated protein. This can be achieved by precipitating the protein in ammonium sulfate ([Padovani et al., 2013](#)). We used a chromatographic method instead. We used *in-vitro* myristoylation to produce the myristoylated Unique Domain of Src (USrc) since the coexpression method failed for this construct. The unfolded character of MyrUSrc allowed for product separation of the products by reverse phase chromatography (since the protein forms a tight complex with NMT, ammonium precipitation might not work). If ^{15}N

labeling was needed, USrc was expressed in minimum medium.

Peptide synthesis constitutes another alternative and can be applied to short myristoylated peptides. Using standard solid phase methods, the myristic acid can be coupled to the last anchored peptide, in a similar method to that described in [Geyer et al. \(1999\)](#). We used this method to synthesize the peptide corresponding to the 17 first amino acids of c-Src, Myristoylated SH4 (MyrSH4).

Degradation

Disordered regions are more easily degraded than folded domains. We experimentally observed that the purified Unique Domain of Src, non-myristoylated, degraded faster than the SH3 domain. Still, no substantial degradation was observed when conserving the non-myristoylated forms of USH3 of USrc during several days or weeks. In contrast, when expressing the myristoylated form, a well-defined cleavage site was observed. In the second part of this chapter, we will study the degradation phenomena. First, we identified the location at which the amino acids chain was principally cleaved, and demonstrated that mutation at that site impeded degradation. We also looked at the factors that can favor or be the source for degradation, in order to try to understand its mechanism. Then we developed a method enabling to obtain degradation-free samples of myristoylated c-Src constructs. Since degradation may have a functional role, we designed an assay to identify a "marker" peptide as a diagnostic for degradation phenomena. The assay was tested *in vitro*, with the aim of extending it to an *in-cell* assay and verify whether the degradation may occur in human cells and has a functional role.

3.2. Coexpression Strategy

3.2.1. Cloning and constructs

- **USH3** The original PetDuet I plasmid, was a gift from Julien Glück, and contained in the first cassette the sequence of yeast NMT, and in the second cassette that of the Nef protein. The Nef sequence was replaced with that of USH3, terminated with a C-terminal HisTag composed of six histidines.
- **UGFP** To obtain the UGFP construct, the sequence of USrc was first inserted in the original plasmid instead of that of Nef. A second digestion and insertion was needed to further insert the sequence of eGFP following that of USrc, with a small linker. The HisTag was added at the GFP C-terminal during the insertion protocole.

3.2.2. Classical expression and purification

Purification of the supernatant (classical purification of soluble proteins) The most straightforward and standard method to express and purify MyrUSH3 and MyrUGFP has been firstly applied in rich medium, as described by [Glück et al. \(2010\)](#). Induction was allowed in presence of myristic acid, and purification of the supernatant was conducted (this method will be called the supernatant purification). Final purification of the protein implied the use of a C-terminal His-tag, retained in a nickel column and eluted with imidazole. By applying the elution product to a size exclusion chromatography (SEC) column, several peaks were obtained (Figure 3.3-A); the fractions of each peak were pooled and analyzed by SDS page and Liquid Chromatography/Mass Spectrometry (LC/MS), which gave insight on the different species present in each peak. The SDS-page indicated that the first peak contained two species, with sizes corresponded to a “USH3” form and NMT respectively; the second peak only a “USH3” form; and the last peak a “USH3” form but also a smaller product. LC/MS clarified which were the actual species:

- The two species of peak 1 were separated by LC (Figures 3.4-A), and one species was found by MS analysis in each peak: a mass of 16628 Da corresponding to MyrUSH3, and a mass of 48140 Da corresponding to NMT. Figures 3.4-B and -C. This indicates that the protein, under solubilization during the sonication step, probably rebound to the enzyme.

- LC of peak 2 gave rise to a single elution peak (Figures 3.4-D), which actually contained several species as revealed by the MS analysis. A first mass of 16432 Da was found, which corresponds to USH3 with the first methionine processed (Figures 3.4-B), but without further acylation. The second mass of 16600 Da corresponds to myristoylated species missing 28 Da. (Figures 3.4-E). This mass is compatible with that of MyrUSH3 having lost two carbons in the acyl chain: we may observe the N-terminal incorporation of lauric acid. The corresponding species would be Laurylated USH3 (LaurUSH3).
- LC of peak 3 gave rise to a single elution peak Figures 3.4-F), containing mainly a molecule of 10542 Da Figures 3.4 G). The SDS-page of the fractions showed the presence of “USH3” form, but no corresponding mass was detected by LC/MS analysis.

Figure 3.5 shows a similar outcome when expressing and purifying the UGFP construct. The SEC profile contained only two peaks (Figure 3.5-A). Indeed, the molecular weight of GFP is much higher than that of SH3 and did not enable to achieve as much separation as for the USH3 construct. The SDS-page (Figure 3.5-B) showed that peak 1 contained two species, while peak two contained a “UGFP” form and a smaller product.

- The two species of peak 1, separated by LC (Figures 3.6-A), contained one specie in each peak: MyrUGFP (37889 Da¹ and NMT (48140 Da) – Figures 3.6-B and -C.
- MC/LS from peak 2 only enabled to detect a smaller product of 31517 Da Figures 3.5 C). The SDS-page of the fractions showed the presence of UGFP, but no corresponding mass was detected by LC/MS analysis.

This result points out several difficulties arising from such a method: MyrUSH3 or MyrUGFP remain intact upon solubilization if they are complexed with NMT, nevertheless they seem to undergo degradation in absence of “protection”. Degradation is treated in a separate part of this chapter (Part 3.5). Obtaining a pure myristoylated sample means that the protein must be further separated from NMT.

¹The experimental mass of UGFP is 22 Da lower than its theoretical mass because of chromophore maturation (See part 5.3). The mass of non processed UGFP is 37828 Da, that of unprocessed MyrUGFP is 39707 Da.

As previously said, NMT can accept different cofactor substrates, especially cofactors possessing shorter acyl chains. Since, MyrCoA is also used in the β -oxidation pathway of the bacteria as one of the carbon sources to produce energy, degradation of MyrCoA into Laurylated CoA (LaurCoA) can take place if this pathway is initiated. The pool of LaurCoA created is ultimately used by NMT. Further evidences of the presence of laurylated species through this process are found in the subsequent parts of this chapter. It is interesting to note that NMT preferentially rebinds to MyrUSH3, which explains why the laurylated species are separated in peak 2 of the SEC.

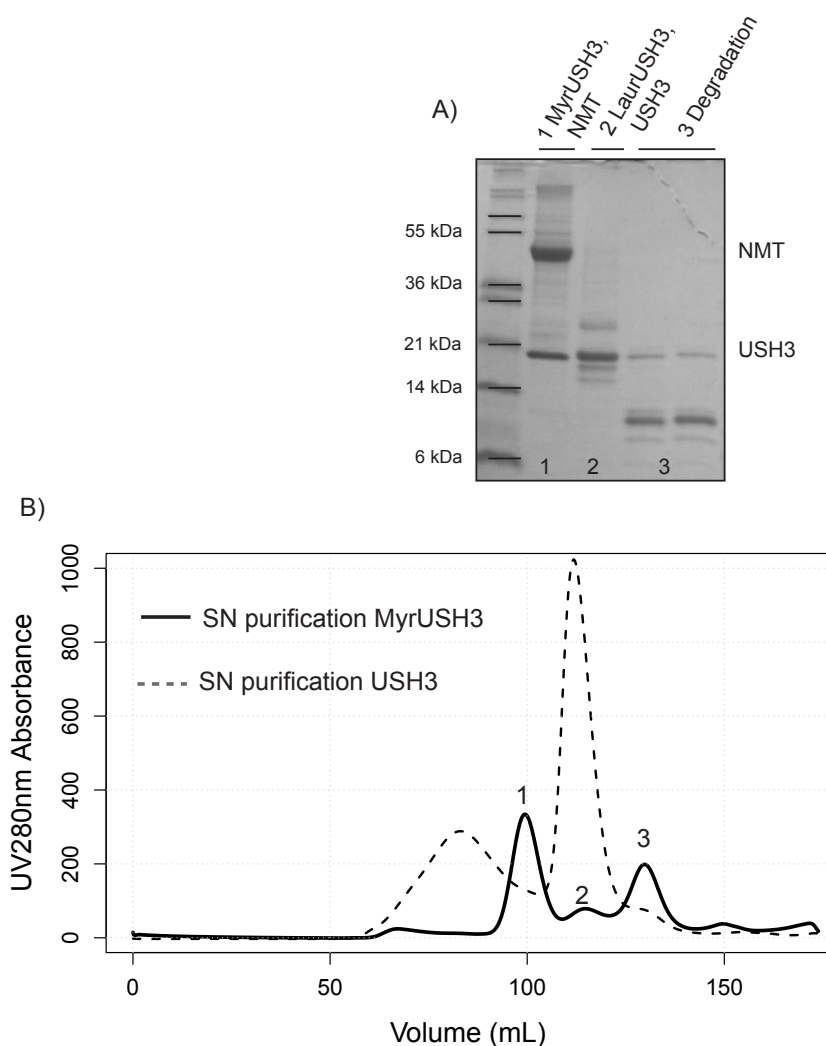


Figure 3.3: *USH3* classical purification. A) SEC profile B) SDS-page of the 3 peaks obtained in the SEC.

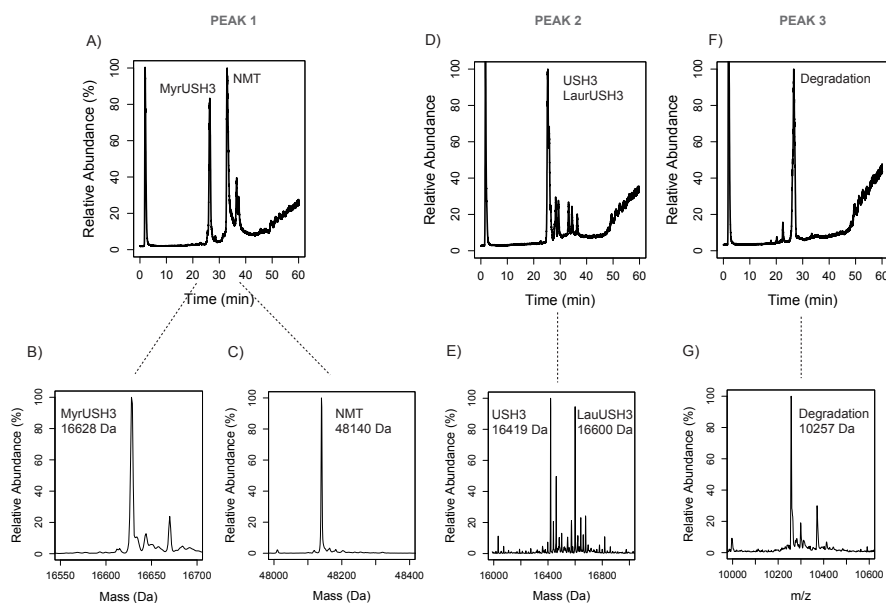


Figure 3.4: *USH3* classical purification. LC/MS of the species obtained in the 3 different peaks of the SEC profile, as shown in Figure 3.3 A. A) LC of peak 1. B) and C) MS of the two species present in peak 1. C) LC of peak 2. D) MS of the species present in peak 2. E) LC of peak 3. F) MS of the species present in peak 3.

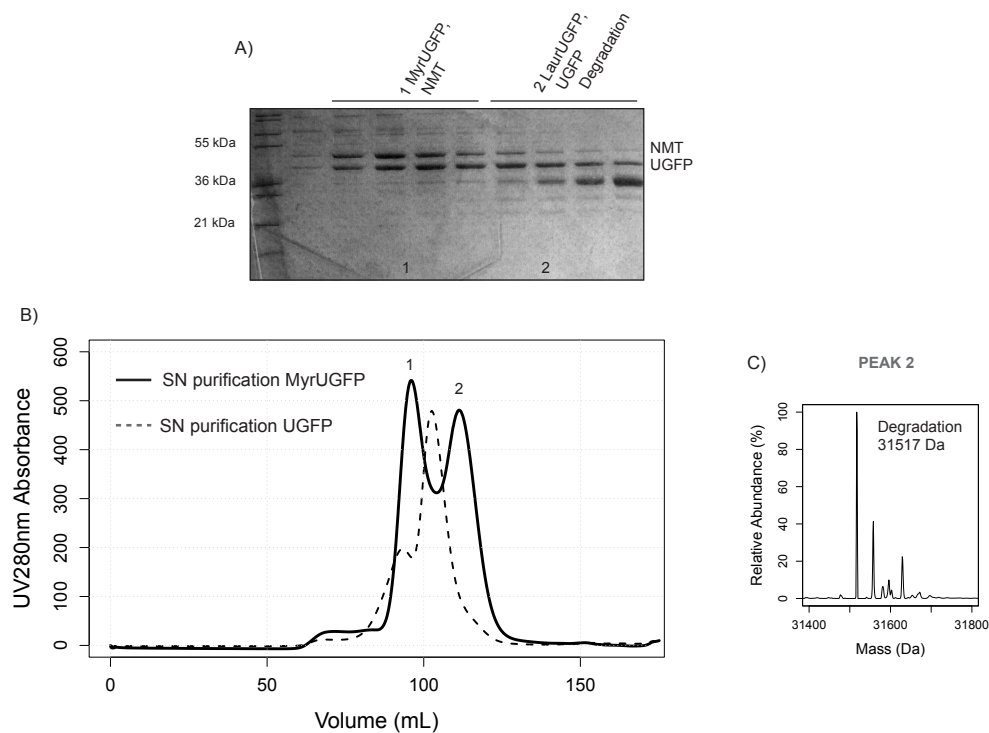


Figure 3.5: *UGFP* classical purification. A) SEC profile B) SDS-page of the fractions obtained in the SEC C) MS of the species present in the second peak of the SEC.

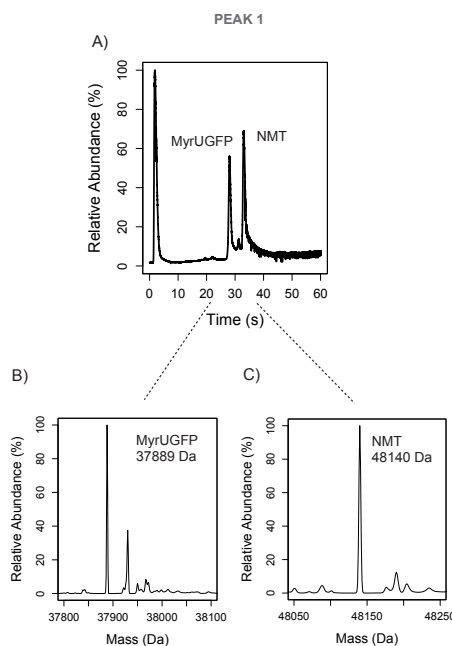


Figure 3.6: *UGFP classical purification. A) LC of peak 1 from the SEC profile of Figure 3.5 A. B) and C) MS of the species present in peak 1.*

Complex separation methods The first peak eluting in the SEC contains the myristoylated protein, together with NMT. The fractions were pooled and attempts have been made to separate the NMT-protein complex. Here are listed some methods in which partial success occurred.

Reverse phase chromatography Reverse phase chromatography successfully achieved enzyme denaturation and complex separation. The data obtained from MC/LS presented previously (Figure 3.4-A and Figure 3.6-A) shows the separation of the myristoylated proteins from the enzyme by HPLC. To further recover the protein sample, the elution fractions of MyrUSH3 or MyrUGFP should be collected, and a subsequent refolding step applied. It might be simple when applied to the SH3 domain, but would not work in case of the GFP domain. Thus this method did not constitute a satisfactory solution.

Hydrophobic Interaction Chromatography (HIC) The complex was applied to an hydrophobic matrix in presence of a high concentration of salt; hydrophobic molecules, depending on the matrix, bind to the column and the non hydrophobic species are not trapped. After trial of several hydrophobic matrices, a isopropyl matrix was chosen and

protein was injected in a 1.5 M ammonium sulfate containing buffer. Resolubilization was achieved with a gradient of salt free buffer. Figure 3.7-B shows the HIC profile obtained when applying steps of decreasing salt concentration. Several elution peaks were obtained, as illustrated in the SDS-page of Figure 3.7-A; the first peak contained NMT, the second still contained the complex, and the last one contained the protein alone. The procedure was also applied to MyrUSH3 with a gradient of decreasing salt. The fractions containing mainly MyrUSH3 still contained some enzyme (first three columns of the SDS-page in Figure 3.7-B). By adding triton as a detergent in the solubilization buffer, the throughput and purity were improved, as demonstrated in the last two rows of the SDS-page of Figure 3.7-B; no trace of NMT was found. After elution and collection of the fractions, the ammonium sulfate, and the Triton if it is the case, had to be removed from the sample, e.g. by dialysis. This separation protocol was only partially successful since some proteins remained complexed; the yield of pure proteins was pretty low and the overall protein purification was very time consuming.

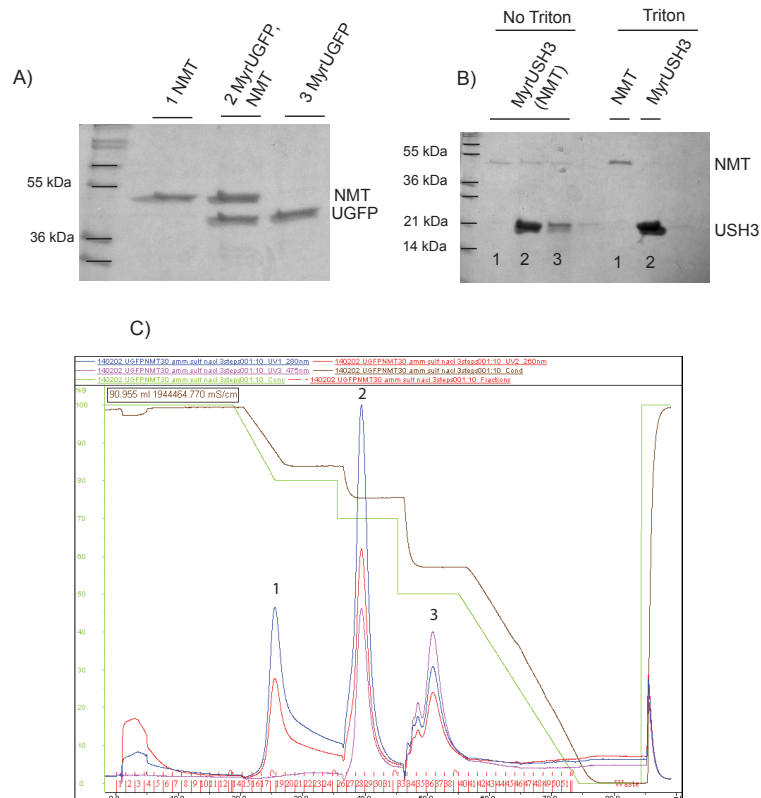


Figure 3.7: SDS-page of the eluted fractions A) and B) HIC profile from the elution of the MyrUGFP-NMT complex. C) SDS-page of the eluted fractions of MyrUSH3-NMT applied to the HIC

Competition with MyrCoA Another method already used by others (Morgan et al., 2011) consisted in using MyrCoA as a competitor. The NMT/protein complex was rebound to the nickel resin, and MyrCoA was applied; as a consequence, a substantial amount of NMT was eluted as a pure product, as shown in the SDS-page of Figure 3.8-A, line 2 to 4. After extensive washing with the MyrCoA solution, the protein was eluted with imidazole, but still contained enzyme contamination (Same gel, Figure 3.8-A, line 5). The same protocol was applied to MyrUSH3 and rendered similar species, pure NMT and non pure MyrUSH3 (same gel, lines 6 and 8). To further purify the MyrUSH3 protein, the product eluted with imidazole was injected in SEC and led to a small amount of pure protein, accompanied with degradation, as shown in the SEC profile and SDS page of the corresponding fractions shown in Figure 3.8-B and -C. This method is costful, time consuming, with low sample yield.

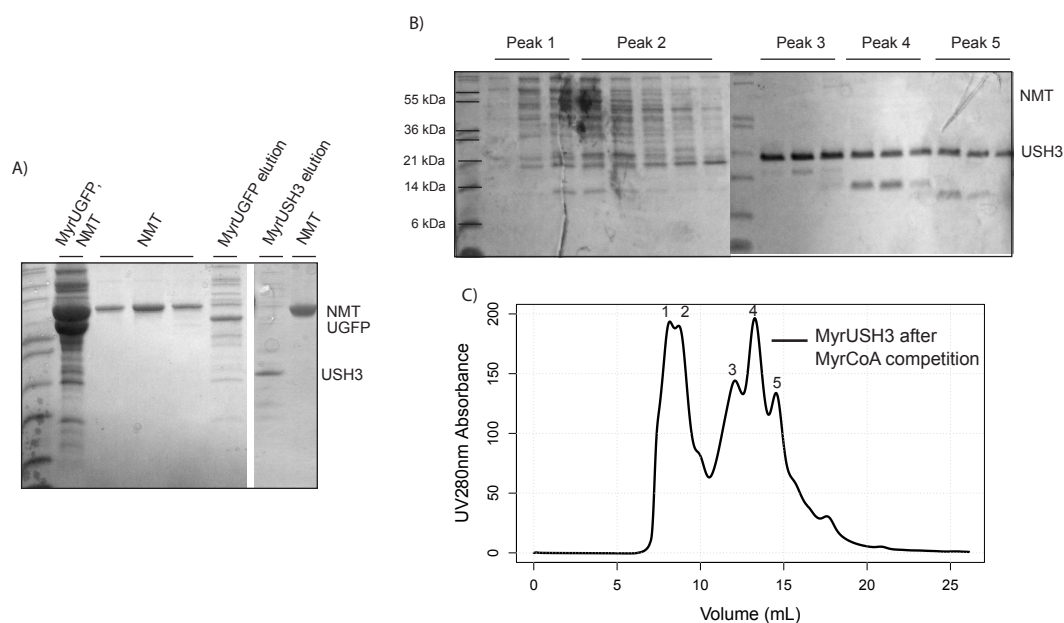


Figure 3.8: A) SDS page of the products obtained during the MyrUGFP or MyrUSH3 separation from NMT by MyrCoA competition, as described in the text. B) SDS-page and C) SEC profile of the non pure MyrUSH3 applied to SEC after MyrCoA competition.

3.2.3. “Triton wash” purification

Extraction from Pellet To overcome the separation issue, another strategy was adopted. Advantage was taken of the binding of myristoylated proteins to membranes: the bacterial pellet (obtained after lysis and centrifugation) was used for further protein extraction. A substantial amount of acylated proteins remained in the lipid phase after lysis and centrifugation. Thus, the supernatant was discarded and the pellet was resuspended in buffer supplemented with 1 % Triton X100. After centrifugation of the resuspended pellet, the supernatant was free of NMT – which had been eliminated in the discarded supernatant – and contained acylated proteins. By repeating the triton wash cycle several times, as much protein as possible was recovered. The proteins were subsequently bound to the nickel resin and Triton X100 could be washed away by passing an extensive amount of buffer. After washing and elution with imidazole, the product was further purified by SEC. This method will be designated as “Triton wash” purification.

Figure 3.9-A, red curve, presents the SEC profile after such a protocol. Two main peaks were obtained; LC/MS analysis reveal that they were pure (Figure 3.9-B and -D) and that they corresponded to the two different acylated species, LaurUSH3 3.9-C and MyrUSH3 3.9-E. With this method, MyrUSH3 and LaurUSH3 could be easily separated by SEC; surprisingly, the laurylated species were eluted with an apparent size higher than that of myristoylated species. NMT was successfully excluded with this method. However, some degradation was still present and eluted in the same fractions as the myristoylated proteins, as illustrated in the SDS page of the SEC fractions (Figure 3.9 B) and MS analysis (Figure 3.9 E).

Though acylated species could be separated by size in the case of USH3, it may not be the case for other acylated proteins, to which similar heterogeneous acylation may occur. In addition, in the case of too high amounts of laurylated forms, the separation was not successful anymore. Figure 3.9 A, green curve, shows a case in which separation was not satisfactorily achieved. Three peaks were obtained, and the two acylated species were mixed. More dramatically, when proteins are expressed in minimum media, this protocol rendered almost only degraded or laurylated species. Since we suggest that laurylation comes from MyrCoA degradation into LaurCoA, several relevant parameters were explored in order to optimize the myristoylation yield.

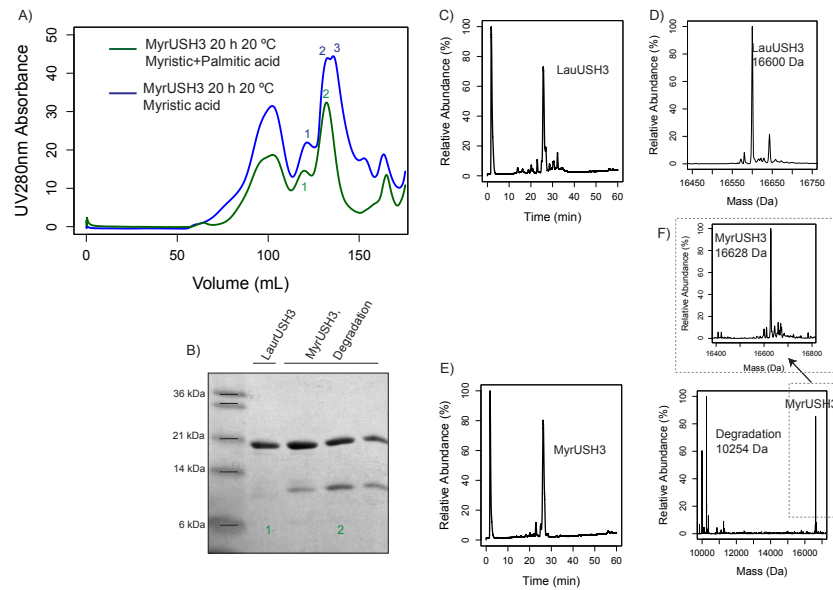


Figure 3.9: A) SEC (Red and Green curves, low and high amount of laurylated species, respectively) and B) SDS page of the corresponding fractions after the “Triton wash” purification of MyrUSH3, red curve. C) LC and D) MS of peak 1 of the SEC, red curve. E) LC and F) MS of peak 2 of the SEC.

Optimization of the conditions of MyrUSH3 expression in rich medium Different parameters were varied to verify their contribution to the type of acylated product obtained, with the final aim of achieving samples containing proteins free of laurylation products. The expression conditions were first optimized in rich medium. Three parameters were explored.

- The cell line; another protein, BASP, was not found to be laurylated when expressed in rich medium, when using T7 cells instead of Rosetta cells. Thus T7 cells were tested to MyrUSH3 expression.
- The addition of another acyl chain, palmitic acid. As discussed in the introduction, NMT can use shorter acyl chains as substrates, but Palmitoyl Coenzyme A (Palm-CoA) is not binding the enzyme; however, PalmCoA may be degraded to MyrCoA in the β -oxidation cycle. The addition of palmitic acid is expected to increase the pool of MyrCoA in the event the cell uses the β -oxydation pathway as mean of energy supply.

- The duration of the expression. This duration is highly variable according to the expression protocol, usually optimized for the particular protein to be produced. Some proteins can be expressed using a short time at high temperature (for instance 4 h at 30 °C), but many proteins need milder conditions (for instance overnight at 20 °C).

These parameters were varied during the expression of MyrUSH3. Expression was done in comparable conditions in the two *E. coli* lines Rosetta and T7. Expression was also always done in presence of myristic acid, to which palmitic acid was added or not. The duration after induction was either 5 h at 28 °C or 20 h at 20 °C. After purification and size exclusion chromatography, the relative amount of the two acylated species was assessed. The results from all the conditions are gathered in table 3.1. The relative amount of myristoylated and laurylated USH3 were determined by integrating the elution peak from the size exclusion chromatography performed on the mixture obtain after the Triton wash purification. The main trends are detailed and exemplified below.

- The cell line had a certain influence. Figure 3.10-A compares the SEC profiles obtained in T7 or Rosetta cells with otherwise similar conditions. The laurylation peak was reduced in T7 cells. In general, laurylation was a bit reduced in T7 cells. Rosetta cells are optimized for rare codons, and the expression of NMT and USH3 may be faster, leading to a faster exhaustion of the energy sources and initiation of β oxidation pathway.
- Addition of palmitic acid in the medium significantly improved the myristoylation yield. Figure 3.10-B compares the SEC profile obtained when palmitic was supplemented to the medium or not during expression. The laurylation was significantly reduced when palmitic acid was use. This results strengthen the hypothesis of LaurCoA coming from MyrCoA degradation in the β -oxydation pathway.
- Expression duration was crucial. Figure 3.10-C compares the outcome from a short (5 h) versus long (20 h) expression. The amount of laurylated species dramatically increased in the long expression time experiment. Long expression time also mean exhaustion of the carbon sources contained in the medium; this result also reinforces the hypothesis under which degradation of MyrCoA in the β -oxydation pathway generates LaurCoA species. Short expression is a key factor to prevent laurylation.

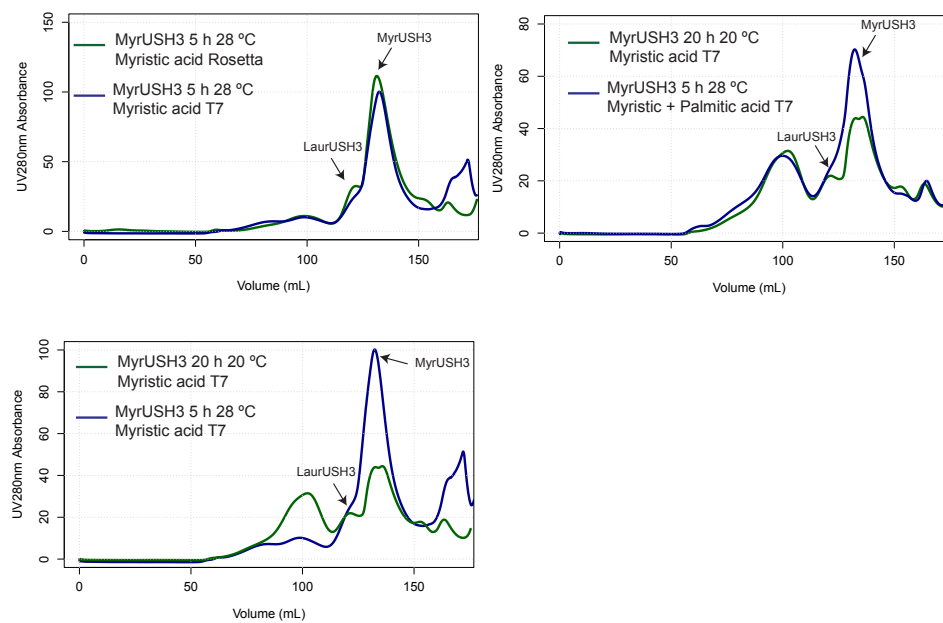


Figure 3.10: SEC from the Triton wash purification of MyrUSH3 A) varying the cell line: T7 (blue curve) or Rosetta cells (green curve) were used; expression for 5 h at 28 °C with myristic acid supplemented. B) varying the additive: Myristic only (green curve) or myristic + palmitic acid (blue curve) were added; expression for 20 h at 20 °C in T7 cells. C) varying the expression duration: 20 h at 20 °C (green curve) or 5 h at 28 °C (blue curve), expression in T7 cells with myristic acid supplemented only.

Table 3.1: Expression factors and their effects on the relative amounts of myristoylated and lauroylated USH3 obtained after Triton wash purification.

Cell Type	Additive	Time	LaurUSH3	MyrUSH3
Rosetta	Myristic acid	5 h 28 °C	20 %	80 %
T7	Myristic acid	5 h 28 °C	20 %	80 %
Rosetta	Myristic + Palmitic acid	5 h 28 °C	5 %	95 %
T7	Myristic + Palmitic acid	5 h 28 °C	2 %	98 %
Rosetta	Myristic acid	20 h 20 °C	80 %	20 %
T7	Myristic acid	20 h 20 °C	65 %	35 %
Rosetta	Myristic + Palmitic acid	20 h 20 °C	18 %	72 %
T7	Myristic + Palmitic acid	20 h 20 °C	3 %	97 %

Altogether, using a short expression (5 h at 28 °C) and supplementing the medium with myristic and palmitic acid enabled to obtain high myristoylation yields. Figure 3.11 and 3.12 show SEC profiles (A), SDS-pages (B) of the fractions in the peak, and LC/MS analysis (C and D) of the pooled fractions, corresponding to the expression and purification of USH3 or UGFP in these conditions. A mass of 16628 Da was obtained for MyrUSH3 and 37888 Da for MyrUGFP. In both cases, some degraded molecules were also present.

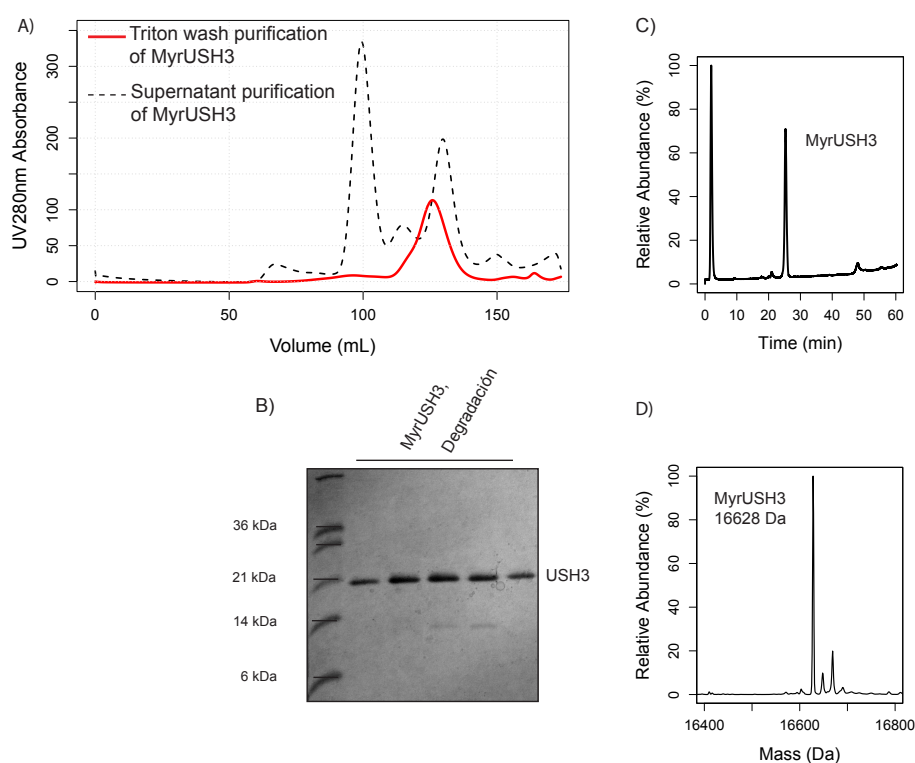


Figure 3.11: A) SEC profile (red curve) after the “Triton wash” purification of MyrUSH3 expressed in optimal conditions, and B) SDS page of the corresponding fractions. C) LC and D) MS of the SEC, pooled fractions from the unique peak of the red curve.

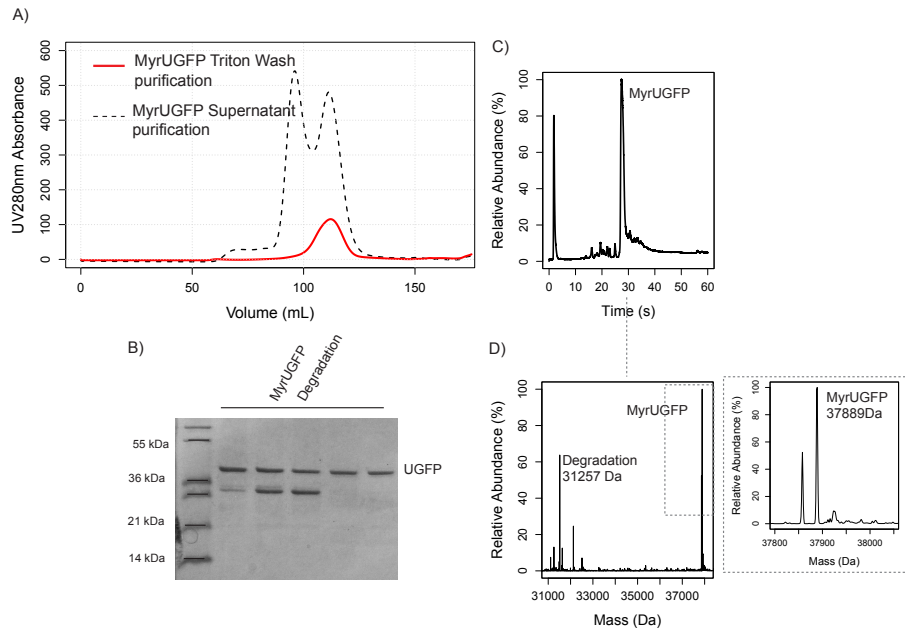
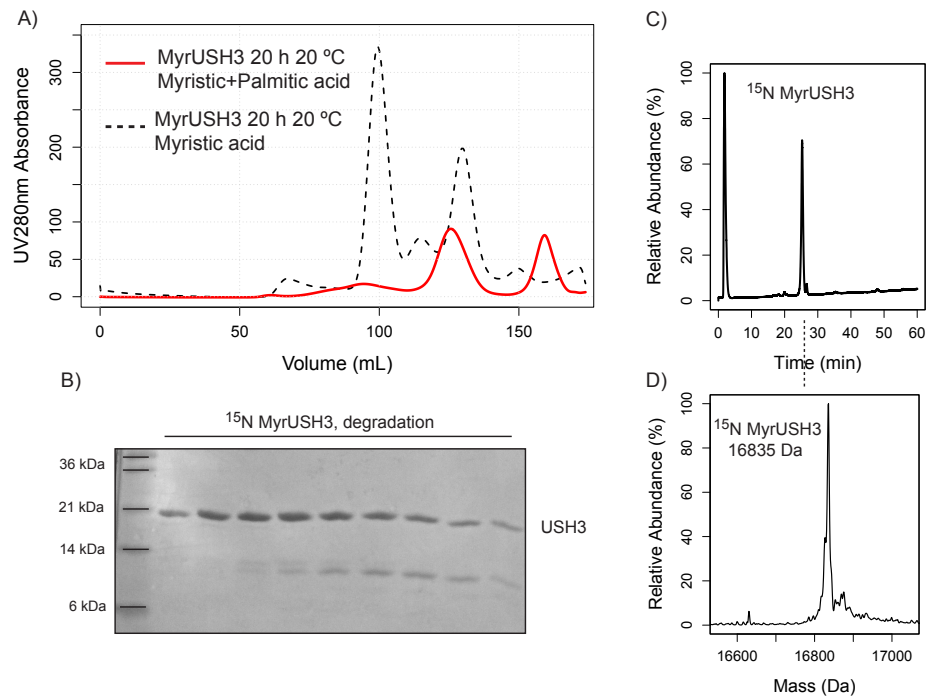


Figure 3.12: SEC profile (red curve) after the “Triton wash” purification of MyrUGFP expressed in optimal conditions, and B) SDS page of the corresponding fractions. C) LC and D) MS of the SEC, pooled fractions from the unique peak of the red curve

Optimization of the conditions in minimum medium Applying the “optimum conditions” to protein expression in minimum medium failed to render MyrUSH3, most probably because of the insufficiency of the energy sources in such a growth medium. To compensate for the very limited carbon sources, the Marley method (Marley et al., 2001) was used, which consisted in generating cell mass in rich medium that was subsequently transferred to a labeled minimum medium, just before induction. The quantity of glucose was also increased.

Figure 3.13 A and B show the SEC profile and SDS-page of the purified MyrUSH3 fractions after expression in minimum medium using the Marley method and the previously optimized conditions.

^{15}N labeling implies that the mass of the protein is larger; since basal expression in rich medium during cell growth may occur, a small part of the proteins may be unlabeled. The theoretical mass of fully labeled MyrUSH3 is 16842 Da, and LC/MS analysis rendered a mass of 16835 Da, 7 Da smaller (Figure 3.13-C). This indicated that probably about 95 % of the population was ^{15}N labeled. In consequence, despite of the basal expression, this method generated a sufficient amount of ^{15}N labeled myristoylated proteins.

Figure 3.13: *MyrUSH3* minimum medium

In fact, it would also be valuable to apply this method in rich medium, especially in case of need for long expression times.

3.3. Importance of using the correct acyl chain length

As introduced in the beginning of this chapter, despite of the very small difference between myristoyl and lauryl (14 carbons and 12 carbons respectively), working with the incorrect acyl chain may affect the binding behavior of the species under study. Thus, binding of myristoylated versus laurylated proteins to vesicles or liposomes was explored in a Lipid Sedimentation Assay (LSA), or in Surface Plasmon Resonance (SPR) experiments.

Even in a very simple assay such as a LSA, a strong difference in the binding to vesicles of the two acylated species was striking. The SDS page of Figure 3.14 compares binding of MyrUSH3 and LaurUSH3 to DMPC:DMPG 2:1 vesicles. All MyrUSH3 proteins were in the pellet fractions, while part of them remained in the supernatant in case of LaurUSH3. In the MyrUSH3 lines, degradation was present and remained in the supernatant, since it is not myristoylated – degradation is not present in LaurUSH3 since it eluted with an apparent size close to that of MyrUSH3.

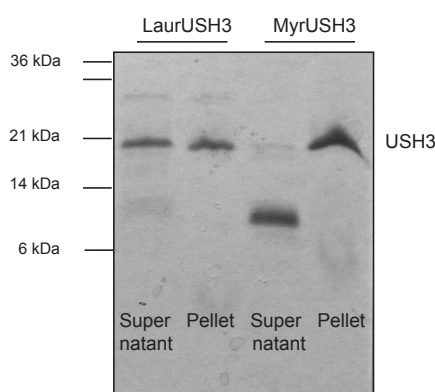


Figure 3.14: *SDS page after a LSA of MyrUSH3 or LaurUSH3 binding to DMPC:DMPG 2:1 MLVs.*

In the SPR assay, binding to liposomes of two laurylated and myristoylated forms of two different proteins (USH3 and BASP) was compared. In Figure 3.15-A, binding to DMPC:DMPG 2:1 liposomes of LaurUSH3 and MyrUSH3 is compared; LaurUSH3 showed significantly reduced binding when compared with MyrUSH3; a 20 μ M injection of LaurUSH3 gave a binding response comparable with that of a 1 μ M MyrUSH3 injection, and had a four times reduced level compared with a 20 μ M MyrUSH3 injection. Laurylated BASP (LaurBASP) also showed a reduced binding compared with Myristoylated BASP (MyrBASP) (Figure 3.15-B), but in a more subtle manner than for USH3. MyrBASP

also attaches to membranes via a dual myristoylation and PCD signal, but formation of an alpha helix upon membrane binding probably balances the effect of the acyl chain, which role might be mainly membrane targeting.

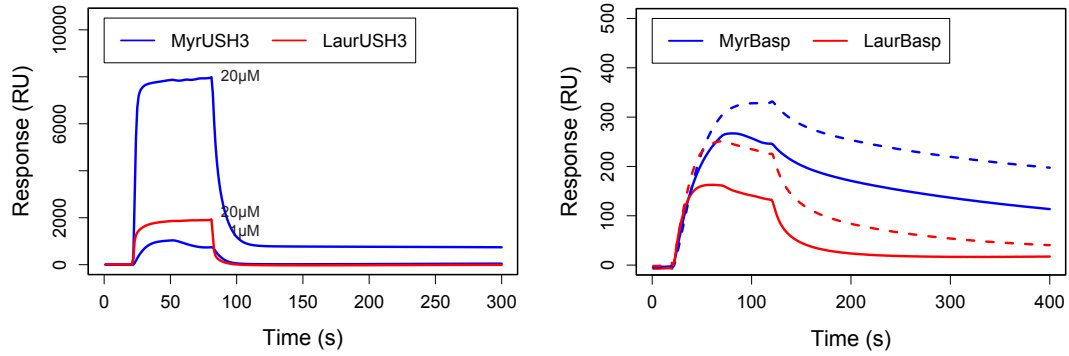


Figure 3.15: *SPR assay monitoring A) the binding of MyrUSH3 and LaurUSH3 to immobilized liposomes of DMPC:DMPG 2:1, monitored by SPR. B) the binding of MyrBASP and LaurBASP to immobilized liposomes of DMPC:PS 3:1 (plain lines) or DMPS:PS 3:1 supplemented with 2 %PI(4,5)P2 (dashed lines).*

3.4. In-vitro myristoylation

Another method leading to pure myristoylated samples is to perform the enzymatic reaction “in-vitro” by mixing the three species previously purified. This procedure was used for the production of MyrUSrc, for which the coexpression method had failed.

The components were obtained separately:

USrc The recombinant USrc protein was obtained by standard methods. Proteins were expressed in *E. coli* and purified with a streptactin resin recognizing a streptag added at the C-terminal of USrc. Figure 3.16-A shows the SDS-page from the fractions of USrc peak eluted after final purification by SEC.

NMT NMT was recovered from the MyrCoA competition assay, as explained in the competition assay of Part 3.2.2. The enzyme itself could be obtained as a tag-free, pure sample, confirmed by the SDS page, but also by MS analysis. Figure 3.16-B shows the SDS-page corresponding to a pure NMT sample.

MyrCoA CoA was chemically coupled to myristoyl as described in [Gaffarogullari et al. \(2011\)](#). Myristic acid was first activated by carboxydiimidazole, and subsequently coupled to CoA in a NaCO₃ buffer. Figure 3.16-C shows the mass analysis by MALDI of the MyrCoA product. A mass of 977 Da, corresponding to MyrCoA, was dominantly measured.

The myristoylation reaction consists in mixing the three components, USrc, MyrCoA and NMT, as illustrated in Figure 3.17-A. NMT was first incubated with an excess MyrCoA. Then an excess protein (relative with NMT, but not with MyrCoA), was added and the reactants were incubated 2 h at 37 °C and subsequently overnight at 4 °C. A simple assay to verify if the reaction had been achieved consisted in a LSA. USrc or MyrUSrc were incubated with DMPC:DMPG 2:1 MLVs. After incubation and ultracentrifugation, the supernatant and the pellet were separated and loaded in an acrylamide gel. Figure 3.17-B shows a SDS page of a LSA performed before and after the in-vitro myristoylation of USrc. In case of USrc, all proteins remained in the supernatant (lines 1 and 2), while in case the myristoylation reaction product, a higher amount of USrc was in the pellet compared with the supernatant (line 3 and 4) . The proteins present in the pellet correspond to the myristoylated species, the ones in the supernatant to non-reacted USrc species. NMT

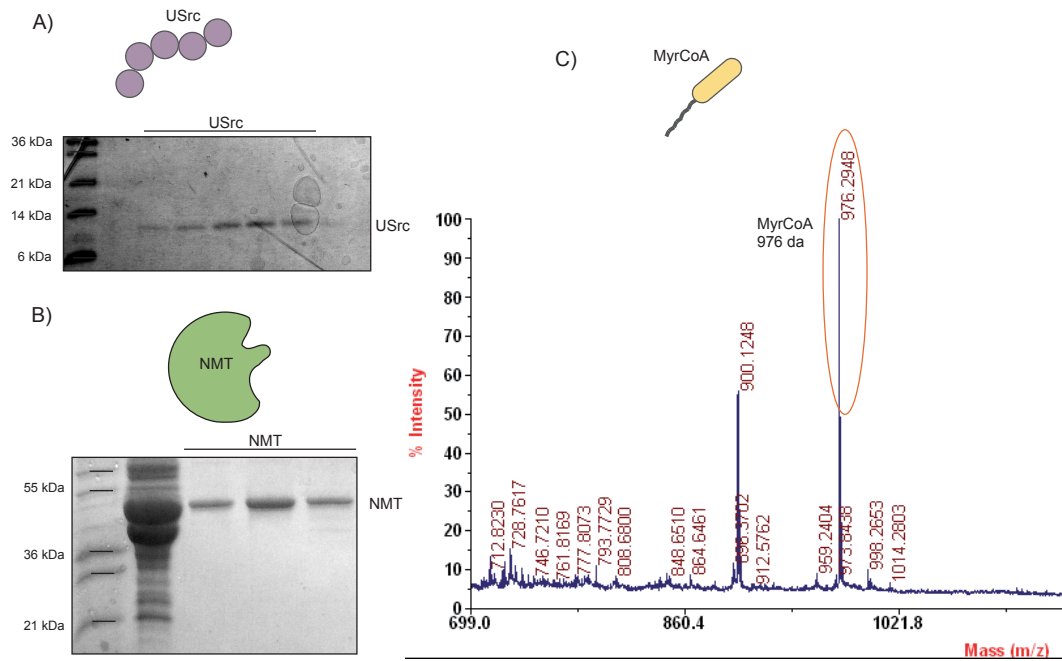


Figure 3.16: A) SDS page of the fractions eluted in the SEC after final purification B) SDS page of the NMT sample obtained in the MyrCoA competition assay described earlier. C) MS analysis by Maldi of MyrCoA.

was also found in the supernatant. This simple assay indicated that the myristoylation reaction was successful, with some unreacted species. Further LC/MS analysis confirmed these results. Figure 3.18-A and -B show the LC and MS results of USrc before the reaction, confirming a mass of 9481 Da. Figure 3.18-C shows the LC of the sample after the reaction MS analysis: three species were separated, USrc, MyrUSrc and NMT. The correct mass of MyrUSrc was confirmed by the MS analysis, 9691 Da (Figure 3.18-D). To separately collect the three species from the reaction product, a C18-LC column was used with a 0-80 % H₂O-acetonitrile. Figure 3.17-C shows that the three species, USrc, MyrUSrc and NMT were separated in the liquid chromatography. MyrUSrc was collected and lyophilized, and subsequently resuspended in the correct buffer.

The methodology was also applied to USH3, and produced a partially myristoylated USH3 sample. Figure 3.19-A shows the LC of the reacted products: The first peak corresponded to USH3 (See MS from Figure 3.19-B) while the second corresponded to MyrUSH3 (See MS from Figure 3.19-C). NMT was detected in the last peak. The reaction was partial, and implied further lyophilization and refolding of the MyrUSH3 product. This method was not used because of the much more straightforward use of coexpression

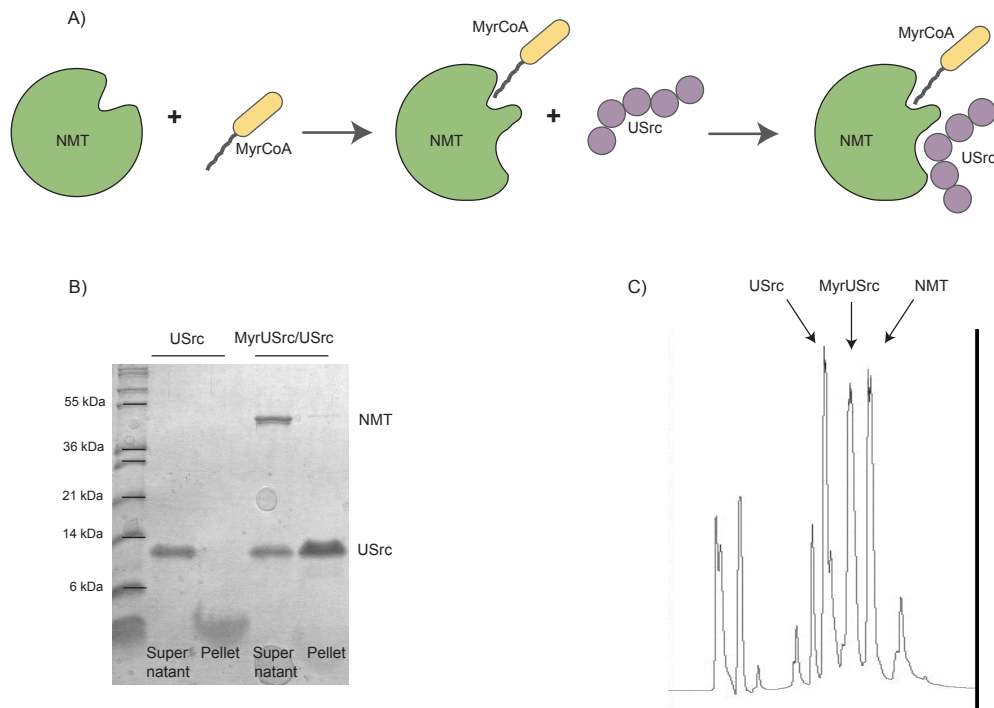


Figure 3.17: A) Scheme of the in-vitro myristoylation reaction B) SDS page after a LSA of the reacted myristoylation product with DMPC:DMPG 2:1 MLVs. C) Separation of the different species in the myristoylation product, using a C18-LC column.

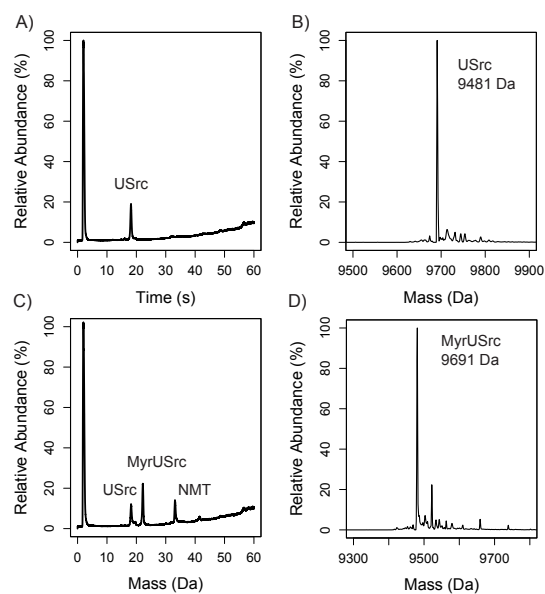


Figure 3.18: A) and B) LC and MS of USrc before the myristoylation reaction C) LC of the reacted myristoylation products D) MS of MyrUSrc present in the second peak of the LC.

methods in optimized conditions.

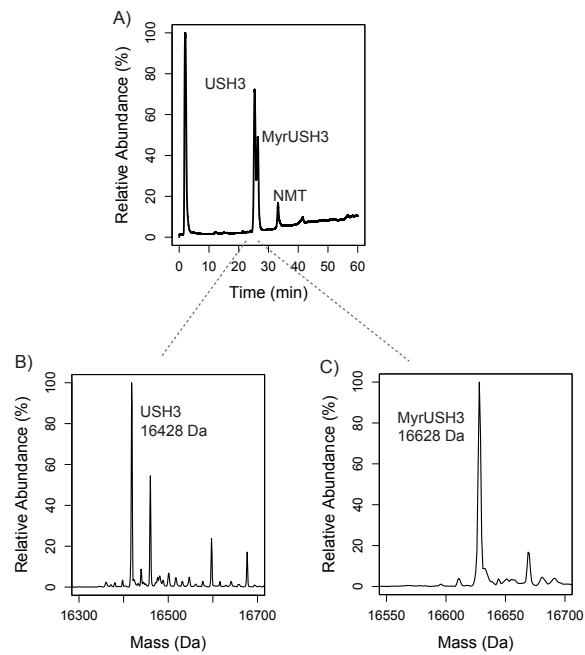


Figure 3.19: A) LC of the reacted products in *USH3* myristoylation B) MS of peak 1 separated by LC C) MS of peak 2 separated by LC.

3.5. Degradation

Degradation: where?

Upon expression of MyrUSH3 or MyrUGFP, a fraction of a degraded product was found. This degradation product was not a generalized protein degradation, but consisted in a well-defined species, as indicated by SDS-page gels of the fractions (Figure 3.3-B or 3.5-B). Since we collected the elution products after a nickel column step, we only obtained a product containing the C-terminal His-Tag. Further characterization of the degradation-containing sample was achieved by performing on one hand a LSA, and on the other hand a western blot with an antiSH4 antibody secondary detection. The LSA clearly showed that the degraded form was not binding to vesicles (Figure 3.20-A), therefore did not contain the myristoyl part. The Western Blot confirmed that the degradation was not detected by the antiSH4 antibody, thus degradation lacked the N-terminal part of the protein (Figure 3.20-B). In consequence, the purified degradation that contaminates our samples is thus a non-myristoylated C-terminal fragment of USH3.

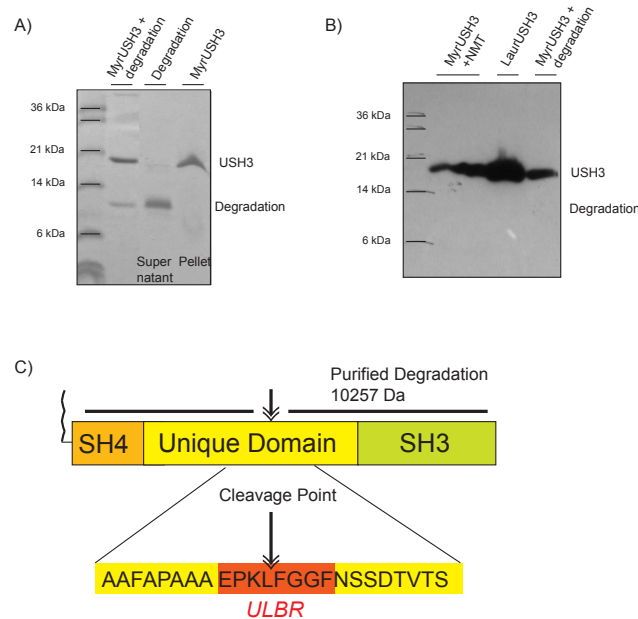


Figure 3.20: A) SDS page before (line 1) and after (line 2 and 3) a LSA of MyrUSH3 binding to DMPC:DMPG 2:1 MLVs B) Western Blot of MyrUSH3 fractions, containing degradation, with antiSH4 antibody detection C) Schematic representation of the cleavage site leading to the degraded MyrUSH3 product.

MS analysis of the degraded sample from MyrUSH3 purification gave a set of masses

that could be related to possible shorter USH3 peptides (Figure 3.4-G). The most abundant mass of 10257 Da exactly corresponded to a peptide starting after lysine 63 until the HisTag, as illustrated in Figure 3.20-C. Therefore, an exact cleavage site exists, and it is probably significant to find that corresponds to the ULBR region. Some additional masses were found in the MS analysis, corresponding to other cleavage points around the main one. The cleavage process observed occurs at a specific amino acid site, but is also found in a minor way in surrounding amino acids.

To further verify the specific amino acid sequence requirement for degradation to occur, a mutant in the ULBR region was expressed. A so-called “A3” mutant (MyrUSH3A3), in which the ⁶⁴LFG⁶⁶ amino acids were replaced with ⁶⁴AAA⁶⁶, was already available as one of the tool used to study the effect of the absence of ULBR in diverse experiments. The mutation replaces the amino acids involved in the cleavage site by alanines. We expressed it in its myristoylated form. Figure 3.21-A compares the SEC profiles of MyrUSH3 and MyrUSH3A3 after purification by the supernatant method (as in Part 3.2.2). The third peak was inexistent in the case of the mutant. The corresponding SDS page of the eluted fractions did not show significant degradation (Figure 3.21-B). This experiment confirms that the degradation takes place at a specific amino acid site and is prevented by modifying the amino acid sequence. Working with this mutant is actually a method to obtain degradation free samples. This mutation also prevented degradation in the MyrUGFP construct, as illustrated in Figure 3.21-C and -D.

When applying the triton wash purification procedure to MyrUSH3 or MyrUGFP (after expression in optimized conditions), a single peak was eluted in the SEC (Figure 3.22-A and 3.23-A). The corresponding SDS page (Figures 3.22-B and 3.23-B) confirmed that these fractions were degradation-free. LC-MS analysis of MyrUSH3A3 confirmed the purity and integrity of the product; a single LC peak was obtained (Figures 3.22-C) rendering a single mass of 16624 Da (Figure 3.23-D).

Mutants screening for neighbouring effects

Degradation might come from proteolysis; nevertheless, protease inhibitors were used during purification, and spontaneous degradation of MyrUSH3 was observed in some instances. Thus a set of mutants in the region adjacent to the ULBR was developed, in an attempt to search for other amino acids that could be responsible for the chemical

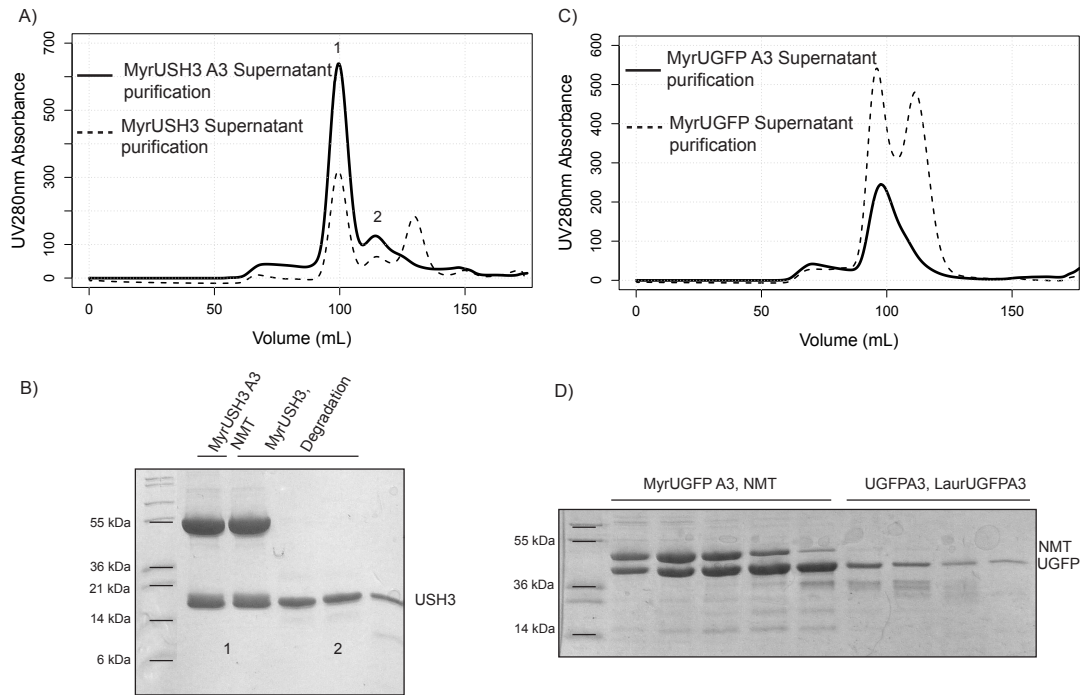


Figure 3.21: *SEC and MS after the supernatant purification A) and B) of MyrUSH3A3, C) and D) of MyrUGFPA3*

proteolysis. We focused on the stretch of polar residues following the ULBR under the hypothesis that proximity of the reactive side chain of aspartic, arginine or serine may promote cleavage. Table 3.2 lists the different mutants used: spanning of the different amino acids next o the ULBR was ensured in this set of mutants. All mutant forms were expressed and purified by the supernatant purification method. None of them showed reduced degradation: both the SEC profile and the SDS page of the corresponding fractions resembled the outcome of that of the wild type (WT). This assay discards the direct chemical role of this region in the degradation phenomena.

Table 3.2: *Mutants around the ULBR of USrc*

Mutant	Amino acid sequence								
USH3 WT	L	F	G	G	F	N	S	S	D
USH3 N68A	L	F	G	G	F	A	S	S	D
USH3 D71A	L	F	G	G	F	N	S	S	A
USH3 N68A D71A	L	F	G	G	F	A	S	S	A
USH3 S69A S70A	L	F	G	G	F	N	A	A	D

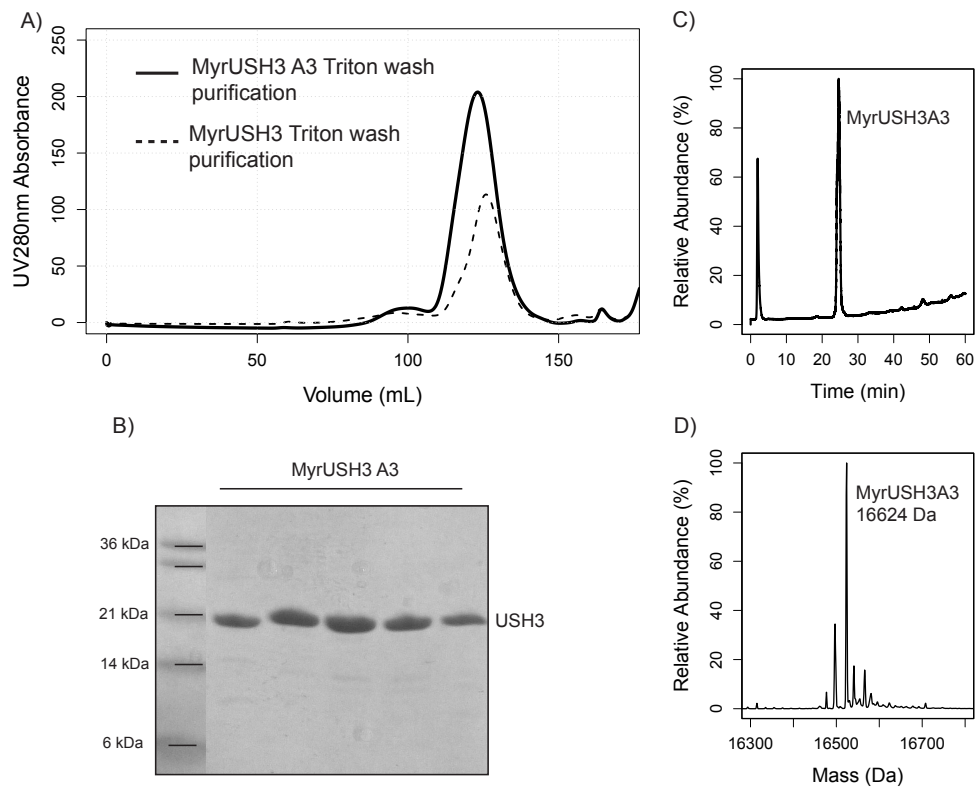


Figure 3.22: A) SEC and B) SDS page of *MyrUSH3A3* by the triton wash purification. C) LC and D) MS analysis of the purified *MyrUSH3A3* fractions.

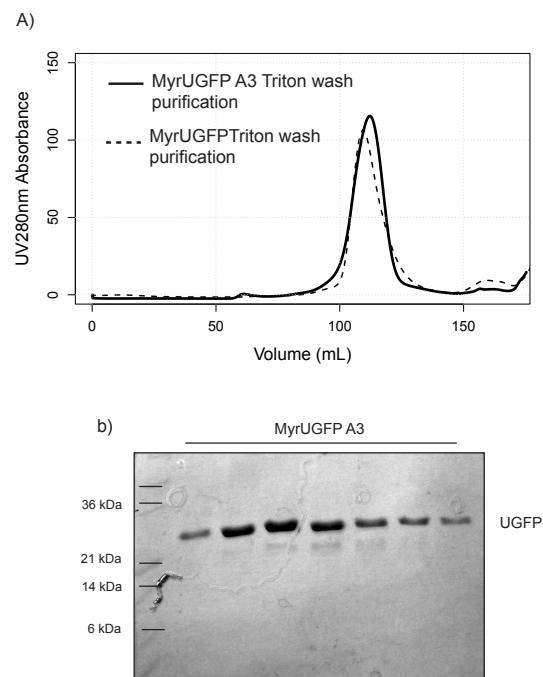


Figure 3.23: A) SEC and B) SDS page of *MyrUGFPA3* by the triton wash purification

Purification of degradation free samples

MyrUSH3 and the degradation products coeluted in the SEC under the standard purification protocol used. Thus, strategies to separate these degradation products from the MyrUSH3 sample have been explored.

pH change

The observation that the SH4 and SH3 domains are interacting in non myristoylated USH3 (Maffei et al., 2015) suggested a possible method to separate MyrUSH3 (where this interaction may still be present) from the degradation products, lacking the SH4 domain.

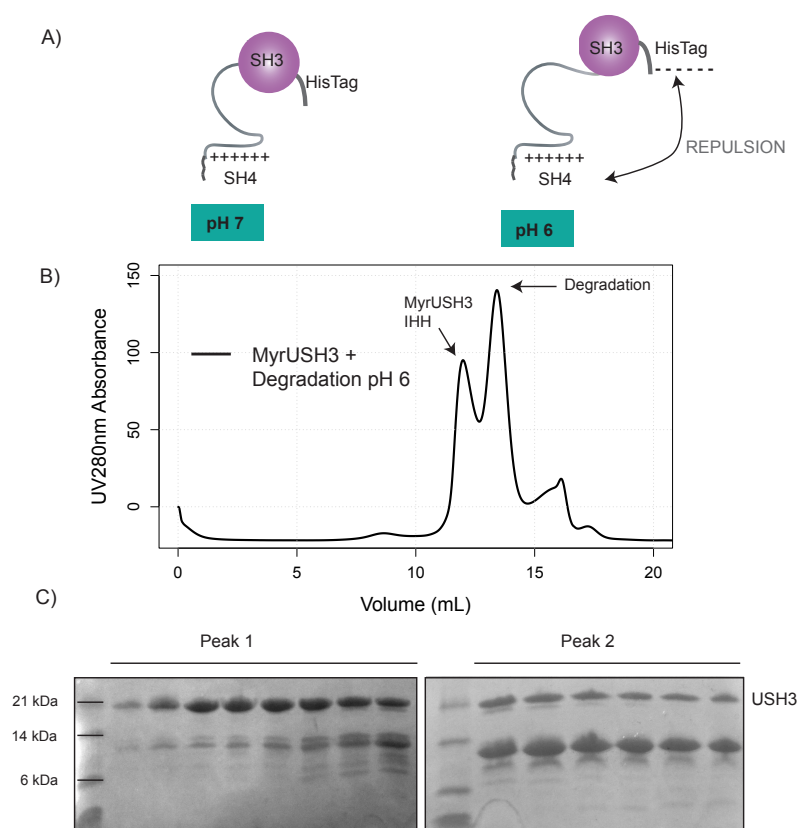


Figure 3.24: A) Schematic representation of MyrUSH3 supposed “conformation” at pH 6 or pH 7 B) SEC and C) SDS page of MyrUSH3 injected at pH 6

In our construct, the C-terminal contained 6 histidines, that are neutral at pH 7.5. By reducing the pH to 6 or lower, the histidine gets protonated. According to Maffei et al. (2015), USH3 is in a “closed” conformation in which the SH3 interacts with the SH4 domain. If the histidine residues get positively charged, they are expected to create electrostatic repulsion with the positively charged SH4 domain which is in turn expected

to favor a more open conformation of MyrUSH3. Figure 3.24-A illustrates the possible conformational change. At low pH, the different apparent sizes between the degraded form and MyrUSH3 may allow separation of the two species by SEC.

A MyrUSH3 sample purified by the triton wash method, which always contained a fraction of degraded forms, was injected in a SEC at a buffer adjusted to pH 6. Two peaks were obtained, instead of a single peak at pH 7. The first peak contained the proteins with a higher apparent size (MyrUSH3 in this case) and the second the protein with a lower apparent size (the degraded form). Figure 3.24 B displays the SEC profile and Figure 3.24 C the corresponding SDSpage of the fractions. This experiment shows that the method is valid, but that perfect separation is not achieved. Optimization of this protocole did not give significant improvement. A lower pH lead to protein destabilization, and lower flow rates did not significantly improve separation. This procedure was not used because of its too low throughput, but gave insights on MyrUSH3 intramolecular interactions.

Imidazole gradient A more effective method was based on a peculiarity occurring during protein elution from the nickel resin when applying the imidazole solution. Imidazole elution is usually very efficient and almost all resin-bound protein species are washed when a reasonable volume of imidazole is passed. It was the not the case for MyrUSH3 or MyrUGFP: large volumes of imidazole solution were needed to elute enough protein, and a lot of protein remained attached to the resin. Increasing the imidazole concentration did elute more proteins but not in a complete manner. This feature might come from the local formation of high affinity clusters of proteins bound to the resin. The benefit of this peculiar behavior was that the non-myristoylated degraded forms of the protein were eluted at low imidazole concentration using a small volume of imidazole solution, therefore could be eliminated. A subsequent increase in the imidazole gradient and resin elution with more buffer enabled to elute degradation free proteins. To collect separately the fractions, a Histrap column was used with an imidazole gradient. The fractions could be collected separately and the protein absorption monitored. In optimized condition, imidazole steps where used instead of a gradient of imidazole. At 200 mM imidazole, all degradation was eluted. At higher imidazole concentrations, the myristoylated protein alone was eluted and collected separately. Figure 3.25-A shows the imidazole steps used, and the elution profile of the protein. Figure 3.25-A shows the corresponding SDS page of the fractions of a MyrUSH3 purification by triton wash. In the first line corresponding

to 200 mM elution, MyrUSH3 was accompanied by a high amount of degradation. Nevertheless, all subsequent fractions, at 400 mM imidazole or more, were degradation free. These fractions were collected; separation of MyrUSH3 from its degradation was achieved by this method.

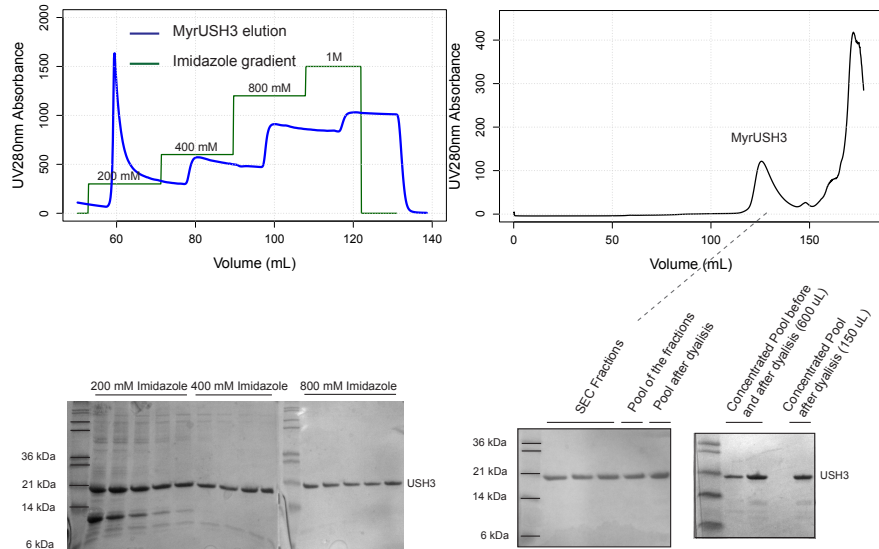


Figure 3.25: A) *Histrap* chromatography column, used with imidazole steps from 200mM to 1 M, used to elute MyrUSH3 extracted with triton. B) SDS page of the eluted fractions. C) SEC of the MyrUSH3 degradation free sample D) SDS pages of MyrUSH3 fractions from the SEC (first gel) and after lyophilization/dyalisis cycles (second gel).

Avoiding concentrators After obtaining a degradation free sample, mainly using the previous method (part 3.5), the obtained volume needed to be concentrated. Use of concentrators is the standard method, but in our case seemed to be able to induce degradation. Indeed, degradation might be a concentration-dependent phenomenon – no direct evidence has been found. The creation of high local protein concentrations may explain why concentrators induce degradation. To avoid the use of concentrators, samples were lyophilized and redissolved in a minimum volume of water given by the solubility of the salts present in the lyophilized product. Imidazole salts were removed by SEC, giving very pure, degradation free fractions (Figure 3.25-C shows the single peak obtained in the SEC and Figure 3.25-D, first gel, the pure MyrUSH3 fractions). If required, further concentration was achieved by lyophilizing the fractions, resuspending them in water, and subsequently removing the excess salt by dialysis. Additional lyophilization/dialysis steps

were performed until the sample was concentrated enough. This method successfully rendered degradation free proteins at sufficient yield. The second SDS page of Figure 3.25-D shows the MyrUSH3 concentrated sample after several lyophilization/dyalysis steps.

Degradation features

Until now, degradation of the myristoylated protein has not been successfully understood and controlled but some interesting features have been observed.

NMT protects the myristoylated protein from degradation As seen in the supernatant purification results, the formation of a complex between NMT and the myristoylated protein happened during proteins resolubilization in the cell lysis step. Thus, preventing NMT to rebound the protein was attempted by adding MyrCoA directly during the lysis, to compete with protein rebinding. After purification of the obtained supernatant, the elution products were separated by SEC, as shown in Figure 3.26-A. Two peaks were obtained, and this time, the peak corresponding to enzyme elution had disappeared. However, the large peak corresponding to the myristoylated product contained in fact a huge amount of degradation, as revealed by the SDS page of the SEC fractions (Figure 3.26-B). This method highlights the protective effect of NMT during lysis, in which the myristoylated species complexed with NMT are not subject to degradation.

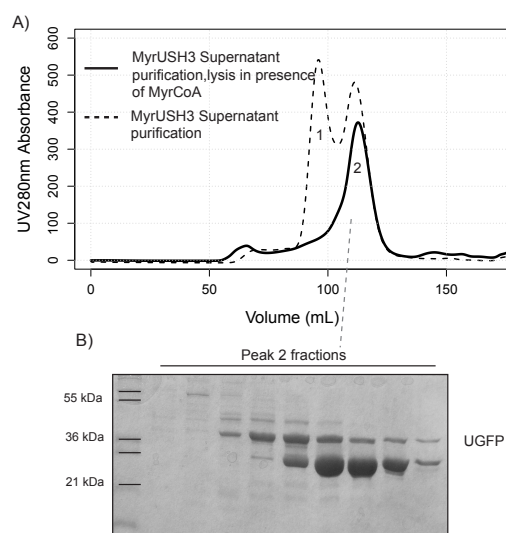


Figure 3.26: A) SEC and B) SDS page of the corresponding fractions of MyrUGFP purification after lysis in presence of MyrCoA

The RT loop in SH3 has a protecting effect A mutant of MyrUSH3 (called MyrUSH3IHH) was designed, in which the $^{98}\text{RTE}^{100}$ amino acids of the SH3 domain were replaced by a $^{98}\text{IHH}^{100}$ sequence (it is a naturally occurring sequence in the SH3 domain of Hck – previous structural studies had shown that the absence of the RT loop in Hck renders the RT-loop much more flexible (Arold et al., 1998)). The expression and purification of this mutant, by the triton wash method, rendered a much higher amount of degradation in the final product. Figure 3.27-A displays the separation of the degradation from MyrUSH3IHH at low pH by SEC (as explained in Part 3.5). A large amount of degradation was obtained, as confirmed by the SDS page of the fractions (Figure 3.27-B). We can see that a change in the RT loop affects the way degradation occurs in the ULBR region.

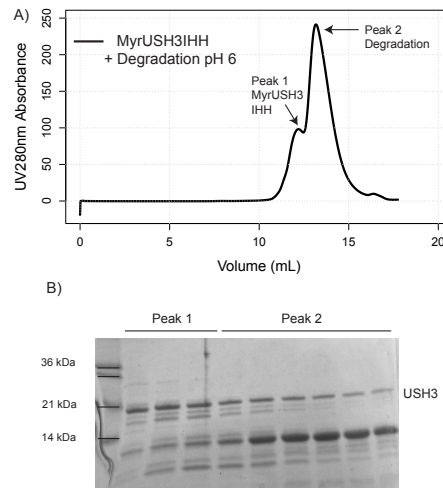


Figure 3.27: B) SEC and C) SDS page of MyrUSH3IHH injected at pH 6

Is degradation functional?

It is striking that degradation only occurs in the myristoylated form, and is very specific: MyrUSH3 is cleaved between amino acids of the ULBR, demonstrated to be a functional region of c-Src.

We developed an assay with the aim of verifying whether the observed degradation occurred as an artifact from the *E. coli* expression, or is also observed in human cells, on the entire Src protein. If it is the case, we could suggest that this degradation is functional within the c-Src regulation mechanisms.

The assay consisted in defining a diagnostic peptide which would be obtained from an enzymatic digestion. The digested peptide includes the cleavage point and would be different in the degraded form and the intact protein. Digestion of the degradation alone, or in the intact protein, or in a mixture of both, was performed with the enzyme GluC. The digestion product was analyzed by MS and all the peptides coming from the digested product that were detected were listed.

Table 3.3: Digestion Results

MyrUSH3	Degradation	Mixture	Amino	Acid Sequence
NO	YES	YES		F G G F N S S D T V T
NO	YES	YES		F G G F N S S D T V T S P Q R
NO	YES	YES		F G G F N S S D T V T S P Q R A
NO	YES	YES		F G G F N S S D T V T S P Q R A G P L
NO	YES	YES		F G G F N S S D T V T S P Q R A G P L A
NO	YES	YES		F G G F N S S D T V T S P Q R A G P L A G
YES	YES	YES	P K L	F G G F N S S D
YES	YES	YES	P K L	F G G F N S S D T V T
YES	YES	YES	P K L	F G G F N S S D T V T S P Q
YES	YES	YES	P K L	F G G F N S S D T V T S P Q R A G P L A

Several peptides were obtained that satisfactorily behaved as expected: they were detected differently in case of a degraded protein or MyrUSH3. Four short peptides which contain the sequence PKLFGGFNSSD were detected both in the MyrUSH3 and in the Mixture (MyrUSH3 + Degradation). Six short peptides which contain the sequence FGGFNSSD were detected among the Degradation and Mixture samples. Table 3.3 shows the list of peptides found. Four diagnostic peptides were thus selected, namely **PKLFGGFNSSDVT** and **PKLFGGFNSSDVTSPQ** for MyrUSH3 and **FGGFNSSDVT** and **FGGFNSSDVTSPQR** for the degraded form.

This protocol is aimed at being applied in the context of human cell lines. By using the selected diagnostic peptides, a protocol in the MS detection can be used to enhance the sensitivity of the assay: only the four diagnostic peptides will be searched for during the MS analysis. Thus a high sensitivity method is now available to apply to *in-vivo* systems, in which human cell lines can be transfected by c-Src and analyzed by MS after cell lysis.

3.6. Conclusion and Discussion

A library of different constructs of N-terminal myristoylated c-Src proteins has been created. It mainly consists in MyrSH4, MyrUSrc, MyrUSH3 but also in a variety of mutants or chimeric constructs, such as the MyrUGFP or the A3 mutants variants. The constructs are listed in Table 3.4, together with the method used to produce them, among the different methods described in this chapter. The list of the constructs with their exact amino acid sequence is available in appendix 9.1.

Table 3.4: Library of c-Src constructs

Protein	Method	Remark
MyrSH4	Peptide Synthesis	
MyrUSrc WT	In-Vitro	
MyrUSrc A3	In-Vitro	Mutant in the ULBR region
MyrUSH3 WT	Coexpression	
USH3 A2G	Standard	Non myristoylated
MyrUSH3 A3	Coexpression	Mutant in the ULBR region
MyrUSH3 IHH	Coexpression	Mutant in the RT loop
MyrUGFP	Standard	
UGFP A2G	Coexpression	Non myristoylated
MyrUGFP A3	Coexpression	Mutant in the ULBR region

Peptide synthesis is straightforward but limited to small peptides. In-vitro methods permit myristoylation of disordered proteins, but are not very appropriate for folded domains. In addition, they are quite costful and time consuming. Coexpression methods are much more interesting in terms of yield, and easiness of the process, but one must be careful with the purity of the final product. In general, laurylation is the most frequent problem that can arise from bacterial expression in *E. coli*. In our specific system, coelution with NMT and degradation also occurred. All our experimental data supports the fact that the source of laurylation is the degradation of MyrCoA to LaurCoA in the β -oxidation cycle. Interestingly, if the medium is not supplied with myristic acid, LaurUSH3 is found to coelute with NMT, and a C:10 acylated protein is also obtained. Thus specificity of NMT is higher for MyrCoA than for LaurCoA, and for LaurCoA to C:10-CoA. This ensures that NMT will not put a third acyl chain type until the other two

are totally exhausted. The situation could be different in other organisms. *E. coli* does not significantly produce shorter than C:10 acylated cofactor in the β -oxydation, but *Salmonella* does (Iram and Cronan, 2006); if *E. coli* would use the *Salmonella* system, a wider mixture of acylated proteins might have been obtained. The palmitic acid addition to enhance the MyrCoA pool is also very efficient in *E. coli* (Iram and Cronan, 2006), but might not have the same effect in other organisms. A universal feature to prevent laurylation consists in ensuring that the amount of energy sources is always large enough so that β -oxydation is not initiated. Exchanging the growth medium to a fresh one and use short times of expression limit the exhaustion of the energy sources.

The elution profile in a SEC of MyrUSH3 versus the laurylated or non acylated forms point out that the presence of the myristoyl chain affects the structure of USH3. In the USH3 protein, interactions between the Unique Domain and the SH3 domain suggest that the disordered Unique Domain is sampling a restricted conformational space. The apparent size of MyrUSH3 in the SEC corresponds to a USH3 lacking a large part of the Unique Domain, thus a constructs resembling the SH3 domain alone. Therefore, the SEC profiles obtained in our experiments seem to reflect an even more restricted configuration of USH3 in presence of myristoyl. The laurylated chain is not sufficient to provide this additional restriction. These results suggest that the myristoyl chain is also participating in intramolecular interactions probably stabilizing the protein conformation. This was later confirmed in our NMR study (See chapter 6). The fact that by playing on electrostatic repulsions between the C-terminal HisTag and the SH4 domain, the MyrUSH3 complex can be "opened" also supports this model. In our specific case, this features enables to separate myristoylated species from laurylated species or degraded forms by SEC. Working with the correct acyl chain seems to be of great importance in case of c-Src, as it was already suggested in other assays presented in the introduction (Heuckeroth and Gordon, 1989). It may not be so crucial for some proteins, and actually reflects the different roles of myristoyl in protein binding. For example, the effect was less striking in BASP than in Src in our SPR experiments. Myristoyl seems to play a crucial role in providing a hydrophobic anchoring of c-Src to the membrane. In the case of BASP, it might only be required to concentrate the protein near the membrane, which triggers alpha helix formation enhancing membrane binding. Laurylation might act almost as good as myristoylation for this purpose too. In fact, studying the effect of the acyl chain

length on protein binding behavior constitutes a biophysical tool to better understand the role of myristoylation in each protein, since as we have seen, it is versatile, even more when the subsequent N-terminal domain is disordered.

The degradation that occurs only in the case of myristoylated form of USH3 constitutes a complication in the purification process. It is still not clear for us what mechanisms triggers it, but we developed some methods in which degradation can be excluded from the sample. We have characterized precisely the cleavage site and used a mutant in which no degradation occurred. Unfortunately, the mutant was designed to eliminate other interactions that involve the ULBR, so the study of wild type USH3 without degradation was challenging, although it could be achieved by the Histrap chromatography method. The fact that degradation occurs in a functional region of the Unique Domain is peculiar, thus efforts have been made to develop a method to test whether degradation also occurs in the full c-Src form in human cells. A MS related assay implying protein digestion and peptide detection was established and a diagnostic peptides has been identified. This assay is ready to apply to transfected human cells transfected with c-Src. This myristoylation-dependent degradation may have a functional role, and a similar phenomena has also been observed in MARCKS protein ([Brauna et al., 2000](#)). A study comparing myristoylated and non-myristoylated forms of c-Src found that the non-myristoylated form exhibited reduced kinase activity but had enhanced stability compared to myristoylated c-Src ([Patwardhan and Resh, 2010](#)). This would be compatible with our finding that the Unique Domain of Src has a favored cleavage site at the ULBR region in its amino acid sequence.

3.7. Experimental Methods

Cloning of USH3 and UGFP The original pETDuet-1 Δ —6His—hNMT-Nef was a gift from J. Glück.

It originally contained the NMT sequence in one cassette, to which the HisTag had been removed, and the Nef sequence in the second cassette. The amino acid sequence of USH3 was PCR amplified from an original pETM30 vector. The C-terminal primer coded for a 6His sequence. The pEtDuet-1 Δ -6His—hNMT—Nef was digested by NdeI and XhoI enzymes and the USH3 amplified product was ligated overnight in place of the Nef sequence in the PetDuet1 plasmid, using the T4 ligase enzyme. The pETDuet-1 Δ -6His—hNMT—USH3—6His plasmid was obtained. The amino acid sequence of USrc was PCR amplified from an original pET-14b vector. The petDuet-1 Δ -6His—hNMT—Nef was digested by NdeI and XhoI enzymes and the USrc amplified product was ligated overnight in place of the Nef sequence in the PetDuet1 plasmid, using the T4 ligase enzyme. The pETDuet-1 Δ -6His—hNMT—USrc plasmid was obtained. Subsequently, the amino acid sequence of eGFP was PCR amplified from its original vector. The C-terminal primer coded for a 6His sequence. The pETDuet-1 Δ -6His—hNMT—USrc was digested by NdeI and PacI enzymes and the eGFP amplified product was ligated overnight just afterwards the sequence of USrc, using the T4 ligase enzyme. The pETDuet-1 Δ -6His—hNMT—UGFP-6His was obtained. After production of the plasmids, they were transformed into *E. coli* X-Gold (BL21) cells (Novagen) and plated over an agarose plate containing ampicillin resistance (plasmids are designed with this ampicillin resistance). Cells were grown overnight and the next day, the DNA was extracted and purified. Minipreps were prepared with the Qiagen miniprep kit. The purified plasmids were sent for sequencing to Macrogen, and the preparations containing the correctly modified sequences were selected.

Site directed mutagenesis to obtain AAA, mutants adjacent to ULBR, and RT mutants Mainly site directed mutagenesis were performed in order to obtain a library of mutants. The original USrc and USH3 vectors contained an alanine in position 2, which was replaced by a glycine to permit myristoylation. In the different constructs, the A3 mutant was obtained by substituting the KLF by AAA amino acids by site directed mutagenesis. The RT mutant of the USH3 construct was also obtained by site

directed mutagenesis. Finally, the mutants in the region adjacent to the ULBR were also obtained by this method. The primers corresponding to the amino acids to exchange were designed and ordered to Sigma. Site directed mutagenesis were performed using the Quik site mutagenesis kit from Strategene. After PCR amplification of the original plasmid with the correct primers, digestion with NdeI was performed to remove the methylated original plasmid. The digested product was subsequently transformed in *E. coli* X-Gold BL21 cells (Novagen) and plated over an agarose plate containing ampicillin. Cells were grown overnight and the next day, the DNA was extracted and purified. Minipreps were prepared with the Qiagen miniprep kit. The purified plasmids were sent for sequencing to Macrogen, and the preparations containing the correctly modified sequences were selected.

USrc expression and purification pET-14b plasmids carrying the sequence of USrc (followed by a StrepTag for purification purposes) were transformed in *E. coli* RosettaTM (DE3)pLysS cells (Novagen). Cells were plated on an agarose plate containing ampicillin and chloramphenicol. Colonies were grown at 37 °C in Luria Broth (LB) medium until reaching an OD₆₀₀ of 0.6-0.8. For ¹⁵N isotopic labeling, cells were grown in M9 minimal medium supplemented with ¹⁵NH₄Cl (Cambridge Isotope Laboratories, UK). Induction was performed by adding 1 mM of IPTG and expression was allowed at 25 °C overnight. Cells were harvested by centrifugation and resuspended in the USrc Lysis buffer 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0,1 % NaN₃, pH 7.5 to which protease inhibitors were added (Protein Inhibitor Cocktail², and 1 mM PMSF, both from Sigma), and 1mM PMSF, both from Sigma). This lysis product was sonicated on ice, treated with DNase (Roche) for 20 min and centrifugated at 25000 rpm for 45 min. The supernatant was subsequently incubated with the Streptag Resin (IBA) and USrc protein was allowed to bind the resin overnight. The resin was subsequently washed with the USrc lysis pH buffer adjusted at pH 8. Elution was achieved with the USrc Lysis buffer pH 8 containing 2,5 mM Desthiotol. The elution product was further purified by Size Exclusion Chromatography (SEC) on a Superdex 75 26/60 (GE Healthcare, Spain) in 50 mM sodium phosphate, 0.2 mM EDTA, 0,01 % NaN₃, pH 7.

²Protein cocktail inhibitor: 23 mM AEBSF, 1 mM EDTA, 20 µM Bestatin, 3µM Pepstatin A, 3 µM E64 (final concentrations in lysis buffer)

USH3 expression and purification PetDuet1 plasmids carrying the sequence of USH3 with an alanine at position 2 was used to expressed non myristoylated USH3. They were transformed in *E. coli* RosettaTM (DE3)pLysS cells (Novagen). Cells were plated over an agarose plate containing ampicillin and cloramphilicol. Colonies were grown at 37 °C in Luria Broth (LB) medium until reaching an OD₆₀₀ of 0.6-0.8. Induction was performed by adding 1mM of IPTG and expression was allowed at 28 °C overnight. Cells were harvested by centrifugation and resuspended in the USH3 Lysis buffer 20 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, 0,1 % NaN₃, pH 8, to which protease inhibitors were added (Protein Inhibitor Cocktail³, and 1 mM PMSF, both from Sigma). This lysis product was sonicated on ice, treated with DNase (Roche) for 20 min and centrifugated at 25000 rpm for 45 min. The supernatant was subsequently applied to a Ni-NTA column (Qiagen) and USH3 protein was allowed to bind the resine for 1 h. The resine was subsequently washed with the USH3 lysis pH buffer. Elution was achieved with the USH3 Lysis buffer containing 400 mM imidazole. The elution product was further purified by Size Exclusion Chromatography (SEC) on a Superdex 75 26/60 (GE Healthcare, Spain) in 50 mM sodium phosphate, 150 mM NaCl, 0.2 mM EDTA, 0.01 % NaN₃, pH 7.5.

Myristoylated USH3 and UGFP expression The bicistronic petDuet 1 plasmids with the sequence of interest were transformed in *E. coli* RosettaTM (DE3)pLysS cells (Novagen) or T7 Express (New England BioLabs). The cells were grown at 37 °C to an OD₆₀₀ of 0.8. Cells were plated over an agarose plate containing ampicillin and cloramphilicol. Colonies were grown at 37 °C. Ten minutes before induction with IPTG (final concentration 1 mM, Melford), freshly prepared myristic and/or palmitic acid (Sigma) were added to the cell culture, at a final concentration of 200 µM; the solutions also contained BSA as a carrier at a final concentration of 0,6 M. In the case of expression in minimal medium, cells were centrifuged at 1000 g at 4 °C for 20 min. The pellet was resuspended in half the volume of M9 minimal medium supplemented with ¹⁵NH₄Cl, in which the glucose was increased to 6 g/L. After 20 min at 37 °C, myristic and palmitic acid were added as described above. The temperature after induction was set to 28 °C for 5 h or 20 °C for 20 h. The cells were harvested by centrifugation at 4000 rpm at 4 °C for 30 min. The pellet was resuspended in USH3 Lysis buffer (20mM TrisHCl, 300 mM

³Protein cocktail inhibitor: 23 mM AEBSF, 1 mM EDTA, 20 µM Bestatin, 3µM Pepstatin A, 3 µM E64 (final concentrations in lysis buffer)

NaCl, 5 mM Imidazole, pH 8), to which protease inhibitors were added (Protein Inhibitor Cocktail⁴, and 1 mM PMSF, both from Sigma), and 1 mM PMSF, both from Sigma).

MyrUSH3 or MyrUGFP Purification The cell lysis was sonicated on ice before centrifugation at 25000 rpm for 45 min at 4 °C. The protein was distributed between the supernatant and the pellet. Thus, two different purification methods were used.

Supernatant purification On the one hand, the supernatant was applied to a Ni-NTA column (Qiagen) and the myristoylated protein was allowed to bind the resin for 1 h. The resin was subsequently washed with the USH3 lysis pH buffer. The elution product was further purified by Size Exclusion Chromatography (SEC) on a Superdex 75 26/60 (GE Healthcare, Spain) in 50 mM sodium phosphate, 150 mM NaCl, 0.2 mM EDTA, 0.01 % NaN₃, pH 7.5.

Triton wash purification On the other hand, the pellet was resuspended in USH3 lysis buffer containing 1 % of Triton X-100. The resuspended pellet was centrifuged again 30 min at 25000 rpm and the procedure was repeated twice or three times. The supernatant of the "Triton-washes" was purified via immobilized metal affinity and SEC as described above. If lauroylated species were present, they eluted at an apparent higher molecular weight than the myristoylated ones from the size exclusion chromatography. This method enabled to separate the two different acylated species and to quantify their respective amount. The purity and identity of the products was established by LC/MS as described in the Mass Analysis section.

MyrCoA competition assay An alternative protocole consisted in sonicating the cell lysis containing MyrUGFP in presence of an excess MyrCoA.

MyrUSH3 degradation Separation

SEC method at low pH After purification of MyrUSH3 by the triton wash methods, protein fractions were collected and concentrated to 500 µL in 50 mM sodium phosphate pH 7.5. They contained MyrUSH3 and its degradation form. The mixture was injected in

⁴Protein cocktail inhibitor: 23 mM AEBSF, 1 mM EDTA, 20 µM Bestatin, 3µM Pepstatin A, 3 µM E64 (final concentrations in lysis buffer)

a SEC (superdex 10/300 GL analytical) in 50 mM sodium phosphate pH 6. Two distinct peaks were obtained.

Histrap Chromatography purification method The triton wash purification protocol was modified as follows: the product extracted from the pellet, containing MyrUSH3, was loaded into a histrap chromatography column. after washing with the USH3 lysis buffer, elution was performed by using steps of imidazole starting from 200 mM, increased to 400 mM, 800 mM and 1 M. The first fractions containing contamination from degradation were discarded. All the subsequent fractions were degradation-free MyrUSH3. These fractions were lyophilised and resuspended in water, and further purified by SEC on a Superdex 75 26/60 in 20 mM sodium phosphate, 50 mM NaCl, pH 7.5. The pure MyrUSH3 fractions were collected and lyophilized before resuspending them in water. This product was then dialyzed against 50 mM sodium phosphate buffer, pH 7. If needed, lyophilization/dialysis cycles were repeated until the desired concentration was reached.

Separation of the NMT-Myristoylated complex The fractions containing MyrUSH3 or MyrUGFP complexed with NMT were concentrated and subjected to one of the following protocols:

HIC protocol The sample was applied to an isopropanol hydrophobic column in 50mM NaP + 150 mM NaCl with or without 1 % Triton X100. The proteins were then eluted by decreasing the $(\text{NH}_4)_2\text{SO}_4$ concentration from 1.5 M to 0 M.

MyrCoA competition protocol The complex MyrUSH3-NMT was applied to the nickel resin. A solution of 5 mM MyrCoA was applied and NMT was eluted. The operation was repeated until almost no more NMT was eluted. NMT was obtained as a pure product and further concentrated. Subsequently, MyrUSH3 or MyrUGFP was eluted with 400 mM imidazole. The elution product still contained NMT. The MyrUSH3 eluted product was concentrated and separated from NMT by SEC, through injection in a Superdex 10/300 GL analytical. Since NMT was not in excess, MyrUSH3 was separated, but a portion of MyrUSH3 also underwent degradation.

Reverse phase chromatography The sample was separated using reverse phase chromatography. The conditions are detailed in the "Mass Analysis" section.

In-vitro myristoylation protocol

MyrCoA synthesis Myristic acid, 6 mg/ml, was dissolved in DCM and carboxy-diimidazole, 4,1 mg/ml in THF, and they were mixed with agitation for 30 min. The solvent was removed in a rotavapor and subsequently resuspended in 2 mL THF. CoenzymeA (Tebubio), 10 mg/ml, was dissolved in 0,5M NaHCO₃. CoA was reacted with myristic-carboxydiimidazole product for 2 h with agitation. The Myristoylated Coenzyme A (MyrCoA) solution was used without further purification. The reaction can be followed via HPLC injections. 50 µl of the reaction product is injected in a Sunfire C18 column with a 0-60 % H₂O:ACN gradient

USrc expression and purification See part 3.7

NMT See part 3.7

Myristoylation protocol USrc, NMT and MyrCoA were obtained as described in the previous sections. NMT was allowed to react with MyrCoA for 20 min at room temperature. The NMT:MyrCoA complex was then reacted with USrc for 2 h at 37 °C followed by an overnight reaction at 4 °C. The products were separated by reverse phase chromatography. 200 µL of product was applied to a Sunfire C18 LC column with a 0-80 % H₂O:acetonitrile gradient. Several injections were performed. The fraction corresponding to MyrUSrc was collected and lyophilised, before being resuspended in 50 mM sodium phosphate, pH 7. The purity and integrity of the product was verified by LC/MS as described in the Mass Analysis section.

Liposome preparation 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-Dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) (DMPG) and L- α -phosphatidylserine (PS) were purchased from Avanti Polar Lipids, Inc. The lipids were dissolved in chloroform or in the case of DMPG in chloroform:methanol:H₂O 65:35:8. Liposomes were prepared by mixing the appropriate amount of lipids in the solvent. The solvent was evaporated in a rotavapor followed by rehydration and vortexing at 40 °C with the buffer used for SPR with a final lipid concentration of 1 mM lipids, and Multilamellar Large Vesicles were obtained. The different liposomes were prepared with a DMPC:DMPG ratio of 2:1 or a DMPC:PS ratio of 4:1. Large unilamellar vesicles were mechanically extruded at 40 °C

using a 10-mL Thermobarrel extruder (LipexTM. Northern Lipids Inc.) with at least ten cycles of extrusion using a 100 nm polycarbonate filter. To verify the appropriate size of the liposomes, the mean diameter was checked using Dynamic Light Scattering (Zetasizer Nanoseries S, Malvern instruments).

Lipid Sedimentation Assay DMPC:DMPG Multilamellar Large Vesicles were obtained as previously described in the "liposome" protocol part. Acylated proteins were mixed with the vesicles and allowed to interact with the lipids at ambient temperature for 30 min. The mixture was ultracentrifuged for 20 min at 55000 rpm. The supernatant was subsequently collected and the pellet was washed three times with buffer before resuspension. The two fractions were loaded in a SDS page in order to visualize if the protein was in the aqueous or in the lipid phase.

Surface Plasmon Resonance (SPR) SPR experiments of BASP were carried out in a Biacore 2000 instrument from Biacore, GE Healthcare using the SPR Sensor chip (L1) from Biacore, GE Healthcare. Liposomes were injected for 500 s at a flow rate of 5 μ l/min. The reference channel was coated with BSA with a 200 s injection of 1 mg/ml BSA (Sigma, fatty acid free) at a flow rate of 10 μ l/min. Protein binding experiments were performed at a 50 μ l/min. The interaction of MyrBASP or LaurBASP with liposomes was followed by observing the SPR response when a solution of protein was injected for 100 s (association phase), followed by a 300 s washing period (dissociation phase). MyrBASP binding to DMPC:PS liposomes was injected at concentrations ranging from 10 nM to 2.5 μ M. LaurBASP binding to DMPC:PS liposomes was injected at concentrations ranging from 20 nM to 7.5 μ M. All experiments were performed in the running buffer, which comprised 20mM NaP, 50mM NaCl, pH 7.4. The surface was regenerated with a series of detergent injections at 100 μ l/min (20 mM CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), each pulse lasting 30 s. Each experiment was started with freshly captured liposomes. Liposome coating was reproducible, with a variation smaller than 4 % between the subsequent coatings, ensuring very reproducible protein binding curves. SPR experiments of MyrUSH3 were performed in a very similar fashion, with slight modifications. The SPR chip (a 2D carboxymethyl dextran surface) was purchased from Xantec and modified by covalent attachment of phytosphingosine (TebuBio) to allow the capture of DMPC:DMPG (2:1) liposomes. Liposomes were injected at 10 μ l/min for 200 s before

protein binding experiment: MyrUSH3 or LaurUSH3 were injected at 50 $\mu\text{L}/\text{min}$ for 60 s and dissociation was allowed for 300 s. The running buffer was composed of 50 mM NaP, 150 mM NaCl, 0.2 mM EDTA, pH 7.5. Liposome coating was reproducible, with a variation of about 1 % between the subsequent coatings, ensuring very reproducible protein binding curves. Data analysis was performed in the Biaevaluation software from Biacore.

Mass Analysis LC Conditions: Chromatograph: Acquity UPLC Binary Sol MGR (Waters Corporation) Autosampler: Acquity UPLC Autosampler MO (Waters Corporation) Column: BioSuite pPhenyl 1000RPC 2.0 x 75 mm; 10 mm Eluents: A: Water 1 % F.A B: Acetonitrile 1 % F.A Gradient: 5 % to 80 % of B in 60 min. Total chromatographic time: 80 min.

MS Conditions: Mass Spectrometer: LCT-Premier (Waters) - Sample introduction: LC-MS coupling - Polarity: ESI positive - Analyzer: V - Capillary voltage: 3000 V - Sample Cone: 80V - Desolvation Temperature: 400 °C - Source temperature: 120 °C - Cone gas flow: 50 - Desolvation gas flow: 750 - Pirani Pressure (mbar): 2.65 e0 - Penning Pressure (mbar): 9.90 e-7 - Argon: 7.0e-3 - m/z 400-4000

Digestion assay

Sample preparation MyrUSH3 was obtained by using the Histrap Chromatography protocol. It was free of degradation. Degradation was obtained by using a sample of MyrUSH3 purified by the standard triton wash protocol. It contained both MyrUSH3 and degradation. A lipid sedimentation assay was performed and the supernatant was collected; it contained only the degraded form. 5 μL of MyrUSH3, 5 μL of diluted Degradation, and a mixture of MyrUSH3 and diluted Degradation (5 μL + 5 μL) were digested (all proteins at 100 μM concentration) with GluC (4 % w/w) at 25 °C during 24 h.

MS method The nano-LC-MS/MS set up was as follows. Digested peptides were diluted in 1% FA. Samples were loaded to a 180 μm x 2 cm C18 Symmetry trap column (Waters) at a flow rate of 15 $\mu\text{L}/\text{min}$ using a nanoAcquity Ultra Performance LCTM chromatographic system (Waters Corp., Milford, MA). Peptides were separated using a C18 analytical column (BEH130 C18 75 mm x 25 cm, 1.7 μm , Waters Corp.) with a 80 min run, comprising three consecutive steps with linear gradients from 1 to 35 % B

in 60 4min, from 35 to 50% B in 5 min, and from 50 % to 85 % B in 3 min, followed by isocratic elution at 85 % B in 10 min and stabilization to initial conditions (A= 0.1 % FA in water, B= 0.1 % FA in CH₃CN). The column outlet was directly connected to an Advion TriVersa NanoMate (Advion) fitted on an LTQ-FT Ultra mass spectrometer (Thermo). The mass spectrometer was operated in a data-dependent acquisition (DDA) mode. Survey MS scans were acquired in the FT with the resolution (defined at 400 m/z) set to 100,000. Up to six of the most intense ions per scan were fragmented and detected in the linear ion trap. The ion count target value was 1,000,000 for the survey scan and 50,000 for the MS/MS scan. Target ions already selected for MS/MS were dynamically excluded for 30 s. Spray voltage in the NanoMate source was set to 1.70 kV. Capillary voltage and tube lens on the LTQ-FT were tuned to 40 V and 120 V. Minimal signal required to trigger MS to MS/MS switch was set to 1000 and activation Q was 0.250. The spectrometer was working in positive polarity mode and singly charge state precursors were rejected for fragmentation. A database search was performed with Proteome Discoverer software v1.4 (Thermo Scientific) using Sequest HT search engine and SwissProt database (Human, release 2015-01 and the common Repository of Adventitious Proteins (cRAP database) and manually introduced the sequence of myristoylated USH3). Searches were run against targeted and decoy databases to determine the false discovery rate (FDR). Search parameters included non-enzyme specificity, myristoyl in N-terminal as static modification and methionine oxidation as dynamic modifications. Peptide mass tolerance was 10 ppm and the MS/MS tolerance was 0.6 Da.

Chapter 4

MyrUSH3 binding to Liposomes

4.1. Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) biosensors have a wide range of applications, among which the characterization of biomolecular interactions. These biosensors permit to monitor *in-situ*, in real time, the interactions between label-free molecules. Information that can be extracted include the kinetics and affinity of the interaction; thermodynamic decomposition into enthalpy and entropy can be obtained if temperature is varied. The biomolecules intervening in these interactions can be proteins, DNA, small molecules, carbohydrates, liposomes, etc ([Bakhtiar, 2013](#)).

The SPR technique is based on Total Internal Reflection (TIR) of an incident beam light at an interface between two media of different refractive index (RI). When the beam propagates from the medium of higher RI to that of lower RI, it can be totally reflected (TIR), as long as its incident angle is greater than a critical angle depending on the RI of the two media ([Zeng and Liang, 2010](#)).

When the incident light interacts with the mobile free electrons at the surface of the metal, surface plasmon polaritons (SPP) arise as transverse magnetic waves that can propagate along the interface between two material of opposite permittivities (a metal and a dielectric material, for example an aqueous medium) ([Daghestani and Day, 2010](#)). SPR conditions are achieved when the angle of incident light is such that its wavevector

component parallel to the metal surface matches that of the SPPs and is detected as a drop in the reflectivity of the incident light occurs (Homola, 2003). In SPR conditions, a change on the dielectric sensor surface will result in a change in the angle of reflectivity, which is the parameter that is ultimately detected. A so-called evanescent field is created in both media, but the majority of the generated field is present in the dielectric medium. This evanescent field decays exponentially with the distance in z ; therefore the depth of the evanescent wave useful for measurements is very limited – about 300 nm of the sensor surface (Akimoto et al., 2000).

Different set-ups can be used to obtain SPR (Homola et al., 1999). A common one is called the Kretschmann configuration, in which a gold layer is placed in the interface of two dielectric media, a glass prism and an aqueous phase, as illustrated in Figure 4.1. The optical light source (often a near-infrared high-efficiency LED) passes through the prism in TIR mode and creates an evanescent wave which propagates into the aqueous phase; the angle is tuned to match the evanescent wave propagation rate with that of the SPP. The resonant angle can be measured very precisely (Akimoto et al., 2000). The sensor is placed in a microfluidic chamber of very small dimensions with the gold layer in contact with a flow of aqueous solution, in which molecules can be injected. If the molecule that is flown in the chamber deposits over the gold surface, a change of the refractive index occurs and is measured as a change in resonance angle induced by the SPR. The change in refractive index on the surface is linear with the number of molecules bound to the sensor surface in most of the cases (Stenberg et al., 1991). A shift in the SPR angle by 0.0001 degree corresponds to one unit shift in SPR signal (Bakhtiar, 2013)

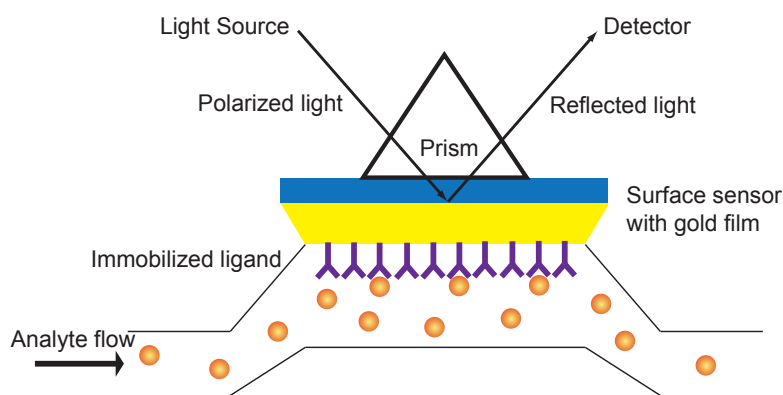


Figure 4.1: *Surface Plasmon Resonance Set Up*

A SPR sensor can be used as a biorecognition transducer, where the gold surface is functionalized by the immobilization of one of the interacting partners, called the ligand. The other interacting partner, called the analyte, is injected in the continuous flow of running buffer and delivered near the sensor gold surface (Myszka, 1997). If the analyte interacts with the ligand, it induces a change of RI near the surface of the gold layer and generates a shift in the resonance angle, that is measured in Response Units, RU. Therefore, the technique enables to monitor over time the association of the analyte to the ligand. When the ligand injection has finished to flow and is replaced by the running buffer, the dissociation of the complex is monitored (as illustrated in Figure 4.3-A). Since the presence of the molecule itself over the surface is what affects the SPR signal, no labeling of the molecules is needed. However, one has to take care that the ligand is immobilized without hampering its biological function.

There are normally several channels in which the analyte can pass simultaneously over the same or different ligands immobilized in each channel. Since changes in refractive index can be generated by other factors than the binding of the analyte it self, one channel is usually set up as a reference and the experimental data are corrected from the SPR responses of the reference (Rich and Myszka, 2000). The gold layer is chemically functionalized to permit ligand immobilization and the ligands can be covalently coupled to the surface (Thillaivinayagalingam et al., 2010); Figure 4.2 shows different immobilization strategies. A carboxylated coated surface can be activated in order to covalently couple the amine group of a protein (Figure 4.2-A). The ligand can also be captured by high affinity binding molecules – a common set up consists in a streptavidin coated surface able to capture biotinylated ligands (Dutra and Kubota, 2007), as illustrated in Figure 4.2-B. Liposomes or liposome-containing membrane proteins (Cooper et al., 2000) can adsorb to a functionalized surface, by e.g. use of biotinylated liposomes binding to streptavidin coated surfaces (Eva-Maria Erb et al., 2000), or through hydrophobic anchors coating (174) Figure 4.2-C and -D).

By following in real time the association of the analyte to the ligand and its dissociation, one can obtain information about the kinetics and the affinity of the interaction. Specifically, the analyte has to be injected over the ligand at different concentrations. The concentration range to be used is given by the equilibrium constant of the interaction K_D . A signal will be detected at concentrations over $0,1 \cdot K_D$, and saturation of all the

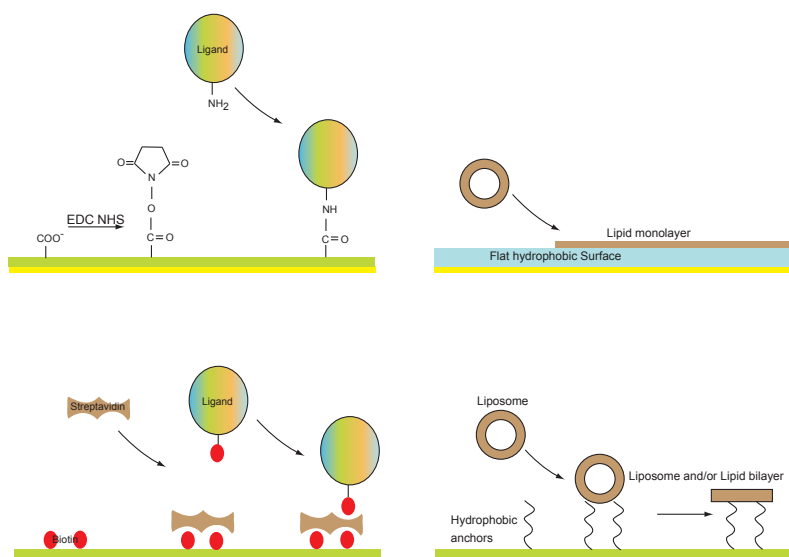


Figure 4.2: *Examples of immobilization schemes. A) Amine coupling mechanism B) Biotin-Streptavidin affinity capture C) Hydrophobic surface leading to the formation of a lipid monolayer D) Hydrophobic anchors capturing liposomes, that may fuse to a bilayer*

immobilized ligands will occur at concentrations about $10 \cdot K_D$. If the dissociation is slow, the analyte can be removed by injecting a solution able to disrupt the interacting complex – mild enough to not affect ligand integrity. By recording the association and dissociation of the analyte at different concentrations a set of so-called binding curves are obtained (Figure 4.3-B). By comparing the experimental curves to simple kinetic models, fitting of the curves finally renders a set up of best-fit parameters corresponding to the equilibrium constant and the association or dissociation rate constants.

The assay has to be optimized for each interacting pair in order to obtain reliable data, by choosing the ligand density, the analyte concentration, the injection time, the flow rate, and the regeneration conditions leading to a good set of binding data. A too high ligand density may favor rebinding effects, especially in the case of fast associating molecules. The analyte concentration must be in the correct range. The injection time must be sufficient during the association phase, so that the binding equilibrium is reached, seen as a plateau. The flow rate should be set as high as a possible to diminish mass transport effects, especially in the case of high association or dissociation rates. Indeed, a constant analyte concentration is assumed in the chamber, but if the transport of the depleted

analytes to the surface is slower than the association rate, the latter is seen with an effective slower rate (Myszka, 1997). If mass transport phenomena cannot be eliminated, a mass transport rate can be included in the kinetics model. A multitude of interactions can be monitored by SPR, but some are very challenging, namely the interactions with low affinity or with very fast kinetics. Low affinity interactions require a high analyte concentration that can favor non specific binding, not always corrected by the reference channel. Fast kinetics can be limited by mass transport phenomena. Very low molecular weight ligands are challenging in term of sensitivity. We used the Biacore instrument, widely spread over the market in SPR, which has a very good fluidics and sensitivity.

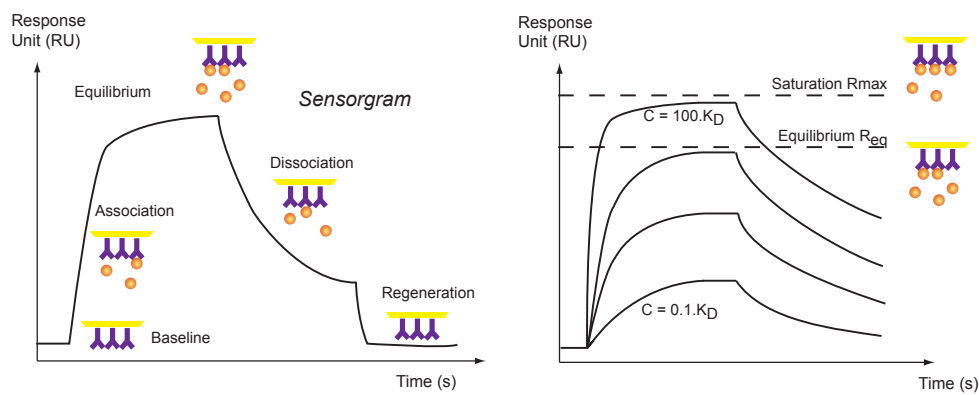


Figure 4.3: A) Example of binding curves of an analyte to an immobilized ligand. During the association phase, the analyte binds to the ligand until equilibrium is reached (recorded as a plateau). Dissociation starts when the analyte is replaced with buffer. B) Example of binding curves recorded at different analyte concentrations, with the same amount of immobilized ligands

The most common model used to monitor a simple interaction is the Langmuir model, in which a protein P interacts with a ligand L (Homola, 2003):



where P represents the MyrUSH3 protein, L the ligand, and PL the protein-ligand complex, the latter forming with the association and dissociation rate constants k_a and k_d respectively.

The SPR response is proportional to [PL], obeying the equation:

$$[PL](t) = L_{eq} \cdot (1 - \exp(-(k_a \cdot [P] + k_d) \cdot t)) \quad (\text{equation 1})$$

with

$$L_{eq} = L_{max} \cdot \frac{k_a \cdot [P]}{k_a \cdot [P] + k_d} \quad (\text{equation 2})$$

The highest protein response possible, proportional to L_{max} , is limited by the number of immobilized liposomes. The rate constants can be estimated by globally fitting the binding curves at various concentrations. The equilibrium constant K_a can be obtained from the ratio of rate constants $K_a = k_a/k_d$.

K_a can also be obtained by fitting the equilibrium SPR responses (L_{eq}) obtained at various protein concentrations $[P]$. In the 1:1 Langmuir model, the relevant equation is a modification of (equation3):

$$L_{eq} = L_{max} \cdot \frac{[P]}{[P] + \frac{1}{K_a}} \quad (\text{equation3})$$

One of the SPR application is the study of the binding of proteins to membranes (Bensenicar et al., 2006), which is the scope of our work: monitor the interaction of MyrUSH3 to lipid bilayers. As already introduced, the membrane models in SPR consist in SLBs. There are several ways of forming SLBs over the sensor chips, but they often consists in liposome deposition. A very standard one consists in the capture of SUVs or LUVs over a chip functionalized with lipophilic anchors – precoated surfaces with such anchors are sold by Biacore. In our set up, we performed the functionalization ourselves, by using a two-dimensional carboxyl coated surface, to which the amine of a lipid (phytosphingosine) was covalently coupled via the “amine-coupling” procedure. Liposome injection was monitored by an increase in the SPR response, and subsequent binding curves of MyrUSH3 to liposomes was recorded. The surface can be regenerated with detergent; liposomes are removed and the surface is free for a next cycle of liposome coating / protein injection / regeneration. Liposomes were either zwitterionic or negatively charged, composed respectively of DMPC alone or DMPC:DMPG 2:1, and the assays were conducted at 25 °C, temperature at which the lipids are in their fluid state.

Though the lipid bilayer can not be described as a discrete ligand as it is the case in many others ligand/analyte interactions, the kinetic modeling has been applied to get

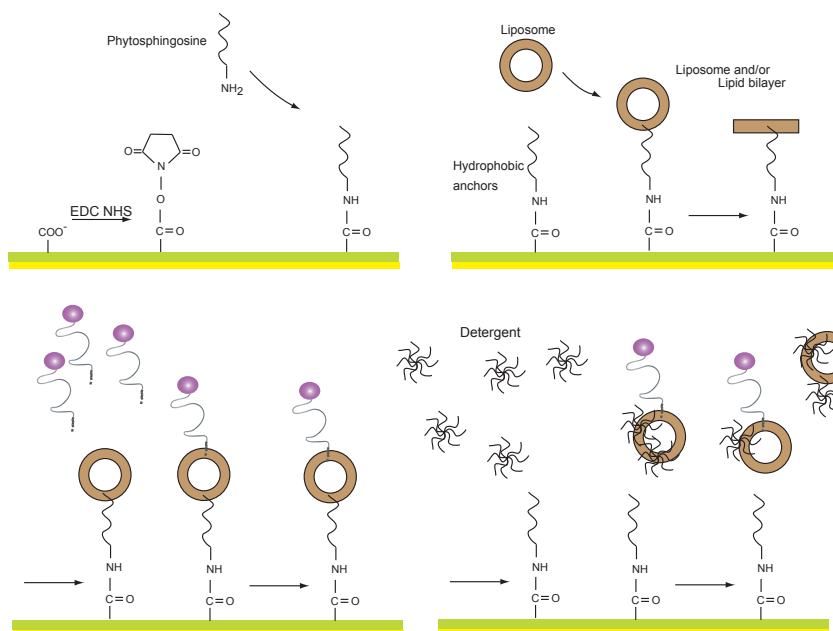


Figure 4.4: *Liposome immobilization scheme: phytosphingosine covalent coupling, liposome capture, protein binding, liposome-protein regeneration with detergent.*

information about the affinity and kinetics of peptides or proteins to membranes ([Eva-Maria Erb et al., 2000](#)), and especially of myristoylated protein ([Lange and Koch, 1997](#)) ([Gohlke et al., 2010](#)).

Here we show that c-Src anchoring involves two kinetically well separated events, namely: a rapid reversible binding step followed by the formation of a persistently bound (PB) species.

4.2. Persistent binding of MyrUSH3 to liposomes

MyrUSH3 binding to immobilized liposomes was followed by SPR. This approach consisted of the following steps:

- I) liposome immobilization
- II) injection of MyrUSH3 (association phase)
- III) washing with a protein-free buffer (dissociation phase)
- IV) antiSH4 antibody injection
- v) surface regeneration by washing away the liposomes.

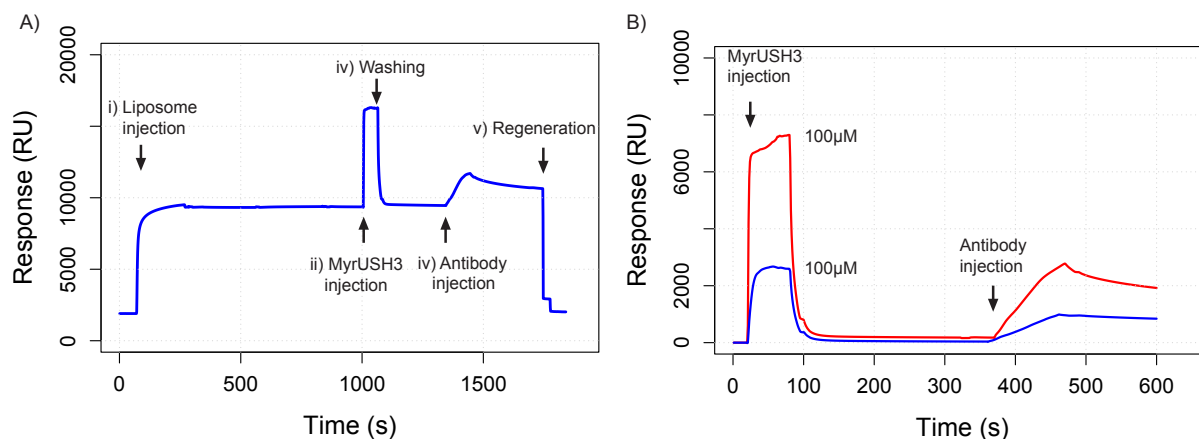


Figure 4.5: A) The cycle consists in (i) liposome coating, (ii) MyrUSH3 associating with and (iii) dissociating from liposomes, and (iv) antiSH4 antibody secondary detection. Complete regeneration of liposomes and proteins is achieved at the end of the cycle (v) B) Association and dissociation of 10 and 100 μ M of MyrUSH3 to DOPC:DOPG 2:1 liposomes, and secondary antiSH4-antibody injection

A complete cycle of MyrUSH3 binding to liposomes is shown in Figure 4.5-A. The association phase revealed rapid binding and equilibrium was reached quickly. The response curve also revealed a rapid dissociation. However, the baseline did not return to its original value (the liposome capture value), but remained stable at a slightly higher level, thereby indicating the presence of PB MyrUSH3 proteins that were not removed by washing with the running buffer. Due to the small population of PB species, a secondary

detection was used to confirm their presence and to quantify them. In this regards, 350 s after the beginning of the dissociation phase, antiSH4 antibody was injected over 60 s. As a control, the antibody was also injected over protein free liposomes. In this case, no response was observed: MyrUSH3 and antiSH4 antibody were completely removed with the lipids when applying the regeneration conditions as described in the method section. Control experiments with the equivalent unsaturated oleyl lipids DOPC-DOPG (2:1) with a transition temperature of -40°C gave very similar results (Figure 4.5-B).

4.3. Components of MyrUSH3 binding to liposomes

The myristoylated N-terminal region of c-Src is known to be essential for binding but the PB population had not previously been reported. In order to check whether persistent binding was caused by the presence of additional lipid binding outside the myristoylated SH4 domain, we measured the binding of a synthetic peptide, containing only residues 2-16 of c-Src with a myristoyl group attached at its N-terminus (MyrSH4), to liposomes. The presence of a PB MyrSH4 population was clearly observed by injection of antiSH4 antibody (Figure 4.6-A). The affinity of the antibody for the two SH4 containing molecules may not be directly comparable, impairing comparison of the relative antibody responses of MyrSH4 or MyrUSH3 PB species. Nevertheless, we can still conclude that formation of the PB species is a property of the MyrSH4 domain and does not require the presence of the additional lipid binding regions in the Unique and SH3 domains. The decrease of the direct SPR response curve during dissociation was slower in MyrSH4 than in MyrUSH3, thereby suggesting that the presence of the secondary lipid binding domains in USH3 enhances the dissociation rate of the MyrSH4 region from the membrane. This apparently contradictory result may be explained by the recently reported interaction between the SH4 and SH3 domains of c-Src (Maffei et al., 2015). Since MyrUSH3 provides a more native-like environment and gives a stronger SPR response than MyrSH4, we focused our study on acylated USH3. The effect of myristoylation on the capacity of c-Src to bind membranes has been extensively discussed. It was concluded that the binding energy provided by the insertion of a single myristoyl chain was not sufficient to explain the stable insertion of c-Src in the membrane. However, the electrostatic interaction between the positively charged SH4 domain and negatively charged lipids, together with myristoyl chain insertion, was enough to ensure stable binding (Buser et al., 1994) (Murray et al., 1998) (Sigal et al., 1994). In order to explore the contribution of the hydrophobic and electrostatic components, we first compared the binding of MyrUSH3 and LaurUSH3 to liposomes. These two species differ only in the length of the N-terminal acyl chain with lauric acid having a 12-carbon chain and myristic acid a 14-carbon chain. Figure 4.6-B compares the binding of MyrUSH3 (1 μ M and 20 μ M) and LaurUSH3 (20 μ M) to DMPC:DMPG liposomes. When the two proteins were injected at the same concentration (20 μ M), the direct SPR response of LaurUSH3 was circa four times lower than that of MyrUSH3, and the secondary detection by antiSH4 antibody gave barely any response

for LaurUSH3. In comparison, the injection of 1 μM MyrUSH3, which provided a direct SPR response half that of the 20 μM LaurUSH3 injection, still gave a clearly detectable antibody response. These experiments show that while shortening of the acyl chain clearly reduced, but did not eliminate, the binding of the fast dissociating form, it abolished the formation of PB species.

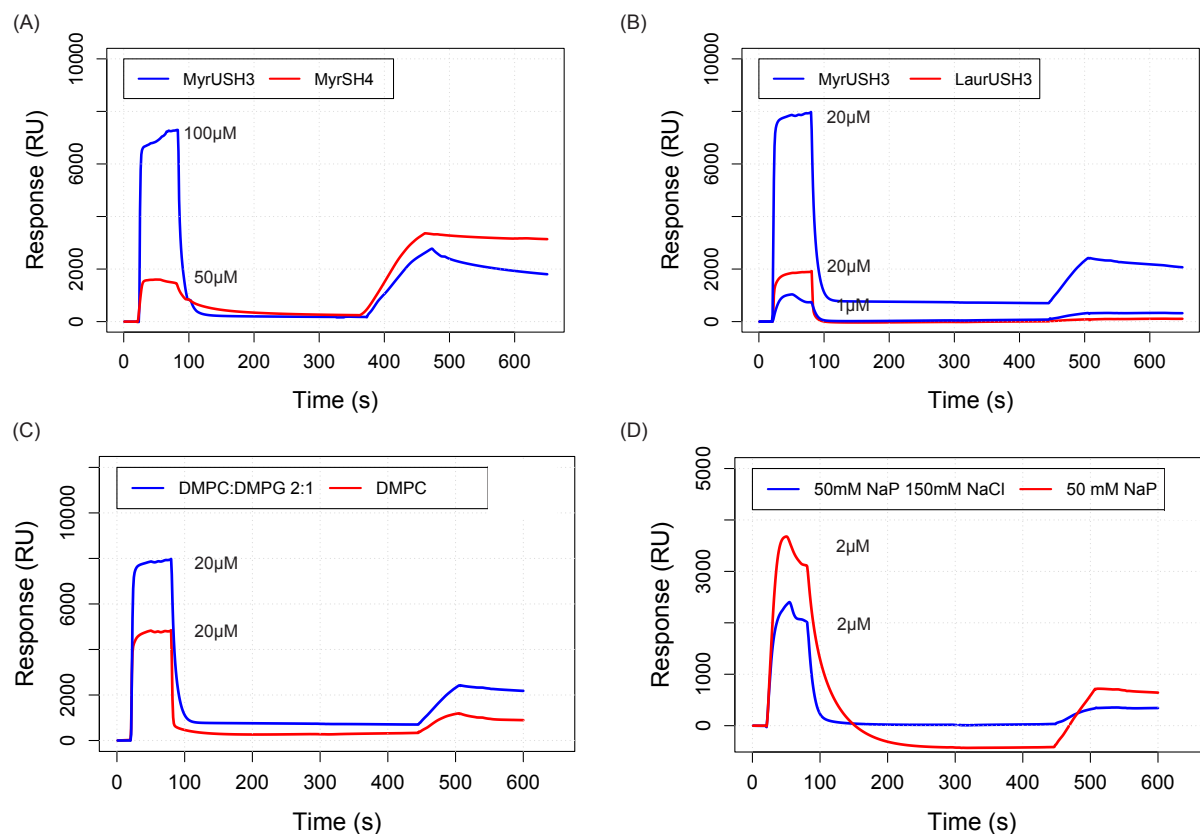


Figure 4.6: *Binding of myristoylated and laurylated peptides to liposomes. (A) Comparison of binding of 50 μM MyrSH4 and 100 μM MyrUSH3 to liposomes. DOPC:DOPG 2:1 liposomes, in 50 mM NaP, 150 mM NaCl. (B) Comparison of the binding of 20 μM LaurUSH3 with that of 1 μM and 20 μM MyrUSH3. DMPC:DMPG 2:1 liposomes, in 50 mM NaP and 150 mM NaCl. (C) Binding of 10 μM MyrUSH3 to DMPC or DMPC:DMPG 2:1 liposomes in 50 mM NaP and 150 mM NaCl (D) Binding of 2 μM MyrUSH3 to DMPC:DMPG 2:1 liposomes in the presence or absence of 150 mM NaCl. In (A-D), the first curve corresponds to direct SPR peptide response, while the second is that of the secondary antibody detection.*

The effect of the electrostatic interactions on the formation of PB MyrUSH3 species was first tested by comparing MyrUSH3 (10 μM) binding to neutral (DMPC) or charged

(DMPC:DMPG 2:1) liposomes. For negatively charged liposomes, the direct SPR response was higher, the dissociation was slower and the baseline recovered after washing was also higher. Secondary detection confirmed that the population of the PB forms was higher for MyrUSH3 bound to negatively charged lipids (Figure 4.6-C). The electrostatic interaction of MyrUSH3 with charged liposomes increased at lower ionic strengths (Figure 4.6-D). The direct SPR response was doubled when the NaCl concentration was reduced from 150 mM to 0 mM, in 50 mM NaP. A markedly slower dissociation was observed at low ionic strength. Consistently, we observed a much stronger response to the antibody, about four times larger, thereby suggesting that the relative population of the PB forms with respect to rapidly dissociating ones is increased at low ionic strength. However, the decrease in the baseline to values lower than before the MyrUSH3 injection suggests that at low ionic strength, MyrUSH3 binding affects the immobilized liposomes. Consequently, we quantitatively analyzed the two binding processes presented below in the presence of 150 mM NaCl, a value close to physiological ionic strength.

4.4. Direct response of MyrUSH3 binding to liposomes

MyrUSH3 binding was initially measured at protein concentrations ranging from 1 μM to 100 μM using the direct SPR response. Below 20 μM , the binding curves had the expected shape, with an association phase leading to a plateau when reaching equilibrium, followed by protein dissociation after washing with buffer. At concentrations higher than 20 μM , the binding curves did not reach a stable plateau after 60 s. Failure to reach a stable equilibrium, at high concentration may be due to slow binding or a cooperative binding of peptides over the lipid surface (White et al., 1998) – see Figure 4.7. We chose to restrict our study to concentrations between 1 μM and 20 μM .

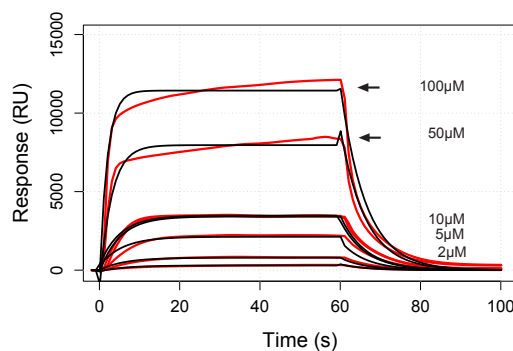


Figure 4.7: *MyrUSH3 direct binding curves to liposomes, ranging from 1 μM to 100 μM . While saturation appears to be achieved at 10 μM , a change in the binding behavior is observed at higher concentrations (50 μM and 100 μM), which showed a very high SPR response and deviation at equilibrium. (red) experimental curves (black) fitted curves*

The direct response of MyrUSH3 binding to liposomes was studied in order to obtain kinetics and affinity information on the fast associating and dissociating population. While the presence of multiple binding modes would in principle prevent the use of a simple model, the contribution of the PB form to this response was very small. We thus first analyzed the data with a simple 1:1 Langmuir model. From a formal point of view, peptides partition to liposomes, and stoichiometry is most often unknown. Nevertheless, simplification using the Langmuir model gives a satisfactory starting point for analyzing kinetics data obtained by SPR (Gohlke et al., 2010) (Hong et al., 2002). A reasonable fit of our experimental curves was obtained, especially for the association phase, although

deviations were observed at the end of the dissociation phase. (see Figure 4.8 A and B).

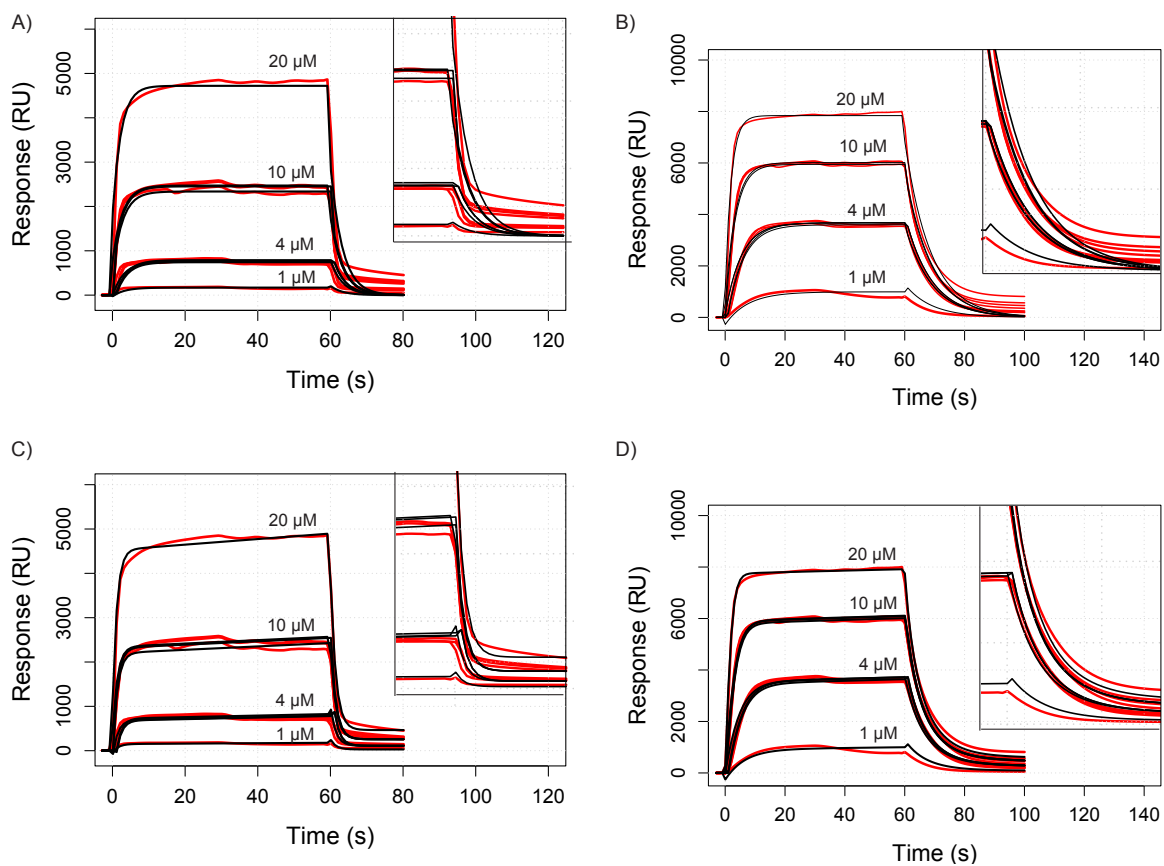


Figure 4.8: *SPR responses (red) after 60 s association of MyrUSH3 to (A) (C) DMPC and (B) (D) DMPC:DMPG liposomes. (A) (B) Best fit curves (black) with the 1:1 model and (C) (D) with the conformational change model. Each image contains a zoom on the dissociation phase in the upper right corner.*

Data can also be treated with a conformational change model in the Biaevaluation software, which assumes two separate bound forms. Initial binding generates one of them (PL). PL may dissociate again or be transformed into a second species (PL*) that does not dissociate directly but has to be converted back to PL before dissociation. The model involves two sets of association and dissociation rate constants: k_{a1} and k_{d1} , k_{a2} and k_{d2} .



The final association and dissociation rates (and their standard deviations) were derived by averaging the results from the global fitting of nine sets of binding curves using

the two models.

The association constant was derived from the ratio of the rate constants. Data for charged and neutral liposomes are presented in Tables 4.1 and 4.2.

Table 4.1: *Kinetics parameters resulting from the fitting of the direct SPR data of MyrUSH3 binding to liposomes applying the 1:1 Langmuir model. Association and dissociation rate constants, as well as association constant, derived from the fitting of MyrUSH3 binding curves to neutral or charged liposomes.*

	$k_a(\text{M}^{-1} \cdot \text{s}^{-1})$	$k_d(\text{s}^{-1})$	$K_D(\text{M}^{-1})$
DMPC	$4.54 \cdot 10^3 \pm 2.18 \cdot 10^3$	$2.81 \cdot 10^{-1} \pm 1.73 \cdot 10^{-1}$	$1.54 \cdot 10^4 \pm 7.01 \cdot 10^3$
DMPC:DMPG 2:1	$2.46 \cdot 10^4 \pm 1.31 \cdot 10^4$	$1.29 \cdot 10^{-1} \pm 8.63 \cdot 10^{-2}$	$1.9 \cdot 10^5 \pm 6.52 \cdot 10^4$

Table 4.2: *Kinetics parameters resulting from the fitting of the direct SPR data of MyrUSH3 binding to liposomes applying the Conformational Change model. Association and dissociation rate constants derived from the fitting of MyrUSH3 binding curves to neutral or charged liposomes.*

	$k_{a1}(\text{M}^{-1} \cdot \text{s}^{-1})$	$k_{a2}(\text{s}^{-1})$	$k_{d1} \cdot (\text{s}^{-1})$	$k_{d2}(\text{s}^{-1})$
DMPC	$5.56 \cdot 10^3 \pm 2.44 \cdot 10^3$	$6.58 \cdot 10^{-1} \pm 2.62 \cdot 10^{-1}$	$3.44 \cdot 10^{-3} \pm 3.05 \cdot 10^{-3}$	$8.16 \cdot 10^{-3} \pm 1.64 \cdot 10^{-2}$
DMPC:DMPG	$3.21 \cdot 10^4 \pm 1.82 \cdot 10^4$	$2.53 \cdot 10^{-1} \pm 1.23 \cdot 10^{-1}$	$1.92 \cdot 10^{-3} \pm 1.59 \cdot 10^{-3}$	$4.9 \cdot 10^{-3} \pm 7.25 \cdot 10^{-3}$

The association constant for binding of MyrUSH3 to charged liposomes was one order of magnitude higher than that of binding to neutral liposomes. Independent determination of this K_a value by fitting the equilibrium responses as a function of protein concentration was possible for charged liposomes and gave a value ($1.35 \cdot 10^5 \text{ M}^{-1} \pm 6.4 \cdot 10^4 \text{ M}^{-1}$) in good agreement with that obtained from the ratio of rate constants. In the case of DMPC liposomes, data at concentrations higher than 20 μM would be required because of the lower affinity of the protein-lipid interaction. The association constant derived from the 1:1 Langmuir model ($1.5 \cdot 10^4 \text{ M}^{-1}$) for the binding of MyrUSH3 to DMPC liposomes is in good agreement with the value ($9 \cdot 10^3 \text{ M}^{-1}$) obtained by Buser et al. for MyrSH4 peptide using partition methods (Buser et al., 1994). The conformational change model provided a better fit to the dissociation phase of the SPR response than that of the 1:1 Langmuir model. Figure 4.8 shows expansions comparing the experimental and best-fit curves for MyrUSH3 binding to neutral and charged liposomes using the two models. While the conformational change model improves the fitting of the (fast) processes that dominate

the direct SPR response (Figure 4.8 C and D) and points to the presence of more than one second form of bound MyrUSH3, the corresponding dissociation rates are too fast to explain the PB form observed after more than 60 s of dissociation.

The existence of a persistently bound form of c-Src has strong biological implications and the characterization of the nature and kinetics properties of MyrUSH3 is thus of great interest.

4.5. PB form of MyrUSH3 binding to liposomes

The observation of the PB form after extensive washing, a procedure that removed the fast dissociating population, offers the possibility to study association and dissociation kinetics of the two species independently. In addition, secondary detection by antiSH4 antibody capture provided the required sensitivity to study the binding kinetics of the PB form, which is much less populated and gives a low SPR signal. By varying the MyrUSH3 association time, and applying the antibody at a constant time after the association phase, we were able to follow the association kinetics of the PB form independently. In a similar manner, by keeping the association time of MyrUSH3 binding to liposomes constant, and applying the antibody at different times of the dissociation phase, we were able to independently study its dissociation. The experimental design is shown in Figure 4.9.

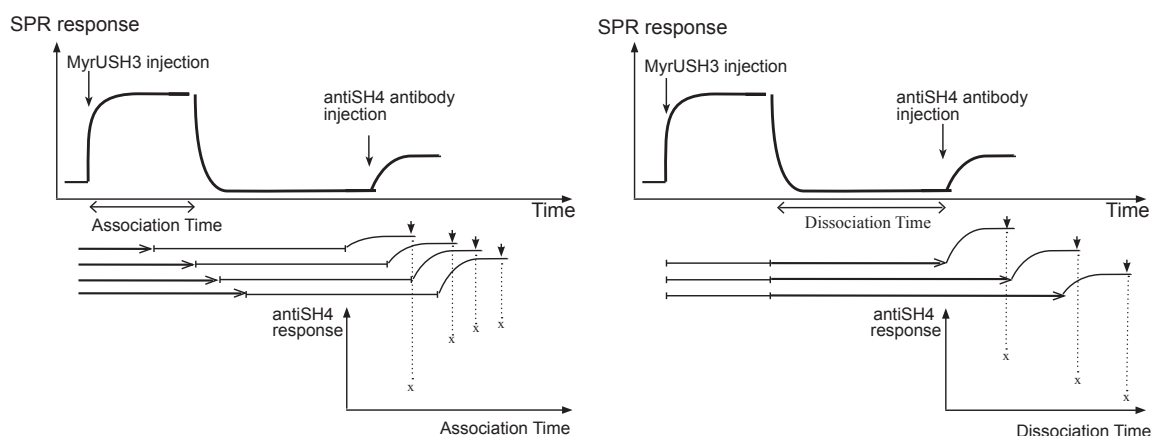


Figure 4.9: *Experimental scheme for the kinetics study of persistent forms of MyrUSH3. (A) The build-up of antibody detectable MyrUSH3 over liposomes is monitored over time by varying the association time using a constant dissociation time. (B) Dissociation is followed by varying the dissociation time after a constant association time.*

Figures 4.10-A and -B show the experimental curves obtained by this method for a given association/dissociation time (60 s/350 s) respectively. The antibody solution was injected at a constant dilution and therefore, the observed SPR response is assumed to reflect the population of MyrUSH3 present on the liposome surface. Indeed, the antibody response was greater with increasing the concentration of MyrUSH3 during the association phase.

Figures 4.11-A and -B shows the association phase of the PB form of MyrUSH3 bound

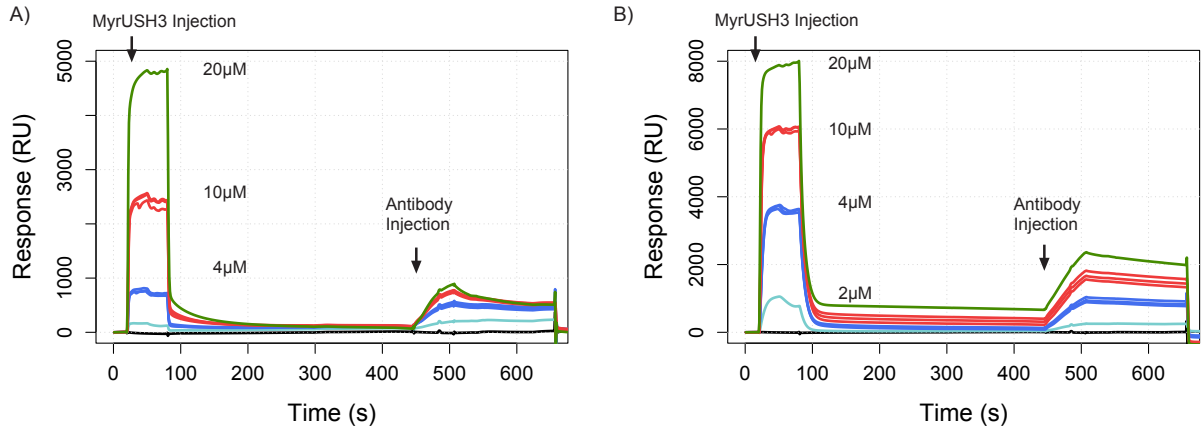


Figure 4.10: *Experimental results for the kinetics study of persistent forms of MyrUSH3. Direct and antibody detected SPR responses of 60 s/350 s association/dissociation of MyrUSH3 (between 2 μ M and 20 μ M) on (A) DMPC and (B) DMPC:DMPG liposomes respectively.*

to neutral and charged liposomes. Figures 4.11 C and D show their dissociation phase. Dissociation from neutral liposomes is negligible in the observed time frame and very slow from charged liposomes. The population of MyrUSH3 that remains bound to neutral DMPC liposomes did not decrease between 400 s and 7200 s after the end of the association phase. In the case of DMPC:DMPG liposomes, the bound population of MyrUSH3 after 400 s was higher than with neutral lipids and slowly decreased between 400 s and 7200 s. After 2 h, the population of MyrUSH3 bound to neutral and charged liposomes reached a plateau, suggesting that a fraction of this form became irreversibly bound to both types of liposomes.

On the light of the above observations, the process leading to the appearance of the PB form was analyzed using a “conformation change”-like model in which PL gives rise to PB in an irreversible manner. The rate constant of the transformation of PL to PB is designated as k'_{on} .



The model is presented using generic lipid containing species [L]; however the actual stoichiometry of the interaction is not known. For this reason we write the derived

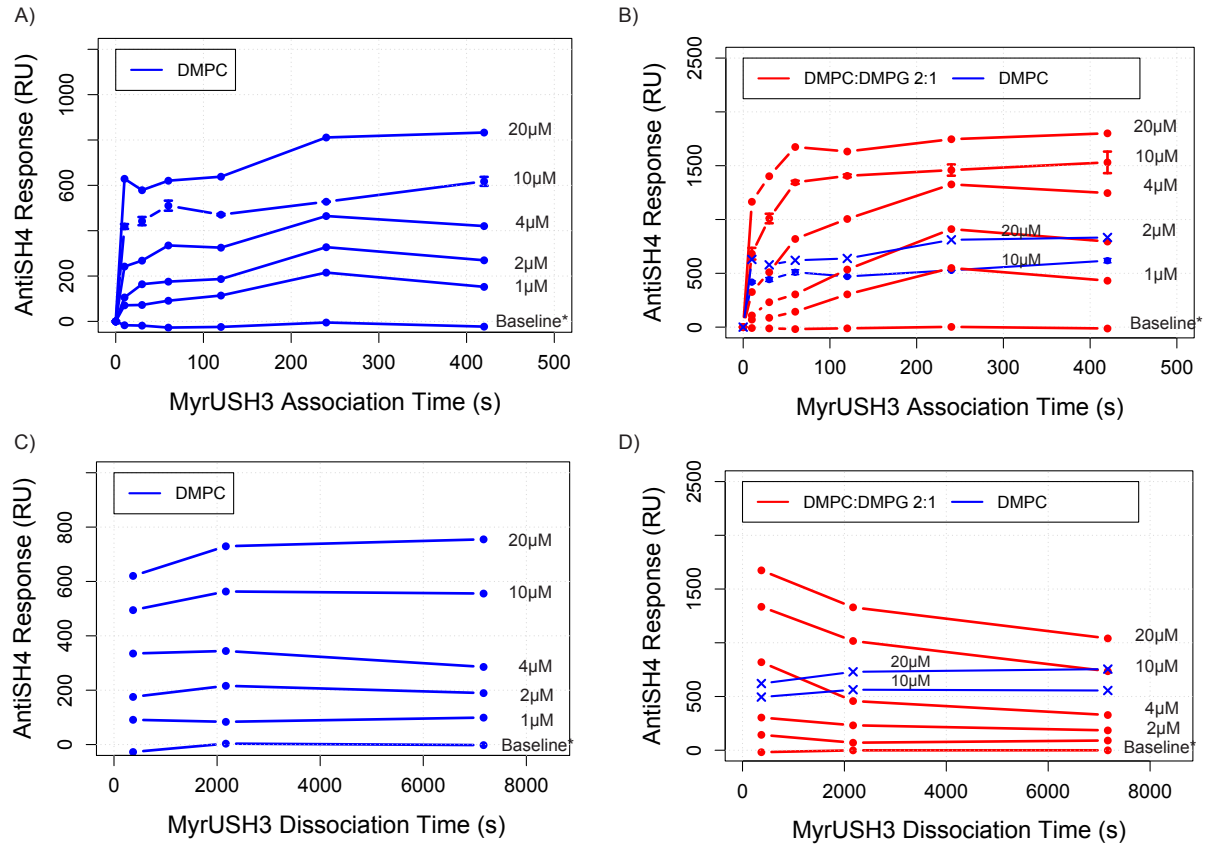


Figure 4.11: *Experimental results for the kinetics study of persistent forms of MyrUSH3. Association to (A) DMPC and (B) DMPC:DMPG, detected by antibody secondary detection after variable MyrUSH3 association times and constant dissociation times. Dissociation from (C) DMPC and (D) DMPC:DMPG, detected by antibody secondary detection, following 60 s MyrUSH3 association time and variable dissociation times.*Baseline corresponds to an identical experiment in which the protein was omitted.*

equations in terms of the fraction of lipids bound to protein, ϑ .

$$\vartheta = \frac{[\text{PL}]}{L_{\max}} + \frac{[\text{PB}]}{L_{\max}} \quad (\text{equation 4})$$

$$1 - \vartheta = \frac{[\text{L}]}{L_{\max}} \quad (\text{equation 5})$$

Since the amount of PB formed is small compared to that of PL, we assume that the kinetics of the formation of PB does not modify the equilibrium of PL:

$$\vartheta \sim \frac{[\text{PL}]}{L_{\max}} \quad (\text{equation 6})$$

This hypothesis explains the (experimentally observed) dependence of the binding levels with protein concentration. Without this hypothesis, the maximum concentration of [PB] would be the same for all concentrations in our equations, due to the irreversibility of the second equation.

Assuming (reaction1) is at equilibrium, we can derive an expression of ϑ .

$$\vartheta = \frac{K_a \cdot [\text{P}]}{1 + K_a \cdot [\text{P}]} \quad (\text{equation 7})$$

We want to solve the kinetics equation corresponding to (reaction 3)

$$\partial[\text{PB}]/\partial t = k'_{\text{on}} \cdot [\text{PL}] \quad (\text{equation 8})$$

In terms of occupation

$$\partial\vartheta_{\text{PB}}/\partial t = k'_{\text{on}} \cdot \vartheta_{\text{PL}} \quad (\text{equation 9})$$

Resolution of equation (equation 9) leads to the following expression:

$$\vartheta_{\text{PB}} = \vartheta \cdot (1 - \exp(-k'_{\text{on}} \cdot t)) \quad (\text{equation 10})$$

We call R the SPR response of the antiSH4 antibody binding to PB, which is proportional to [PB].

$$R = C_1 \cdot (1 - \exp(-k'_{\text{on}} \cdot t)) \quad (\text{equation 11})$$

with C1 proportional to $\vartheta \cdot L_{\max}$.

We fitted (equation 11) locally to the experimental curves obtained at various concentrations. The best-fit values are displayed in Figure 4.12 A for DMPC and Figure 4.12 B for DMPC:DMPG. Table 4.3 gives the values of the best-fit parameters.

Table 4.3: *Results from the fits (as shown in Figure 4.12-A and B), giving values for C_1 and k'_{on} (and the standard deviations of the fit coefficients) at the different protein concentrations, for DMPC and DMPC:DMPG 2:1 liposomes*

Concentration (M)		10^{-6}	$2 \cdot 10^{-6}$	$4 \cdot 10^{-6}$	10^{-5}		$2 \cdot 10^{-5}$
DMPC	C_1 (RU)	175.1 ± 26.9	272.1 ± 32.5	383.4 ± 32.8	512.3 ± 31	520.2 ± 23.3	697.3 ± 47
	k'_{on} (s ⁻¹)	0.01462 ± 0.004	$0.02269 \pm 0,006$	$0.06505 \pm 0,022$	0.1688 ± 0.077	0.1480 ± 0.046	0.2256 ± 0.155
DMPC:PG	C_1 (RU)	512.3 ± 75.6	889.3 ± 83.3	1223 ± 48.2	1484.1 ± 65	1419.8 ± 27.7	1678 ± 36.1
	k'_{on} (s ⁻¹)	0.00808 ± 0.002	0.00877 ± 0.001	0.01829 ± 0.002	0.04481 ± 0.009	0.05 ± 0.005	0.1050 ± 0.020

We observe that k'_{on} depends linearly on protein concentration (Figure 4.12-C and -D).

This concentration dependence of the apparent rate constants for the conversion between the initially (and reversibly) bound form of MyrUSH3 to the PB form indicates that the conversion between the two forms of is a bimolecular process and suggests that the PB species of MyrUSH3 are, at least, dimeric forms. Dimerization can be explicitly included in the binding model, assuming that PL can interact with a second P molecule to form PB, which we now explicitly label as PB₂ to stress its dimeric nature, with an association rate constant k_{on} :



In this case, ϑ_{PB_2} can be expressed as:

$$\vartheta_{PB_2} = \vartheta \cdot (1 - \exp(-k_{on} \cdot [P] \cdot t)) \quad (\text{equation 12})$$

Fitting using (equation 11) is equally applicable but in this case

$$k'_{on} = k_{on} \cdot [P] \quad (\text{equation 13})$$

The bimolecular k_{on} (unit M⁻¹ · s⁻¹) can be obtained from the slope of the linear fit between k'_{on} and the protein concentration [P]. For DMPC liposomes, $k_{on} = 1.2 \cdot 10^4 \text{M}^{-1} \cdot \text{s}^{-1}$.

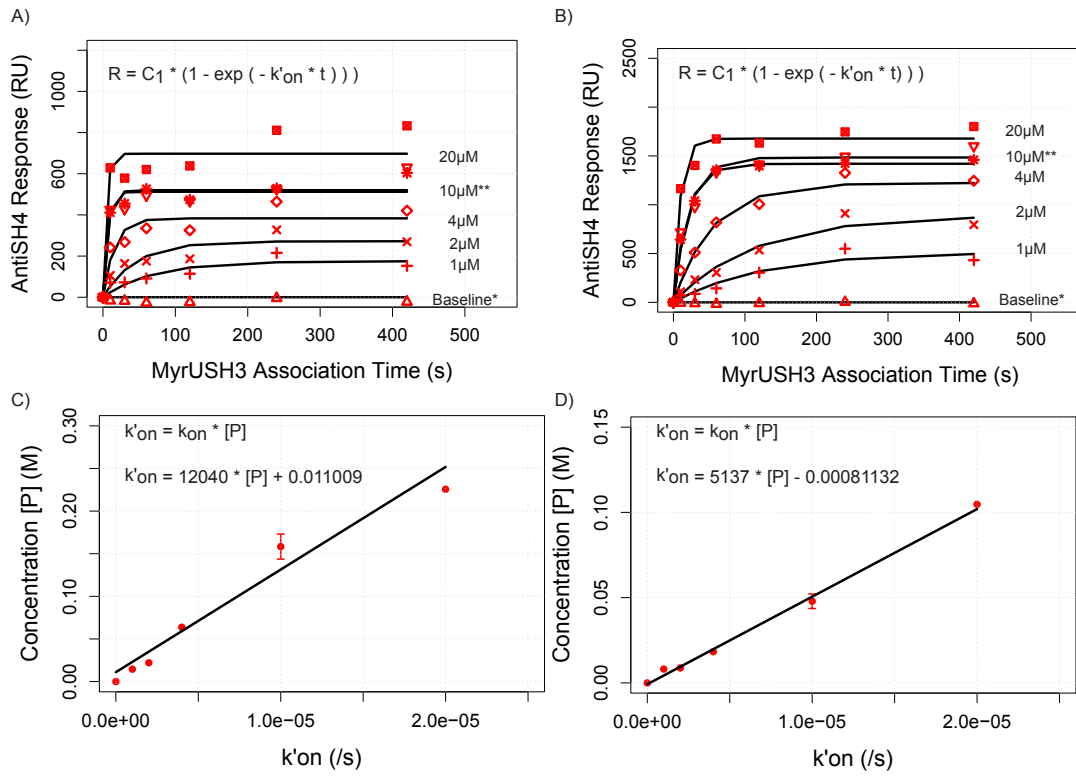


Figure 4.12: Indirectly detected binding curves of persistent forms of MyrUSH3. Build-up of persistent MyrUSH3 on (A) DMPC and (B) DMPC:DMPG, detected by antibody secondary detection, using a constant dissociation time of 350 s. The continuous curves correspond to the best-fits of the equation shown. Linear concentration dependence of k'_{on} for binding to (C) DMPC and (D) DMPC: DMPG liposomes. *Baseline corresponds to an identical experiment in which the protein was omitted. ** Duplicated results at 10 μ M are shown; the error bar is shown in (c) and (d).

s^{-1} , while for DMPC:DMPG liposomes, $k_{on} = 5.1 \cdot 10^3 M^{-1} \cdot s^{-1}$. The k_{on} value obtained with this model for neutral liposomes is larger than for charged ones. From the fitted k_{on} values it is possible to estimate and compare the true velocity of the PB species formation on both types of liposomes and at different protein concentrations. According to our model, the velocity v is given by:

$$v = k'_{on} \cdot R_{eq} \quad (\text{equation 14})$$

where R_{eq} is the direct SPR response at equilibrium (in Response Units)

The ratio of velocities of PB species formation in DMPC:DMPG versus DMPC alone, plotted as a function of protein concentration $[P]$, can be found in Figure 4.13. At low concentration, the formation is faster in charged liposomes, but the ratio reaches a plateau close to 1 at $10 \mu M$ and higher concentrations, suggesting that the inherent rate of dimer formation is the same in both cases. The difference observed at low concentration may come from a higher concentration of bound monomers and/or the accumulation of free protein with their positively charged N-terminal region oriented towards the surface.

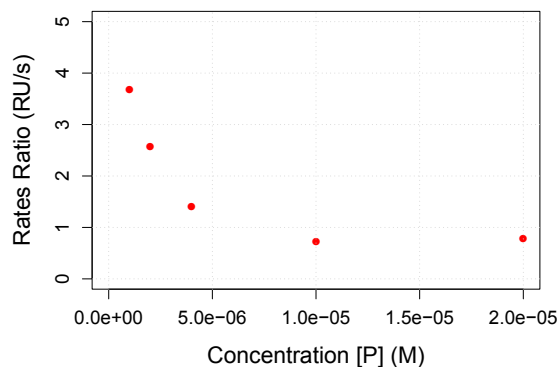


Figure 4.13: Plot of the Ratio $\frac{(k'_{on} \cdot R_{eq})_{DMPC:DMPG}}{(k'_{on} \cdot R_{eq})_{DMPC}}$, as a function of the protein concentration $[P]$

The model presented is only a first approximation to the binding process, which is probably more complex. The bound species are assumed to be either fast dissociating monomers or PB forms. While the second order rate constant needed to fit the experimental rate of formation of the PB species strongly points to the formation of dimers, the presence of higher oligomers cannot be ruled out. Also the model assumes that the [reaction 4](#) is irreversible, which would ultimately lead to an infinite concentration of PB_2 ,

since P is supplied in excess at a constant concentration. This is obviously not the case and the maximum concentration of PB_2 must be limited, possibly by the increase in lateral pressure induced by the inserted proteins (or their mutual electrostatic repulsion), which reaches a maximum level beyond which no further protein monomer or dimer can be inserted.

4.6. Conclusion and Discussion

All the members of the SFK have a myristoyl group attached to their N-terminal glycine residues. In addition, all of them, with the exception of c-Src, contain additional palmitoyl group(s) attached to cysteine residues in their SH4 domain. The hydrophobic acyl groups are essential for anchoring SFKs to membranes. The rest of the protein remains soluble and is connected to the membrane-anchored SH4 domain through the intrinsically disordered Unique domain. In the case of c-Src, insertion of a single myristoyl group does not provide enough hydrophobic energy to ensure stable binding. This problem has been extensively discussed in the literature (Buser et al., 1994) (Murray et al., 1998) (Sigal et al., 1994) and the consensus view is currently that permanent anchoring of c-Src is achieved by the combination of hydrophobic insertion of the myristoyl group and electrostatic interaction of the strongly positively charged SH4 domain with negatively charged membranes. Here we have used SPR to characterize the kinetics of binding of Myristoylated USH3, a construct that contains the main lipid binding region of c-Src, to immobilized liposomes. Surprisingly, two kinetically well-defined binding events were observed. The first one has fast association and dissociation rate constants and can be followed directly by the SPR response of MyrUSH3 injections. At low protein concentrations, a standard analysis of the binding curves gives an association constant that is in good agreement with that obtained by partition methods in similar systems. Most of the bound species can be washed away from the immobilized liposomes with buffer. However, a small population of a PB form of MyrUSH3 was observed. While the corresponding SPR response was only a small shift from baseline level, the presence of the PB species was confirmed by capture of an antiSH4 antibody by the proteins remaining on the liposome surface. The antibody SPR response is proportional to the PB population. The latter was retained for more than 2 h and could be eliminated only by detachment of the immobilized liposomes by means of detergents. When liposomes were not exposed to protein, no antibody was captured.

A PB form was also observed using a synthetic myristoylated peptide containing only the SH4 domain (MyrSH4). In contrast, USH3 with a C12 lauryl chain instead of the C14 myristoyl chain showed no PB form, though fast association and dissociation processes were observed (with lower affinity than MyrUSH3). Thus from the structural point of view, MyrSH4 is a necessary and sufficient requirement for the observation of persis-

tent binding. The electrostatic contribution was tested by comparing MyrUSH3 binding to neutral and negatively charged liposomes, and by the effect of the ionic strength. MyrUSH3 affinity and also the rate of formation of the PB form increased in negatively charged lipids. The effect is consistent with an electrostatically driven approach of MyrSH4 to the liposome surface. The very slow dissociation kinetics and the secondary detection using antiSH4 antibody allowed an independent analysis of the kinetics of PB species formation. The association kinetics depends on the population of the labile form of bound MyrUSH3 and the concentration of free MyrUSH3 during the association phase. Thus, PB formation follows an effective second order law, thereby suggesting that the PB form contains more than one MyrUSH3 molecule.

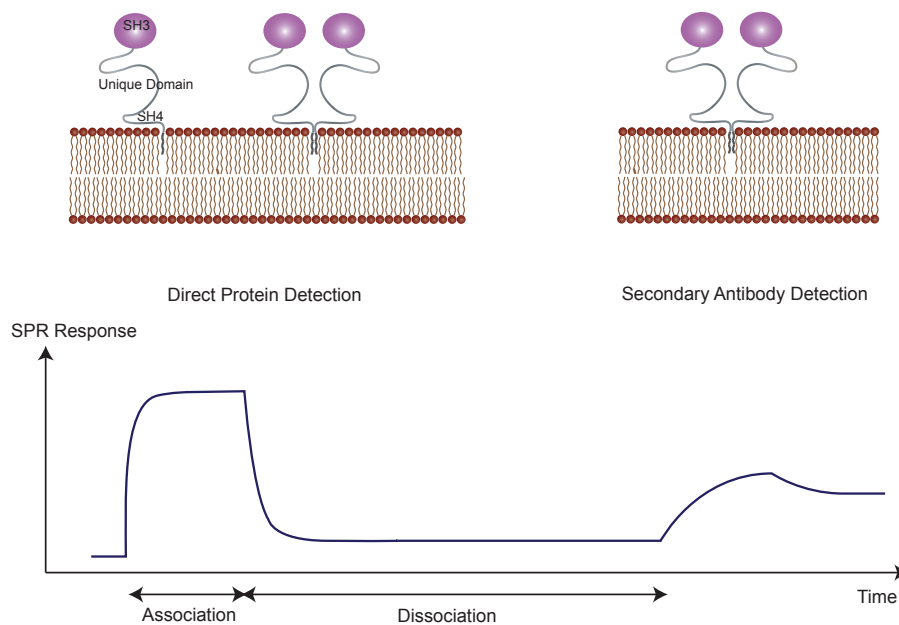


Figure 4.14: *Illustration of the possible dimer formation during the SPR assay*

While membrane-integrated receptor kinases are known to oligomerize, usually in response to ligand binding, and this process triggers signaling events, non-receptor kinases, like c-Src, are commonly believed to be essentially monomeric, even when anchored to membranes. However, c-Src phosphorylation by a second c-Src molecule is known to occur, therefore at least transient contacts between c-Src molecules are functionally important. A super resolution spectroscopy study, in which the first 15 residues of the c-Src SH4 domain fused to photoswitchable (PS-GFP2) were expressed in HeLa cells, provided evidence of the formation of clusters of MyrSH4 containing proteins on the membrane

surface (Owen et al., 2010). Neither non-myristoylated USH3 nor myristoylated USH3 show evidence of dimerization in solution, therefore, we conclude that the dimerization of MyrUSH3 is directly related to membrane binding. Figure 3.5 shows that replacing the C14 myristoyl chain by a C12 lauryl also abolished dimerization, although LaurUSH3 retains the capacity to interact with lipids albeit with a lower affinity. These results suggest that myristoyl insertion is essential for MyrUSH3 dimerization. The strict requirement for a myristoyl group suggests that the insertion of the fatty acid chain in the lipid bilayer is necessary for dimerization. We speculate that myristoyl insertion induces a conformation of the SH4 domain that allows the dimerization to occur. In addition, MyrUSH3 dimerization is observed in the presence of either neutral or negatively charged lipids and the dimer formed is irreversibly bound in both systems. The slow dissociation of MyrUSH3 dimers is consistent with the effectively irreversible binding of other SFKs in which two acyl chains are simultaneously inserted in the membrane. The enhanced binding and dimer formation observed in negatively charged lipids probably reflects the additional binding affinity, which results in a larger concentration of bound MyrUSH3 monomer as well as the increased local concentration of soluble MyrUSH3 close to the membrane. The biological relevance of c-Src dimerization remains to be demonstrated but we suggest a possible functional role of the coexistence of a fast and a slow dissociation form of bound c-Src. In its resting state, inactive c-Src is found predominantly in the perinuclear region of the cell. However, upon activation c-Src accumulates in specific regions of the membrane. Switching between monomeric and dimeric forms of c-Src would facilitate the fast exploration of the membrane surface until the target site is located, at which point the binding mode would switch to the persistent form and signaling would start. At low concentrations, myristoylated c-Src can probably attach and dissociate rapidly from the membrane surface, allowing fast diffusion on the 2D membrane surface and efficient exploration of large membrane areas. However, a local increase in c-Src concentration, for instance by high local concentrations of negatively charged lipids such as phosphoinositides, may result in the formation of c-Src dimers, which will remain in this particular membrane site and trans-activation will be facilitated, thus triggering the relevant signaling pathway. A similar strategy is used by transcription factors to explore large regions of DNA. These systems switch from non-specific low affinity binding mode, to retain the transcription factor close to the DNA surface and facilitate fast one-dimensional diffusion,

to specific, high affinity binding at the target site. The observed dual lipid binding mode of c-Src suggest a similar mechanism in which fast dissociation allow diffusion close to the 2D surface of the membrane, while dimerization fixes c-Src at the target site to initiate downstream signaling. The problem of c-Src deactivation arises if the binding of the dimer to membrane is as strong as the one we measure here. Nevertheless, in the previous chapter, we found a location in the amino acid sequence of the Unique Domain that is more labile and easily subject to cleavage. It is not clear how this cleavage is triggered. One hypothesis could be crowding effects, since the degradation may be concentration dependent. Another one would be conformational restriction (a forced conformation might induce mechanical constraints favoring rupture). Such a degradation mechanism would enable to stop the signaling process, by detaching the kinase from the membrane.

4.7. Experimental Methods

Liposome preparation All lipids were from Tebubio. 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC, and 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt), DOPG were dissolved in chloroform. 1,2-Dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DMPG) was dissolved in chloroform:methanol:H₂O 65:35:8. Three lipid compositions were used, DMPC alone, DMPC:DMPG 2:1 or DOPC:DOPG 2:1. The lipid solution were evaporated in a rotavapor, and lipid films were rehydrated with buffer (50mM NaP, 150 mM NaCl, 0.2 mM EDTA, pH 7.5) by warming the lipid film at 40 °C and vortexing, rendering a final total lipid concentration of 1 mM. Large unilamellar vesicles were prepared by mechanical extrusion using a 10-mL Thermobarrel extruder (LipexTM. Northern Lipids Inc.). The lipid suspension was extruded repeatedly (10 times) through a 100 nm polycarbonate filter at 40 °C. To verify the appropriate size of the liposomes, the mean diameter was checked using Dynamic Light Scattering (Zetasizer Nanoseries S, Malvern instruments).

Direct SPR binding assay SPR experiments were carried out in a Biacore 100 instrument from GE Healthcare. The SPR chip (a 2D-carboxymethyl dextran surface) from Xantec was modified by covalent attachment of phytosphingosine (TebuBio) to allow the capture of neutral or negatively charged liposomes containing DMPC or DMPC-DMPG (2:1), respectively. Experiments were carried out at 25 °C where these lipids and lipid mixtures are in the liquid crystalline phase. Control experiments with the equivalent unsaturated oleyl lipids DOPC-DOPG (2:1) with a transition temperature of -17 °C were also conducted, by using injections of MyrUSH3 at 10 or 100 µM. The reference channel was blocked with BSA, with a 1 mg/ml injection at 10 µl/min for 100 s. The immobilization protocol and subsequent liposome coating were performed in the instrument chamber, at a flow rate of 10 µl/min. To minimize mass transport phenomena, a flow rate of 50 µl/min was used to monitor the interaction of MyrUSH3 with liposomes. The SPR response of MyrUSH3 binding to liposomes was followed for 60 s (association phase), while the subsequent washing lasted 200 s (dissociation phase). Protein concentration ranged from 1 µM to 20 µM, and all experiments were performed in the running buffer, which comprised 50 mM NaP, 150 mM NaCl and 0.02 mM EDTA, pH 7.5, unless indicated otherwise. The surface was regenerated with a 10-mM HCl pulse followed by a 20-mM

CHAPS pulse, both for 30 s at 10 μ l/min. Each experiment was started with freshly captured liposomes. The liposome coating itself was reproducible, reaching an average value of 6785 RU (standard deviation 625 RU) for DMPC liposomes, and 8193 RU (standard deviation 608 RU) for DMPC:DMPG liposomes, guaranteeing reproducible protein responses as shown in Figure 3.7.

SPR detected antibody capture MyrUSH3 dissociated very quickly from liposomes, but the presence of a residual population of lipid bound MyrUSH3 was detected by the SPR response observed after injecting antiSH4 antibody (Abcam) at a 1:10 dilution in the running buffer. The association and dissociation kinetics of these PB species were studied separately: dissociation was measured by recording the antibody response at increasing times after a constant association time of 60 s, while association was monitored by detecting the antibody response at a fixed time after the end of increasingly long association times. The flow rate was kept at 50 μ l/min during protein association and dissociation, and decreased to 30 μ l/min just before antibody injection.

Model and data analysis Direct SPR responses of MyrUSH3 binding to liposomes were analyzed using the Biaevaluation software from Biacore using two alternative models: the 1:1 model and the conformational change model. Data of the PB fraction, obtained by antiSH4 antibody secondary detection, were analyzed using Igor software, with the models described in the results section. All data were double referenced (reference channel and baseline subtraction).

Chapter 5

MyrUGFP binding to monolayers and SLBs

5.1. The Monolayer Technique

Amphiphilic molecules deposited at low concentration on top of a water surface arrange in a single layer at the air/water interface. If lipids are used, they are in practice dissolved in a solvent and deposited on top of an aqueous solution, and they spread and form a monolayer at the interface, in which the phospholipid head is in contact with the water and its tail points to the air. In this configuration, at the air/water boundary, there is a change in interfacial free energy, resulting in the existence of a surface pressure ([Maget-Dan, 1999](#)), or lateral pressure (defined as the first derivative of the monolayer free energy with respect to the surface area).

In the case of membrane bilayers, the concept of lateral pressure is not relevant since they are in a tension free state. A lateral pressure manifests only if the membrane is subject to a lateral stress, and in this case the concept of lateral compressibility can be applied. In the case of a monolayer, the lateral compressibility is defined as the slope of the surface pressure against area isotherm. This enables to derive a monolayer pressure equivalent to a bilayer, which has been found to range from 30 to 35 mN/m ([Marsh, 1996](#)) depending on lipid composition, aqueous buffer composition, temperature, etc.

The surface pressure can be easily measured, for instance using a Wilhelmy plate. A thin plate is vertically immersed at the interface. The force acting on this plate is

measured by a tensiometer, or an electrobalance, and is directly proportional to the surface pressure (Maget-Dan, 1999).

Once the monolayer is formed, it can be used to study how the peptide (or protein) partitions to the monolayer, and in particular how it inserts into the monolayer, by injecting of the peptide in the subphase. After spreading lipids at a desired constant pressure P_i , a change in the surface pressure will occur, caused by peptide insertion, and can be monitored over time. The surface pressure will ultimately reach a stable equilibrium pressure P_e (Calvez et al., 2009). Figure 5.1 shows the experimental set up, as well of typical pressure changes curves recorded through the Wilhelmy method.

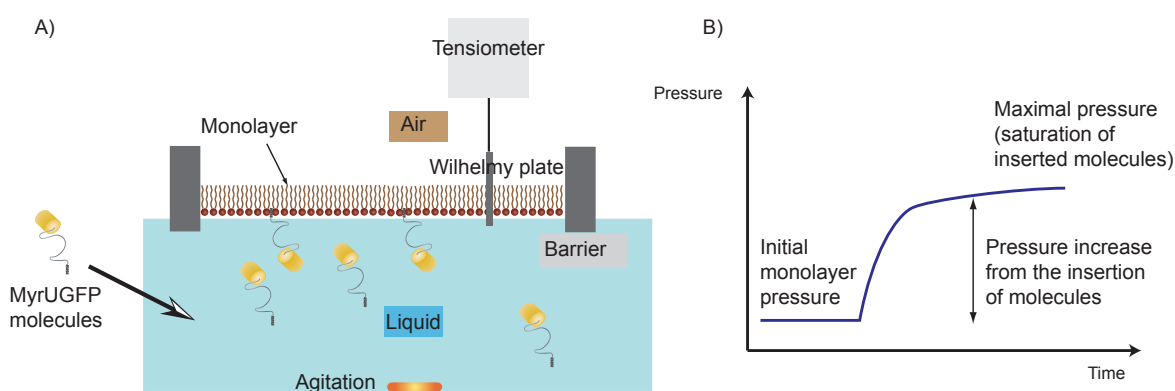


Figure 5.1: A) Monolayer set up; lipids are organized at the air water interface. Pressure is measured by a Wilhelmy plate. B) If a molecule inserts into the lipid bilayer, pressure increases from the initial monolayer pressure P_i until the equilibrium pressure P_e .

One parameter that can be extracted from such a set up is the maximum insertion pressure (MIP) of a peptide in a monolayer of specific composition. The change in surface pressure $P_e - P_i$ can be recorded when injecting a constant concentration of peptide at different initial pressures. At low concentration, this change is linear with initial pressure P_i (Marsh, 1996). If the experimental data render a linear plot of $P_e - P_i$ versus P_i , then linear regression permits to interpolate the maximum insertion pressure (or critical insertion pressure (Rafalski et al., 1990)) of the peptide in the monolayer $P_{\max}$. By knowing the equivalent pressure for a bilayer, this experiment gives insight on the insertion capability of a peptide in a bilayer. If $P_{\max}$ is higher than the equivalent lateral pressure of 30-35 mN/m, the peptide is potentially enable to insert into the bilayer. This technique is very often used to measure the insertion properties of peptides to lipid mono-

layers (Sánchez-Martín et al., 2011) (Alay et al., 2013). However, several studies have been conducted which involve myristoylated proteins, for instance Calcineurin or Recoverin. The values of MIP obtained with myristoylated and non-myristoylated forms of Calcineurin were comparable, suggesting that the myristoyl group does not contribute to monolayer binding in the conditions used (probably due to the recruitment of the myristoyl chain in the pocket) (Calvez et al., 2009). On the contrary, binding of recoverin to monolayers was significantly different in the myristoylated form (Desmeules et al., 2007)

Myristoylated proteins are amphiphile entities, therefore, if they are injected in the subphase in absence of lipids, they will have a tendency of going at the air/water interface. Such an experiment enables to determine the activity of the protein; at low concentration, a small pressure change will be noted at the interface. The pressure change will increase with protein subphase concentration until a plateau is reached, in which no more proteins can fit at the surface. Determining the activity of the protein and the concentration at which saturation occurs permits to choose the concentration at which the protein will be injected in the MIP experiment. Indeed, this experiment must be conducted at constant protein concentration in the subphase, which should be high enough to obtain sufficient signal, but not too high to prevent artifacts. The concentration rendering half of the pressure change at saturation is usually chosen.

In the case of c-Src, most of the studies have been performed with the N-terminal MyrSH4 peptide. We measured the MIP obtained for MyrSH4 inserting into a DMPC: DMPG 2:1 monolayer, but also for a bigger construct, constituted of the myristoylated Unique Domain followed by a fluorescent reporter (enhanced Green Fluorescent Protein). We compare the insertion characteristics of both constructs, and showed that both can insert into a bilayer, but the values obtained in case of MyrUGFP were closer to biologically relevant values.

5.2. MyrUGFP binding to lipid monolayers

The surface properties of two myristoylated constructs (MyrSH4 and MyrUGFP) were initially explored, and their minimum insertion pressure (MIP) was subsequently determined for a monolayer composed of DMPC:DMPG 2:1 monolayer at room temperature.

Surface properties The surface properties of MyrSH4 were explored from 100 nM to 10 μ M. At 100 nM, a surface pressure increase of 4 mN/m was observed (Green curve in Figure 5.2-A). Nevertheless, higher concentrations did not result in higher pressure changes. At 500 nM and above, a small pressure change of about 1 mN/m was observed, followed by a pressure decrease indicating peptide desorption from the interface (Red and blue curves in Figure 5.2-A). This abnormal behavior suggests that at concentrations of 500 nM and higher, MyrSH4 forms micelles in solution. The typical “activity curve” plotting the pressure increase as a function of concentration could not be determined.

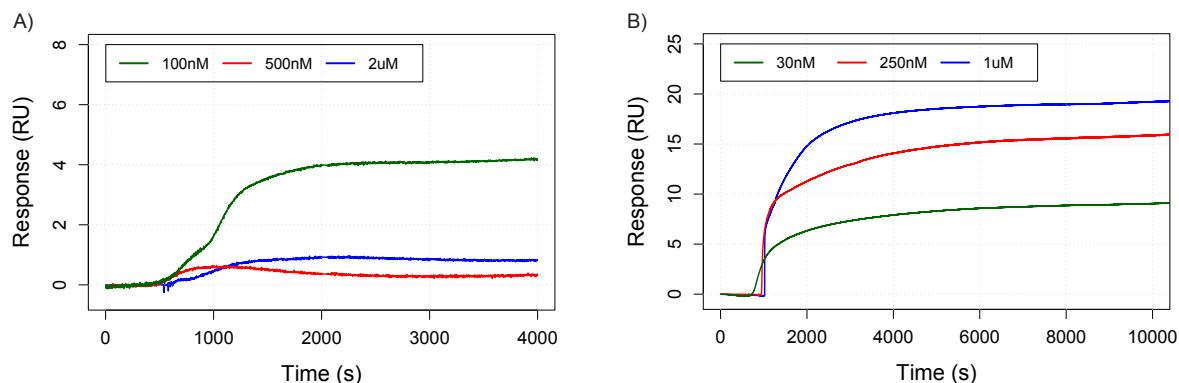


Figure 5.2: *Surface pressure increase due to the adsorption at the air-water interface A) of MyrSH4 at 100 nM, 500 nM and 1 μ M B) of MyrUGFP at 25 nM, 250 nM and 1 μ M*

On the contrary, MyrUGFP displayed a normal behavior, with surface pressure increasing with concentration, until saturation of the surface with the proteins was reached. Figure 5.2-B shows examples of pressure increase at the interface due to MyrUGFP adsorption at three different concentrations. Figure 5.3 shows the corresponding surface activity plot for MyrUGFP (pressure change versus protein concentration).

The pressure changes obtained with the peptide or with MyrUGFP can be compared at 100 nM concentration, given the hypothesis that MyrSH4 does not form micelles at this concentration. The 100 nM-MyrSH4 injection induced a pressure change of 4 mN/m,

while the 100 nM-MyrUGFP injection induced a pressure change of about 13 mN/m. This difference can be explained by the increased number of exposed hydrophobic amino acids of the Unique Domain. The GRant AVerage of hYdropathy (GRAVY) of MyrSH4 is -2.553, while that of MyrUGFP is -0.608, indicating that the latter is much more hydrophobic than the peptide. The disordered Unique Domain possesses indeed several exposed hydrophobic amino acids.

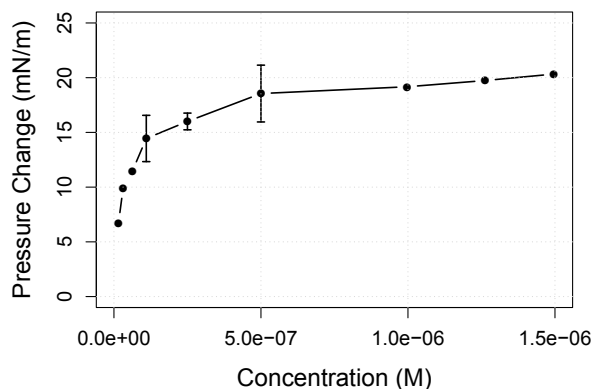


Figure 5.3: *Activity curve of MyrUGFP*

We next studied the insertion of MyrSH4 or MyrUGFP into a DMPC:DMPG 2:1 monolayer. The peptide or the protein were added at a constant concentration but at different initial pressures. The working concentration for MyrSH4 was chosen arbitrarily to be 1 μ M. In the case of MyrUGFP, the activity curve in Figure 5.3 was used to determine the working concentration – a concentration rendering about half of the maximal pressure was chosen, that is to say 100 nM.

Here again, the pressure changes induced by MyrUGFP were higher than for the peptide. After injection of 1 μ M MyrSH4, the pressure increased continuously until stabilizing to a plateau – the abnormal behavior monitored in absence of lipids was not observed. Figure 5.4-A and -B shows examples of peptide or protein insertion in the monolayer, at two initial pressures. In both cases, the pressure change is higher when the initial pressure is lower. The plot of the pressure change versus initial pressure was linear, as illustrated in Figure 5.4-C and -D. Linear regression of the data enabled to determine the MIP for both species. A MIP of 50 mN/m was obtained for MyrSH4, way above 35 mN/m, indicating that MyrSH4 can certainly insert in membranes. The MIP of 34 mN/m for MyrUGFP was much lower, and also closer to typical values obtained from other proteins (Calvez

et al., 2009).

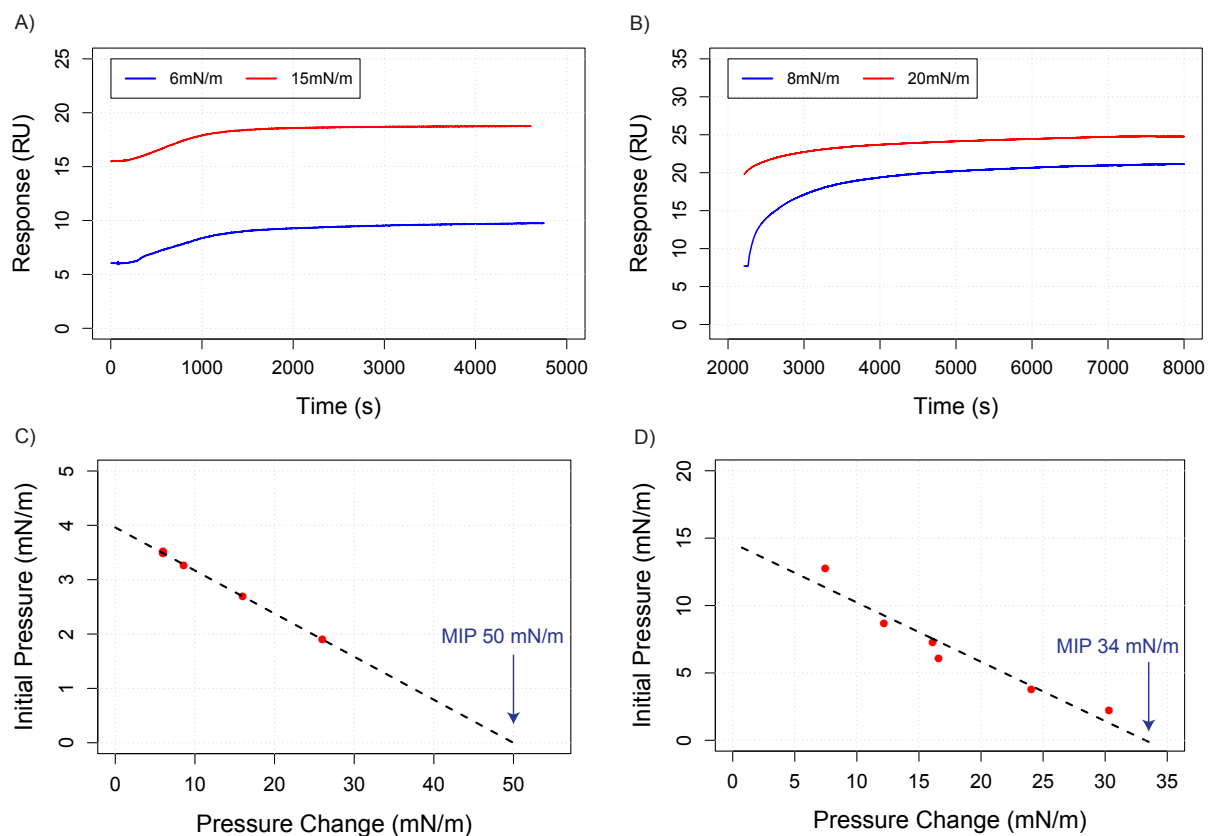


Figure 5.4: Surface pressure increase due to the insertion in the monolayer A) of $1 \mu\text{M}$ MyrSH4 at two initial pressures, 6 and 15 mN/m B) of 100 nM of MyrUGFP at two initial pressures, 8 and 20 mN/m. MIP plots for C) MyrSH4 and D) MyrUGFP

These experiments suggest that MyrUGFP, including the Unique Domain and a folded domain, is a system much more representative of the biological reality than the peptide alone. The micelle formation is prevented in this bigger construct, and the MIC is more realistic.

5.3. Single Molecule Photobleaching

Fluorescence is a process in which certain photoexcited molecules emit light of a longer wavelength than that of the light causing excitation, in a fast manner that do not involve intersystem crossing. This process takes place in three steps, as illustrated in Figure 5.5. **Excitation:** A photon of energy $h\nu_{\text{ex}}$ is absorbed by a molecule initially in its ground state. **Excited-state lifetime:** the molecule remains in the excited state a certain time, during which the energy is partially dissipated vibrationally to a lower energy excited state. **Emission:** a longer wavelength photon is emitted, $h\nu_{\text{em}}$, and the molecule returns to its ground state. The process can be cyclical, unless photobleaching occurs, in which the fluorescence is irreversibly lost in case of prolonged light exposure. Reversible loss of fluorescence may also occur upon quenching or blinking. Quenching is due to short-range interactions between the fluorophore and the local molecular environment (including other fluorophores). Blinking occurs randomly, the fluorophore switching between an ON (bright) and an OFF (dark) state under continuous excitation.

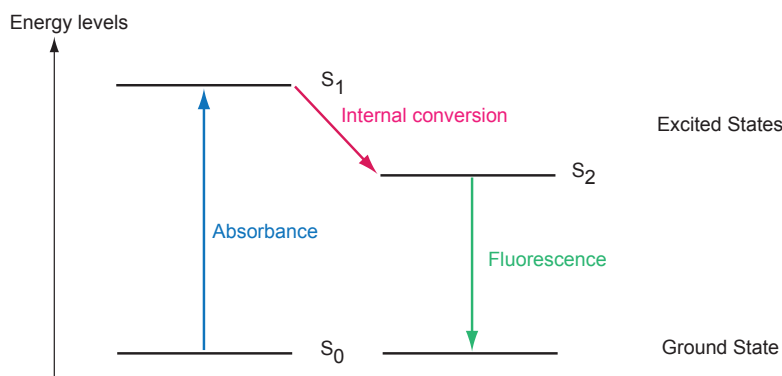


Figure 5.5: *Diagram illustrating the fluorescence phenomenon.*

Fluorophores can be excited for instance by laser illumination at the excitation wavelength, and fluorescent light is collected at the emission wavelength. In biological studies, fluorescent probes are used as reporters for other molecules. Fluorophores possess an extinction coefficient, related to their capacity to absorb light at a specific wavelength, that can be related to the molecule concentration. In our study, we used two fluorescent probes. One is a GFP mutant, directly attached at the N-terminal of MyrUSrc. The other one is a lipid functionalized with a fluorophore at the lipid head, namely 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) or LissRhode PE.

eGFP GFP acquires its fluorophore properties upon formation of a chromophore during protein folding. The chromophore maturation consists in a Cyclization-Oxidation-Dehydration Mechanism between 3 amino acids (Rosenow et al., 2004a) (Rosenow et al., 2004b) at positions 64-65-66; this process results in a loss of 21 Da with respect to the theoretical mass (calculated from the amino acid sequence). During protein expression, misfolding may occur, in particular if GFP is cloned after a non folded domain. Enhanced Green Fluorescent Protein has been engineered from GFP. It has faster folding and brighter fluorescence emission (Goossens et al., 2006) (Yang et al., 1996). Its excitation maximum is in the blue, at 488 nm, and its emission is in the green, with a maximum at around 507 nm (Tsien, 1998). Figure 5.18 shows the absorption and emission spectrum of eGFP. The molecule is most efficiently excited at 488 nm but can be excited over a wide range of wavelength, and fluorescence can be collected between 500 and 600 nm.

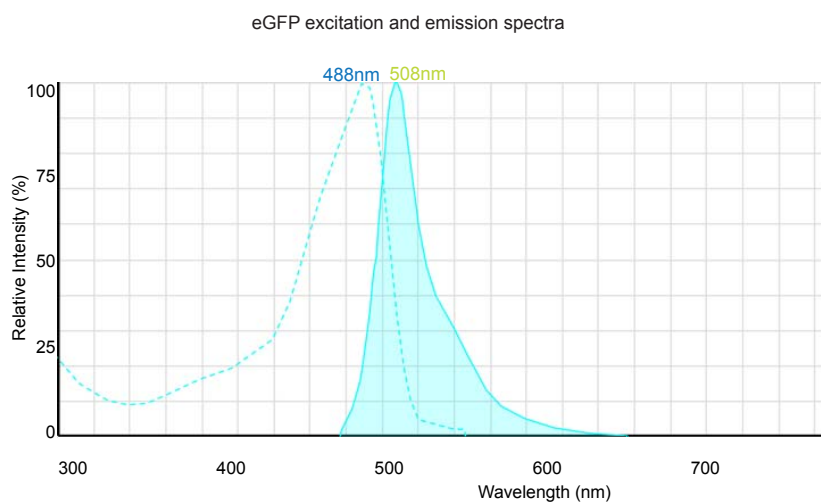


Figure 5.6: *Absorption and emission spectrum of eGFP.*

eGFP has an extinction coefficient of $56000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Lambert and Thorn) at 488 nm absorption. The construct in which an Enhanced Green Fluorescent Protein (eGFP) was appended after the Unique domain of c-Src was used. The amount of correctly folded GFP can be deduced by comparing the protein concentration calculated with the Beer-Lambert Law, in which

$$c = \frac{A}{\varepsilon \cdot l} \quad (\text{equation 15})$$

with A being the measured absorbance, ε the extinction coefficient and l the path

length of the cuvette. The concentration can be measured either by using the classical protein absorbance at 280 nm and the related extinction coefficient ($27515 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in case of UGFP), or by using the protein absorbance at 488 nm and the related extinction coefficient $56000 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

LissRhode PE DMPE is functionalized at the lipid head with LissRhode, as illustrated in Figure 5.7 A. Liss Rhode has a maximum absorption at 560 nm and a maximum emission in the red, at 583 nm. The absorption and emission spectrum is displayed in Figure 5.7 B. LissRhode is used as a fluorescent reporters to identify the supported lipid bilayer and characterize its homogeneity.

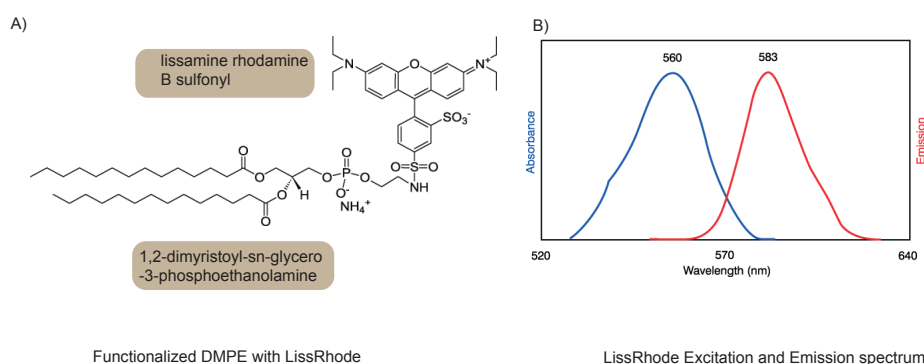


Figure 5.7: A) Chemical structure of LissRhode PE B) Absorption and emission spectrum of LissRhode. Data are from Avanti Polar Inc website.

Single Molecule Fluorescence Microscopy Use of fluorescence microscopy has grown exponentially in the field of cell biology, in particular since the discovery of fluorescent proteins such as the Green Fluorescent Protein from jelly fish. Recombinant techniques have allowed optimization of the fluorescence emission and excitation of such proteins and expanded the wavelength range available (Cormack et al., 1996). These fluorescence reporters can be cloned within the protein sequence and a recombinant protein terminated by a fluorescent domain can be obtained. The can also be used in cells as a reporter of gene expression and protein localization (Chalfie et al., 1994) (or colocalization with another protein derived with a second fluorescent marker). Use of such reporters allows fluorescent imaging in cells or in model systems with low background and high specificity

Nevertheless, conventional fluorescence microscopy is limited to study such molecules, because of optical diffraction. Optical systems will introduce distortions in which the

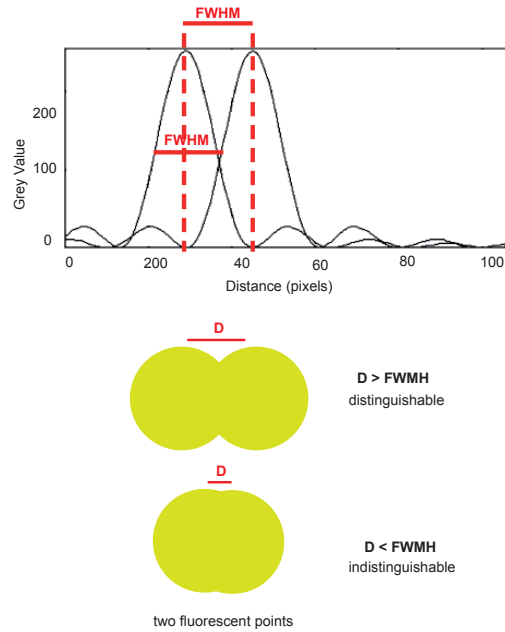


Figure 5.8: *Resolution criteria of two small sources, which diffracted image is a Airy function. The Rayleigh criteria corresponds to a separation of one FWHM between the points.*

image of an infinitely small light source is a typical 3D-pattern called the Point Spread Function (PSF), which can be modeled by a Airy function, and limits the lateral resolution of an image.

Image resolution is achieved when two points are far enough to be distinguished. The Rayleigh criterion is used to define resolution. It roughly states that, in order to distinguish between two points, the minimum distance between them corresponds to the the full width at half maximum (FWHM) of the PSF. It means that two points can be resolved if they are separated by at least the distance of a FWHM, which is about $\frac{\lambda}{2 \cdot \text{NA}}$, where λ is the wavelength of light and NA the numerical aperture. In practice, point sources need to be about 200 nm apart in the visible wavelength region to be distinguished with modern high-quality fluorescence microscopes.

There is also a limitation in axial resolution, of about $\frac{2\lambda \cdot n}{\text{NA}^2}$ with n the index of refraction, corresponding to a depth of field of 500 nm in the visible wavelength region with modern microscopes (Prasanna Pavani et al., 2009).

Since the size of proteins is below the diffraction limit, they cannot be resolved in an image by standard microscopy. If a fluorescent signal is imaged, it is not possible

to know how many molecules are included. Nowadays, several techniques have been developed to overcome the diffraction limit and obtain fluorescent images of very high resolution. One general concepts consists in selectively imaging only a few fluorophores within a sample. The center position of the PSF is considered to be the position of the excited molecule. Small populations of fluorophores are sequentially excited and the center positions of their PSF are recorded. By repeating the operation, accurate position of all fluorophores can be obtained and used to reconstruct a high resolution image (the protocol of such techniques is illustrated in Figure 5.9. To selectively excite the fluorophores, two techniques are mainly used. In Stimulated Emission Depletion (STED), fluorescence is depleted in specific regions of the sample while leaving a center focal spot active to emit fluorescence (Hell and Wichmann, 1994). In PhotoActivated Localization Microscopy (Zhong, 2010) (PALM) and STochastic Optical Reconstruction Microscopy (Rust et al., 2006) (STORM), photoswitchable fluorophores are used: the fluorophores are driven by light between an active-ON and an inactive-OFF state. In both cases, the fluorophores have to be specifically chosen in order to be manipulated according to the needs of the techniques.

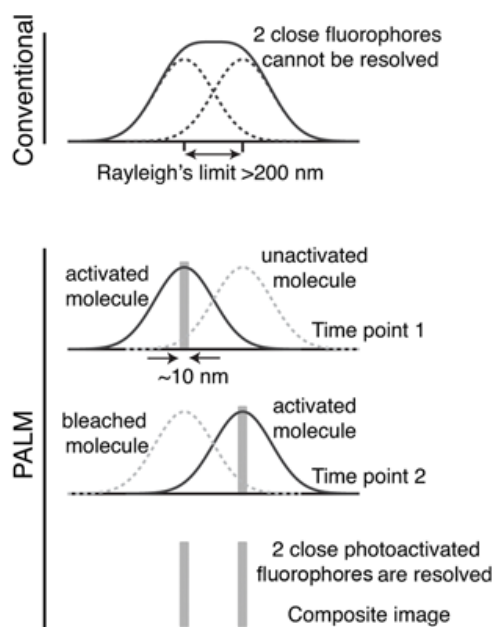


Figure 5.9: *Schematic representation of the protocol used when recording fluorescence of only a few chromophores in a sample. Image from Zhong (2010)*

In addition to dramatically improving image resolution, these techniques enable to image individual objects which size and distance smaller than the diffraction limit. Thus single protein imaged in a cell can be resolved, and if they are clustered, the number of protein in a cluster can be evaluated (Owen et al., 2010).

There are simpler ways of indirectly overcoming the diffraction limit and inferring the stoichiometry in a protein cluster. One of them consists in a single molecule photobleaching experiment (Garcia-Parajo et al., 2001) (Zijlstra et al., 2012). In this case, the fluorophore does not need to possess specific properties. The diffraction limit is not directly overcome either.

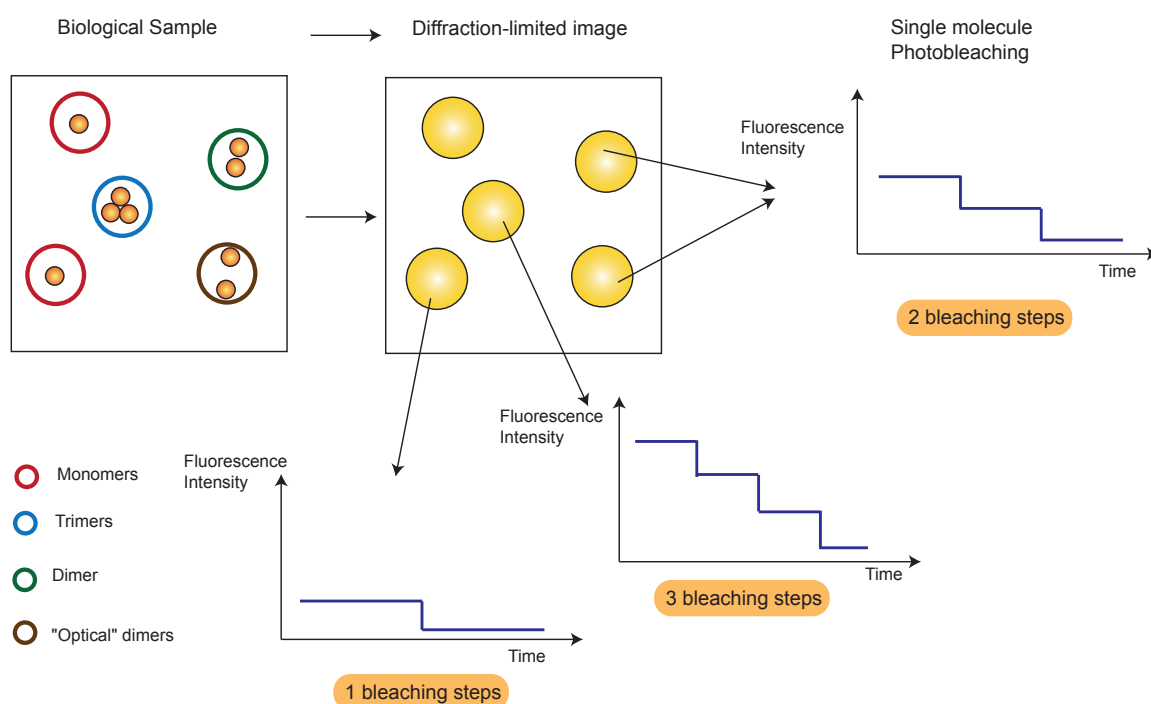


Figure 5.10: *Single Molecule Photobleaching protocol*

The image is performed with a scanning confocal microscope, rendering very high resolution. The fluorophore concentration of the sample needs to be adjusted in order to obtain a very low surface density. If the image size and the number of pixel are correctly adjusted, the image resolution permits to distinguish separate spots which size is close to the diffraction limit. In this configuration, single molecules are imaged. Nevertheless, if these molecules are in complex, many subunits are present in it. To know how many molecules are in the complex (one or several), each spot is subjected to a high laser power inducing bleaching of the molecule(s). Fluorescence recorded as a function of time will

show photobleaching steps, each step corresponding to one subunit. By simply counting the number of these bleaching steps, one can observe how many fluorophores were imaged and thus how many subunits are associated in the observed complex. The protocol of such an experiment is illustrated in Figure 5.10.

We applied single molecule photobleaching to test the formation of membrane-induced formation of c-Src clusters, by using eGFP as a fluorescent reporter. eGFP was included in the myristoylated c-Src construct as explained in 8.3, and the resulting molecule was called MyrUGFP. The fluorescent construct was bound to a supported lipid bilayer (SLB) formed by dipalmitoylphosphatidyl choline (DPPC), and dipalmitoylphosphatidyl glycerol (DPPG) (supplemented with LissRhode PE as a fluorescent probe). The association state of MyrUGFP bound to supported lipid bilayer (SLB) was probed using single molecule photobleaching (Figure 5.11).

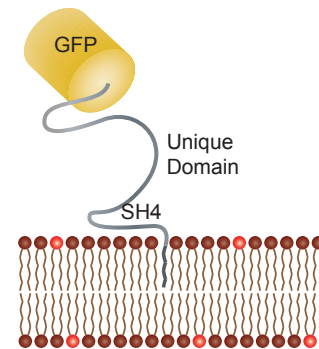


Figure 5.11: *Set up of the single molecule photobleaching of MyrUGFP bound to SLBs.*

5.4. Single Molecule Photobleaching of MyrUGFP binding to SLBs

We used the MyrUGFP construct bound to SLBs made of DPPC:DPPG 2:1 in a single molecule photobleaching experiment. Supported lipid bilayers (SLB) were obtained by depositing 100 nm-liposomes on glass. After 1-h incubation of the liposomes at 45 °C (DPPC and DPPG are fluid above 41 °C), excess lipids were rinsed and MyrUGFP was incubated over the SLB for 30 min, at 45 °C. The excess protein was rinsed with 3 mL of cold buffers. The sample was mounted in a chamber enabling imaging in buffer solution at room temperature, in which the lipids are in gel phase, thereby limiting protein diffusion in the bilayer.

Figure 5.12 shows a two-color image of the fluorescent SLB and bound proteins. The protein concentration was adjusted for single molecule detection. Individual, well-separated spots of GFP signal, randomly distributed, were identified, indicating the successful incorporation of MyrUGFP in the SLB.

Figure 5.13 shows a confocal image of the fluorescence emitted by MyrUGFP after incubation of 200 pM protein over the SLB. Visual inspection of the discrete fluorescent spots in Figure 5.13 indicated a heterogeneous distribution, ranging from low (gray arrows) to very high brightness (red arrows), with a majority of the spots having intermediate intensity values (white arrows). Since brightness is directly related to the number of molecules, these results already suggest that incorporation of MyrUGFP on the SLB was heterogeneous.

We used single molecule photobleaching to accurately quantify the number of proteins per spot. Statistics were gathered at three different incubation concentrations, in which individual spots could be distinguished, namely 200 pM, 500 pM and 750 pM. MyrUGFP was imaged

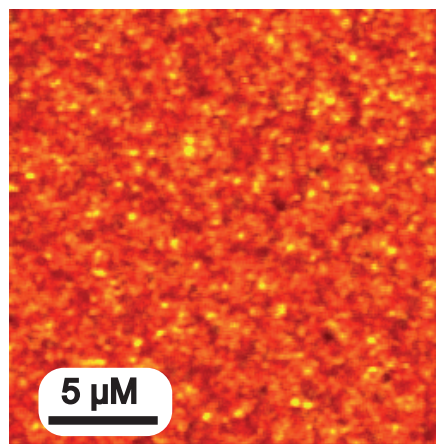


Figure 5.12: *Merge of the fluorescence images obtained from the Liss Rhode embedded in liposomes (red) and from MyrUGFP (yellow)*

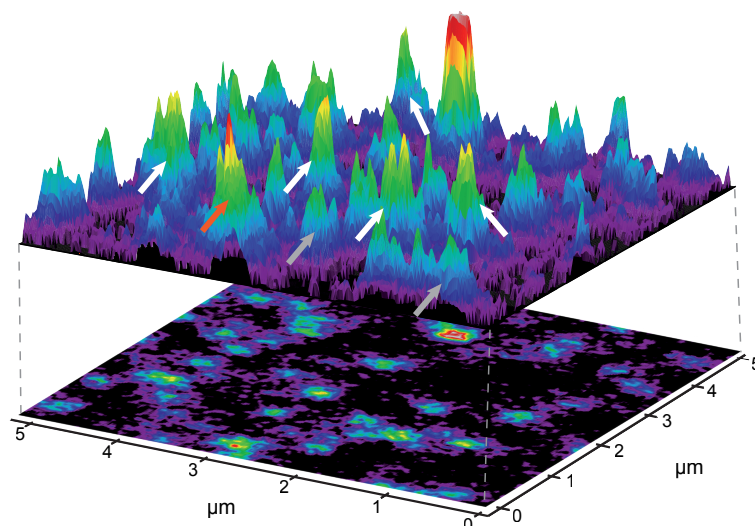


Figure 5.13: *Fluorescent image of MyrUGFP proteins bound to deposited liposomes, after protein incubation at 200pM, ensuring a single molecule regime; 5*5 μm area, 256 pixel resolution. The arrows show different intensities, low (grey), medium (white) and high (red), possibly corresponding to different numbers of molecules present in each spot.*

at low power to avoid bleaching during image recording. Subsequently, a higher power was applied to each spot to induce photobleaching. Fluorescence recorded as a function of time showed discrete photobleaching steps, from which the number of monomers present in a particular spot could be determined. Figure 5.14 shows examples of time traces, in which one (Figure 5.14-A), two (Figure 5.14-B) or three (Figure 5.14-C) molecules were bleached.

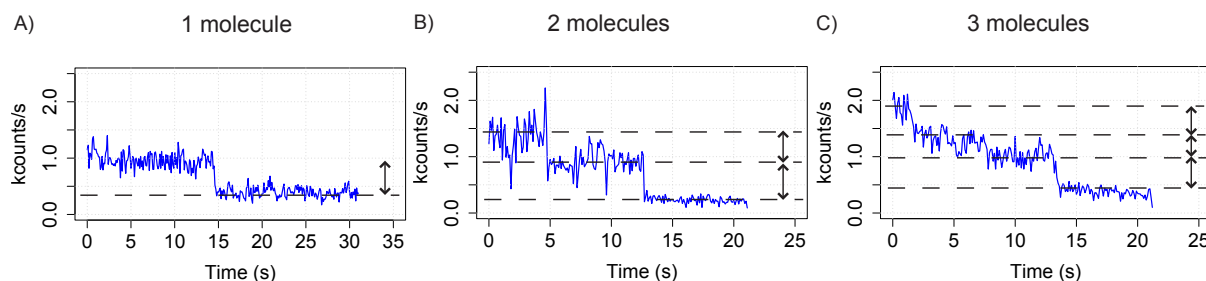


Figure 5.14: *Time resolved fluorescent traces of MyrUGFP photobleaching when the bleached fluorescent spot is composed by A) one B) two and C) three molecules.*

Traces were classified according to the number of bleaching steps, corresponding to 1, 2, 3 or more molecules in one spot. Figure 5.15-A shows the histograms corresponding to the classification obtained for these experimental data at the three distinct concentrations. The number of monomers (16 % at 200 pM, 24 % at 500 pM and 12 % at 750 pM) was lower than that of dimers (57 % at 200 pM, 46 % at 500 pM and 48 % at 750 pM) at all concentrations. Trimers are also found (21 % at 200 pM, 24 % at 500 pM, and 28 % at 750 pM) and their number steadily increased with concentration. Spots containing four or more molecules represented less than 6 % at 200 and 500 pM, and 12,5 % at 750 pM. These data show that a majority of MyrUGFP species dimers was found (about 50 %) when bound to the SLB, at all concentrations used.

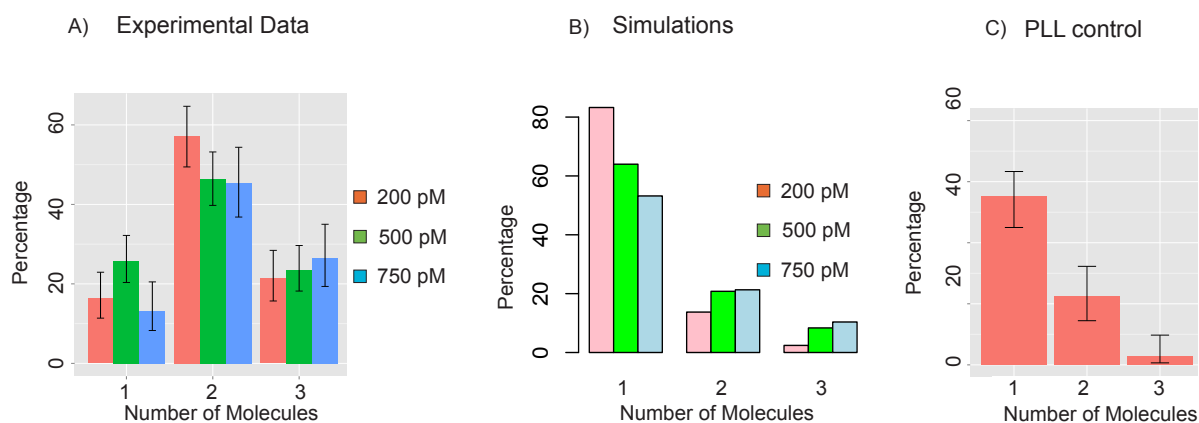


Figure 5.15: *Histogram of the number of bleaching steps observed for each discrete spots at the different concentrations A) experimental data (159 fluorescent trajectories at 200 pM, 220 at 500 pM and 115 at 750 pM) B) simulations (100 spots were simulated) C) control on PLL (62 fluorescent trajectories).*

To exclude that the oligomerized species counted in the experimental data were not “optical dimers” (due to the probability of counting adjacent monomers as associated species when crowding occurs at higher concentration), the experimental data were compared with simulations of random distribution of particles. The experimental densities obtained at the three concentrations and the spatial resolution of the confocal microscope were taken into account. The experimental densities were derived by counting the density of fluorescent spots and the experimental distribution of clusters of various stoichiometry. Spots were convoluted to mimic a diffraction-limited sample and statistics were derived. A majority of monomers was found, from 83 % at 200 pM to 53 % at 750 pM and a

much lower number of dimers (from 14 % at 200 pM to 21 % at 750 pM) and trimers (Figure 5.15-B). Expectedly, the number of higher order oligomers increased with concentration. Thus, a significant deviation between the experimental data and the simulations was found, which clearly shows that the observed dimers are “biological dimers”. To rule out that dimers were not due to eGFP dimerization, a control was performed on a cover slip coated with polylysine, in absence of SLB. MyrUGFP was incubated over the polylysine surface, at concentration rendering a protein density comparable with that of a 500 pM-SLB coated sample. A very distinct distribution of the association state of MyrUGFP was found, as shown in Figure 5.15-C. Monomeric species were the most abundant (68 %), while only 27 % of dimers and 5 % of trimers were found. This distribution is consistent with a random deposition of monomeric particle.

A statistical test was performed to verify that the difference between the fraction of monomers of the experimental data and of the control data was significant. The test was repeated for the fraction of dimers. All tests were performed after adjusting a linear model with "day" as covariate, since the data acquisitions have been repeated at different days. Table ?? shows the resulting p-values; p-values below 0.05 indicate that the difference between two values is statistically significant. All p-values were significant except when comparing the dimer fractions at 750 pM; crowding effect occurring at this high concentration probably explains this absence of statistical significance.

Table 5.1: p-value

Concentration	Monomers	Dimers
200 pM	$9.8 \cdot 10^{-10}$	$7.1 \cdot 10^{-5}$
500 pM	0.00017	0.036
750 pM	0.00089	0.18

Another control experiment consisted in measuring the fluorescence emission of MyrUGFP in solution of different concentrations, and plot a titration curve of intensity emission as a function of the concentration. Figure 5.16-B shows the corresponding plots. The measurements were performed between 125 nM and 6 μ M, including the concentrations used in the single molecule photobleaching experiments. To obtain data over a larger concentration range, the fluorimeter configuration has to be set, thus the experiment was repeated separately with different instrument settings, at lower and higher

concentration ranges. Linearity was observed at very low concentrations, from 1 nM to 500 nM (Figure 5.16-A), and also at the experimental range (Figure 5.16-B, from 125 nM to 6 μ M, indicating that at these low concentrations, no association tendencies between the molecules are observed. At much higher concentrations (Figure 5.16-C), a quenching of the fluorescence is observed (for concentrations above 20 μ M), which possibly come from association of the molecules (GFP has a known tendency for dimerization). These controls excluded in principle eGFP dimerization as the cause for the high percentage of dimers formed on the SLB. MyrUGFP self-association results from its binding to the SLB.

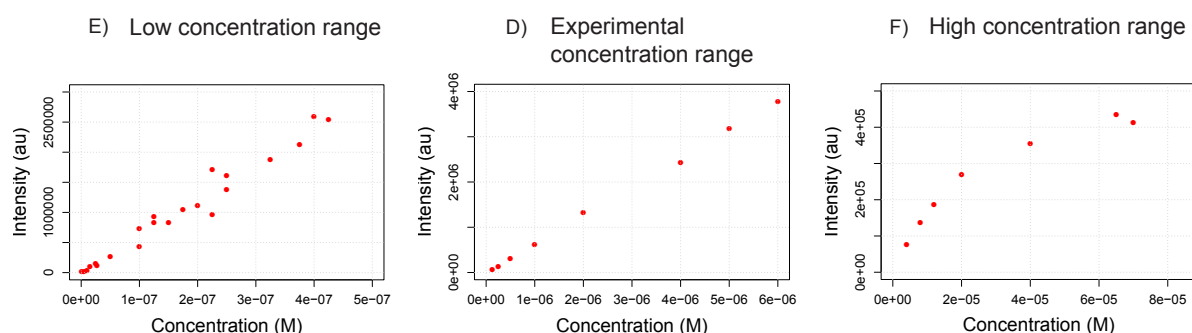


Figure 5.16: *Plot of MyrUGFP fluorescence emission at 210 nm as a function of the concentration, A) from 25 nM to 6 μ M, B) from 10 nM to 400 nM C) from 2 μ M to 60 μ M*

Altogether, these data demonstrate that a dominant population of MyrUGFP proteins persistently bound to lipid bilayers are in a dimer state, and that dimerization does not take place in solution but is an assisted-diffusion process occurring upon lipid binding. To further infer on the association mechanism of MyrUGFP proteins, a competition experiment was designed, in which 200 pM of MyrUSH3 was added to 200 pM of MyrUGFP. The experimental scheme is shown in Figure 5.17-A. In this experiment, both proteins were mixed before incubation over the SLB. By adding to MyrUGFP a non fluorescent protein, the probability of detecting monomers or dimers is expected to increase if mixed complexes can be created between MyrUGFP and MyrUSH3. The statistics of the fluorescent trajectories obtained for 200 pM MyrUGFP in presence of 200 pM MyrUSH3 was compared with that of 200 pM of MyrUGFP alone, as shown in Figure 5.17-B. They were significantly displaced when compared with the ones obtained in absence of MyrUSH3. A

higher amount of monomers was found (52 % instead of 16 %), a lower amount of dimers (42 % instead of 57 %) and a lower amount of trimers (6 % instead of 21 %). This result first confirmed that eGFP did not promote MyrUGFP dimerization in solution. More importantly, it demonstrates that the oligomers can be formed by a MyrUGFP/MyrUSH3 complex. Figure 5.17-C illustrates the different complexes that the two proteins may form.

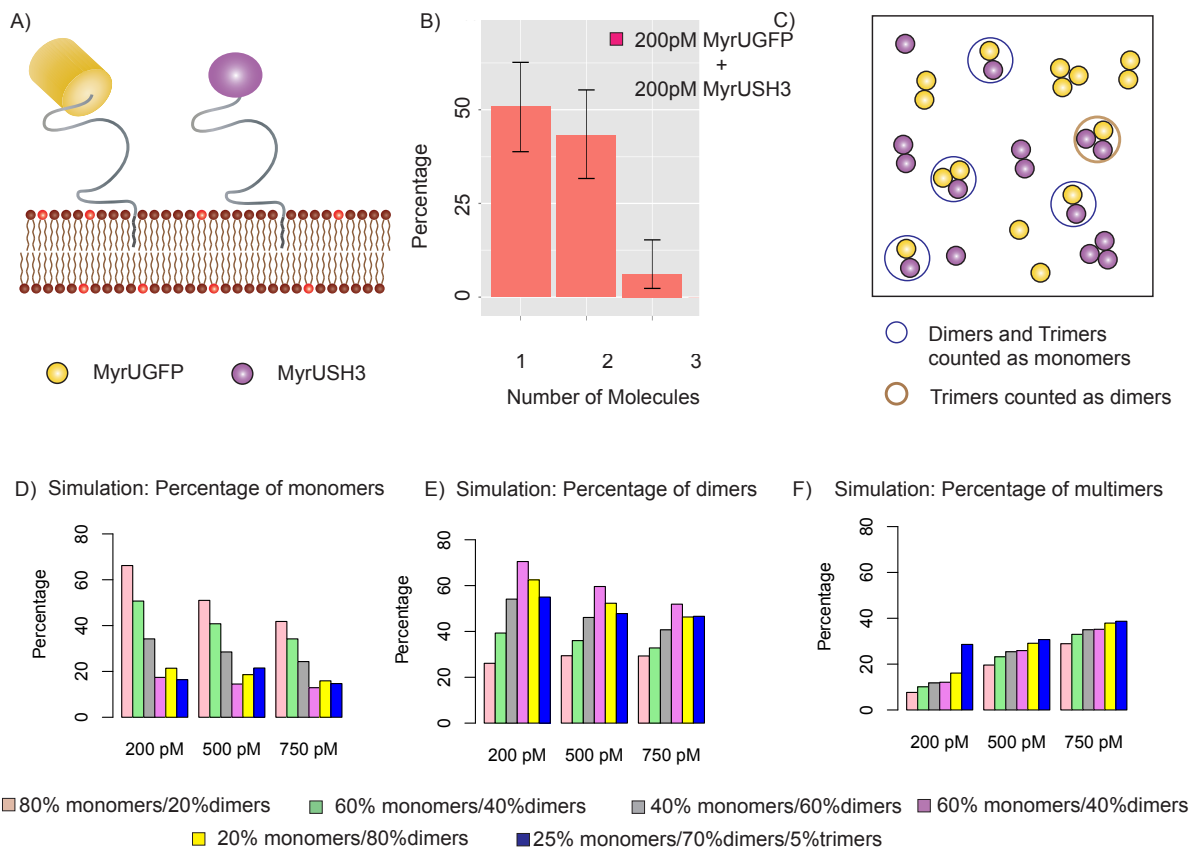


Figure 5.17: A) Scheme of the two-proteins assay, in which MyrUGFP and/or MyrUSH3 are anchored to the lipid bilayer B) Histogram of the number of bleaching steps observed for each discrete spots. The histogram compares the statistics when a protein mixture MyrUGFP/MyrUSH3 is used or when MyrUGFP is use alone (64 trajectories) C) Scheme of the different configurations when a protein mixture is used, in which MyrUGFP and MyrUSH3 can form a complex of higher order species. Histograms from the simulations in which heterogeneous population of monomers, dimers or trimers are assumed as the starting point. Statistics of the number of D) monomers E) dimers F) oligomers.

To infer whether the observed trimers were “biological trimers”, and not optical trimers

appearing because of the probability of having a monomer close to a trimer, simulations were performed with the same densities as previously, but this time, a heterogeneous population was assumed (monomers, dimers and/or trimers). Different situations were modeled. The simulation that approached the experimental data most closely was for a population of 25 % monomers, 70 % dimers and 5 % trimers, as shown by Figure 5.17-D, -E and -F. Thus, the experimental trimers observed may be real trimers. Though dimerization is found in most of the cases, complexes of 3 or more molecules are probably also formed by MyrUGFP when bound to SLBs.

5.5. Conclusion and Discussion

By using these single molecule photobleaching experiments, we found for MyrUGFP bound to SLBs a stoichiometry deviating from the monomeric one, with a specific capacity to form dimers. In Surface Plasmon Resonance (SPR) experiments, we showed in the previous chapter that two distinct kinetics modes of MyrUSH3 proteins binding to liposomes exist. A dominant form has a very fast dissociation kinetics, while another population of persistently bound species was detected, with a very long residence time. This population is certainly the one studied in this work. The SPR data also showed that MyrSH4 was sufficient to induce persistently bound species, and in addition, MyrSH4 was found to cluster *in vivo*, upon binding in the cell membrane (Owen et al., 2010). The monolayer experiments showed that MyrSH4 is capable of forming micelles at low concentrations, already at 500 nM. This could explain why clusters of MyrSH4, and not dimers, are found in the above mentioned in-cell experiments. Micelle formation is not observed in MyrUGFP, but we postulate that MyrSH4 is the promoter of the autoassociation observed in MyrUGFP and that it is responsible for the stabilization of these specific dimers. As a side comment, the fact that the MIP of MyrUGFP is in the range of 30-35 mN/m indicates that the insertion features of c-Src are probably tuned in a subtle way.

All other members of the c-Src family possess one or two palmitoylated chains, in addition to the myristoyl, which provides strong anchoring to membrane (Leo et al., 1971). c-Src does not, and currently, the consensus view is that c-Src anchoring to membranes is achieved by cooperative hydrophobic and electrostatic interactions (in which the strongly positively charged SH4 domain strongly interacts with the negatively charged liposomes enhancing binding). This mechanism would be compatible with the fast dissociating species found by SPR, for which there is no evidence of clustering phenomenon. Here we find a population of dimerized proteins with a very stable binding to the bilayer, probably via cooperative binding of two

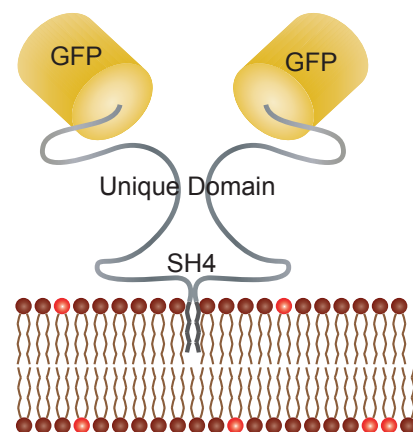


Figure 5.18: Scheme of MyrUGFP dimerized over the bilayer.

myristoylated chains; as for dually myristoylated and palmytoylated proteins, two fatty acids are better than one and considerably increase the residence time of the acylated protein over the membrane (Martin et al., 2011) (Resh, 1999). Myristoyl chain has the minimum length to induce micelle formation, but only at high concentration (Pool and Thompson, 1998), thereby a local concentration increase upon lipid binding could favor this phenomenon and induce dimerization. Other myristoylated proteins are known to form trimers upon myristoyl exposure and membrane binding (Resh, 2004).

c-Src family members are targeted to the plasma membrane, but Src is distributed in both rafts and non rafts regions (Seong et al., 2009), while the other members of the c-Src family, are targeted to lipid rafts because of their dual acylation (especially and the presence of the saturated palmitoyl chain) (Martin et al., 2011). Dimer forms of c-Src would conserve non specific targeting to membrane, providing a stably bound kinase with a broadened distribution over the plasma membrane, possibly fine tuned by other factors (such as lipid composition, phosphorylation events in the SH4 domain, etc). Or, on the contrary, c-Src could be displaced to raft through dimerization, as occurs for other species (Simons and Sampaio, 2011), and be specifically targeted to rafts in a more active state. Transient contacts between two c-Src molecules are functionally important since full activation of the kinase is achieved by autophosphorylation (Sun et al., 2002) (Ingleby, 2008). Thereby, dimerization of two c-Src molecules, by increasing contacts, could favor the autophosphorylation step and directly influence triggering of the signaling cascade.

5.6. Experimental Methods

Monolayers

Monolayer Set up

The surface properties of MyrSH4 were analyzed in a NIMA (Conventry, UK) film balance equipped with a Wilhelmy platinum plate and a round Teflon dish (surface area 19.6 cm², 27.2 cm³ of capacity)

The surface properties of MyrUGFP were analyzed in a rectangular teflon cuvette (surface area 31 cm², 14 cm³ of capacity) equipped with a Wilhelmy filter paper plate attached to a Kibron DeltaPi 4x balance (Kibron Inc.), controlled by the software DeltaGraph4X 1.13 (Kibron Inc.).

MyrSH4 and MyrUGFP activity determination The Teflon trough and the plate were thoroughly cleaned before each run with chloroform and boiling water to avoid carryover of lipid. The cuvette was filled with the buffer (50 mM NaP, pH 7 in case of MyrSH4, 50 mM NaP, 150 mM NaCl, pH 7.5 in case of MyrUGFP). Pressure baseline was established and set to 0 before peptide or protein injection in the subphase. Increasing volumes of a concentrated solution of the peptide or the protein were injected beneath the surface through a lateral hole. During the experiments, the subphase was continuously stirred. MyrSH4 was injected from 0.5 to 8 μ M, MyrUGFP from 25 nM to 1.5 μ M. Surface pressure with time was monitored until a steady-state value was obtained, and the equilibrium pressure P_e was recorded.

MyrSH4 and MyrUGFP MIP experiments The kinetics of peptide or protein binding to the lipid monolayer was monitored using the same conditions as above (Teflon trough and buffer conditions). A few microliters of DMPC:DMPG 2:1 solution dissolved in chloroform:methanol were deposited on the top of the subphase with a microsyringe; the deposited volume was varied in order to form monolayers at the required initial pressures. After 10 min of solvent evaporation, a stock solution of the peptide or protein (in the same buffer as in the subphase) were injected in the subphase, rendering final concentrations of 1 μ M of MyrSH4 and 100 nM of MyrUGFP. The subphase was stirred

continuously to ensure a homogeneous distribution and a good interaction of the peptide with the monolayer. Pressure increases ΔP were recorded with time until no further changes occurred indicating that equilibrium was reached; the equilibrium pressure P_e was recorded. The experiment was repeated at different initial pressures P_i , which ranged from 6 to 26 mN/m in case of MyrSH4 and from 7.5 to 30 mN/m in case of MyrUGFP.

Single molecule photobleaching

Fluorescence titration measurements MyrUGFP samples at different concentrations were prepared, from 25 nM to 60 μ M. Fluorescence emission was measured in a customized fluorimeter from PTI and analyzed with the software Felix 32. Excitation was set to 488 nm, and emission was scanned from 505 to 600 nm. Plots of fluorescence intensity versus concentration were established by taking the fluorescence emission value at 510 nm. Titration was performed in the total concentration range by varying the aperture of the slit (adjusting it for the different concentration ranges).

Liposome preparation Lipids were from TebuBio. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and Liss RhodE PE were dissolved in chloroform, 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) sodium salt (DPPG) in chloroform:methanol:H₂O 65:35:8. Lipids were mixed in a DPPC:DPPG 2:1 ratio, with addition of 0.015 % Liss RhodE PE, and the solvent was evaporated in a rotavapor. Lipid film was rehydrated with phosphate buffer by warming the lipid film at 55 °C and vortexing, at a final total lipid concentration of 2 mM. Large unilamellar liposomes were prepared by mechanical extrusion using a 10-mL Thermobarrel extruder (LipexTM, Northern Lipids Inc.). The lipid suspension was extruded repeatedly (10 times) through a 100 nm polycarbonate filter at 55 °C. To verify the appropriate size of the liposomes, the mean diameter was checked using Dynamic Light Scattering (Zetasizer Nanoseries S, Malvern instruments).

Liposome deposition over microscope coverslips Glass cover slips (circle cover slip 30 mm, thickness 1, Menzel Gläser) were sonicated for 15 min in 50 % ethanol, and further cleaned for 10 min in a ozone plasma cleaner and used immediately. 200 μ L of liposomes (pre-warmed at 45 °C) were deposited on the cover slip and incubated for 1 h at 45 °C. Liposomes were manually rinsed with 2 mL MilliQ water and 1 mL phosphate buffer. 200 μ L of MyrUGFP protein were deposited over the supported lipid bilayer (SLB)

formed on the cover slip and incubated for 30 min at 45 °C. Three protein concentrations were used (200 pM, 500 pM, 750 pM). Final wash was performed with 3 mL cold phosphate buffer before mounting the cover slip in the chamber and covering it with 1.5 mL cold phosphate buffer. Imaging was performed at room temperature, after 10 min incubation at 4 °C (DPPC and DPPG are in the gel phase at room temperature, slowing down protein diffusion in the bilayer). SLB homogeneity was verified by imaging the Liss Rhod PE fluorescence.

Fluorescence microscopy Set Up All measurements were acquired using the Microtime200 from PicoQuant, consisting of an inverted IX73 microscope from Olympus. Excitation was achieved by a picosecond pulsed diode laser LDH-P-C-470 (470 nm, 40 MHz) for eGFP and Rhodamine-PE. Linear excitation light from the diode passed through a $\lambda/4$ plate to obtain circular polarized light, which was focused onto the sample by a 60x water (UPlanSApo, 1.2 NA) or oil (ApoN, 1.49 NA) immersion objective from Olympus. Emission light from the sample was collected through the same objective and spectrally separated from the excitation light by a dichroic (Di01-R405/488/561/635, Semrock), passing through a 50 nm pinhole and detected by single photon counting APD (PDM series from MPD). Emission light from eGFP and Rhodamine-PE was spectrally separated using a dichroic (600DCRX, Semrock) and further filtered using bandpass filters (520/35 for eGFP and 692/40 for Rhodamine-PE). Images were acquired by scanning the sample over the objective, using a computer controlled 2-dimensional XY piezo stage from Physics Instruments (P-733) with sub-nanometer resolution. A PIFOC system from Physics Instrument ensured that the changes in focus (z) were minimal.

Image acquisition For single molecule photobleaching measurements, a 256 x 256 pixels image corresponding to a 5 x 5 μm area of the sample was acquired at a 300 W/cm² excitation power density (after the objective) and 1 ms integration time. Regions that show signal were then selected for acquisition of photobleaching traces. Intensity trajectories over time were then recorded on these spots using 1 Kw/cm² of excitation power density.

Statistical analysis Data was fitted with a generalized linear model using the date of experiment as covariable. In order to fit a model to the proportion of monomers (dimers)

of controls and the various concentrations we used the function "glm" from the R statistical language (Team, 2015) with the binomial family and logit link function. P-value for the effect of the concentration variable was calculated with a X-square test using the "drop1" function in R. Individual p-values for the comparison of concentrations vs the two controls were computed through a wald-test (function "wald.test" in the "aod" package (M and R., 2012) in R).

Simulation parameters The protein density used in the simulations was derived from ten images recorded at 200 pM. The number of spots was manually counted and an average of the total number from the different images was used. This number was corrected with the experimental statistics of the multimeric species, and the final density was deduced. Linear concentration dependence was assumed to calculate the density at the higher concentrations (this assumption is justified by surface plasmon resonance experiments in which binding of MyrUSH3 at different concentrations was recorded and specific detection of the bound proteins followed a linear trend in the low concentration range).

Table 5.2: *Densities*

concentration (pM)	200	500	750
average (/μm ²)	3.12	7.8	11.7

Chapter 6

NMR study

6.1. NMR for biomolecules

Very few techniques allow for protein structure determination with atomic resolution. X-ray crystallography is the most widely used, but is limited to the study of proteins that have been crystallized, which does not occur with IDPs. Nuclear Magnetic Resonance is the other technique; it has the potential to study the structure, but also the dynamics, of proteins of limited size, when they can be sufficiently concentrated. It is thus perfectly suitable for the study of small and partially disordered myristoyl constructs of c-Src.

NMR is based on the existence of a nuclear spin angular momentum, giving rise to a nuclear spin quantum number I . I can be equal to 0, 1/2, 1, 2, etc. If I is different from zero, the nucleus possesses a nuclear magnetic moment μ . Spins 1/2 nuclei are especially convenient for NMR, and the most used are ^1H , ^{13}C , ^{15}N , ^{19}F and ^{31}P . Their nuclei in a magnetic field (B_0) present only two states with different energy, corresponding to magnetic moments of opposite orientation. The energy difference results in different populations of the two states and a small bulk magnetization. B_0 is usually very intense and its direction represents the longitudinal (z) direction ([Cavanagh et al., 2007](#)).

The existence of this bulk magnetization, $M = M_0 \cdot z$, parallel to B_0 , is exploited in NMR spectroscopy. M can be perturbed by applying a radio frequency (rf) pulse at a suitable frequency, corresponding to the energy level difference between the two spin states of the nucleus (thus it is different for different nucleus types), which induces magnetic resonance of the nuclei; in the simplest examples, M is moved to a plane perpendicular to B_0 . After the pulse, M will relax back to the Boltzmann equilibrium by precessing around

B_0 at the Larmor frequency ω_0 . This time dependent magnetization induces an electric current, which is measured as a Free Induction Decay (FID). Nevertheless, each nuclei, even of the same type is surrounded by different electronic environments. The electronic cloud has a shielding effect in which it slightly modifies the resonance frequency of each specific nucleus, by creating a specific local magnetic field different for each of them. This so-called chemical shift enables to distinguish between each nucleus within a molecule, since each nucleus will resonate at a specific frequency. The time domain FID of a nucleus is Fourier transformed to obtain its specific resonance frequency peak which will have with a certain linewidth.

Relaxation is described by two processes. First, the spin-lattice relaxation R_1 comes from the interactions of the spin with the environment; it is also called longitudinal relaxation time $T_1 = 1/R_1$, since it measures how fast the magnetization relaxes back along the z-axis. Second, the spin-spin relaxation R_2 comes from the interactions between the adjacent spins; it is also called transverse relaxation time $T_2 = 1/R_2$, since it measured how fast the spins loose coherence in the transverse plane (Cavanagh et al., 2007) (James, 1998). Relaxation occurs though various processes. All of them require fluctuating interactions at specific frequencies related to the existing energy between spin levels. The most important interactions are dipole dipole and chemical shift anisotropy. Longitudinal relaxation influences the rate at which experiments can be repeated. Repeated excitation may result in decreased population differences between spin states (or even equalize them at saturation), and therefore lower sensibility. Transverse relaxation is directly related to the line width. Fast R_2 results in broad lines. Since the peak integral is constant, broad lines have lower intensities and can even disappear in the noise level. Dynamic properties have a strong influence in relaxation. In the case of transverse relaxation, slowly rotating molecules relax slowly and give broad lines, while small, fast reorienting molecules give sharp lines. Longitudinal relaxation rate also increase when the molecules tumble slower but a minimum is reached after which relaxation rates decrease. Thus very large molecules suffer from broad lines and easy saturation setting a limit to the size of proteins that can be studies in solution. Temperature is an important parameters since temperature increase will enhance molecular dynamics. Some of the barriers can be overcome by solid state NMR methods.

Some nuclei may be submitted to more than one chemical environment, for instance if

intramolecular interactions occur within a molecule, or if an interacting partner is present in solution. Two different chemical environments will result in two resonance frequencies. If a nucleus is in exchange between two environments, the shape of the spectrum will depend on the exchange rate. If the exchange is slow, two peaks will be observed. If the exchange is fast, a chemical shift corresponding to a weighted average of both frequencies will be observed. In intermediate situations, broadening and eventual coalescence of the peaks is observed (James, 1998). Figure 6.1 illustrates the effect of chemical exchange on the spectral shape.

In order to obtain information from NMR spectroscopy, sequences of rf pulses are used, in order to measure the desired parameters. For protein NMR, one-dimensional spectra, in which only the resonance frequencies of the protons are measured, are not sufficient to characterize and differentiate all the nuclei in a molecule. Multidimensional NMR was developed and enabled to enlarge NMR spectroscopy to molecules above 10 kDa. There are several NMR experiments that enable to connect the resonance frequencies of directly bonded atoms. The main experiments use ^1H - ^{13}C and ^1H - ^{15}N coupling. In order to perform such experiments, the protein ex-

pression medium needs to be enriched in either ^{15}N or ^{13}C isotopes because of their low natural abundance. Several pulse sequences exist, the most common ones being HSQC (Heteronuclear Single Quantum Correlation) and HMQC (Heteronuclear Multiple Quantum Correlation). The pulse sequence of 2D experiments includes four steps. A “preparation phase”, in which the proton is allowed to relax – in HSQC or HMQC experiments, the polarization of the proton is transferred to the other nuclei (^{15}N or ^{13}C). The labeling, in which the evolution of the magnetization is followed during a time t_1 . Then comes the mixing, in which another group of pulses results in transfer of the magnetiza-

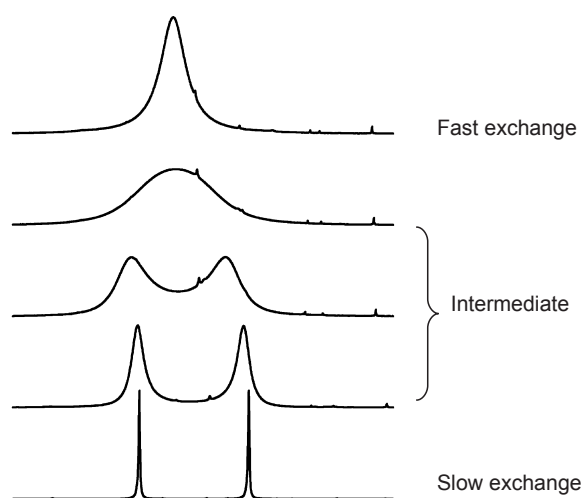


Figure 6.1: *Chemical Exchange affects the chemical shift of a nuclei according to the exchange rate, as explained in the text. Figure from Bain (2003)*

tion – in HSQC or HMQC, magnetization is transferred to the adjacent bonded atom (^{15}N or ^{13}C) via J coupling. The FID of the proton is subsequently measured during a time t_2 . The experiment needs to be repeated by varying t_1 , which means that many scans are needed to perform this experiment. Only the chemical shifts of the bonded atoms give a cross peak in the 2D spectra, which contains ^1H frequencies on one axis and the other atom frequencies (^{15}N or ^{13}C) on the other axis (Vasavi et al., 2011). In case of ^{15}N - ^1H correlation spectra, one peak is obtained for each backbone amide proton, so one peak corresponds to one residue (except for proline). The ^{15}N - ^1H spectrum constitutes a fingerprint of a protein at the atomic level. The disordered state of a protein can be assessed in such a spectrum. If all amino acids are exposed to a similar environment, it will result in a poor signal dispersion in the spectrum, which is the “signature” spectrum of an IDP. If alpha helix or beta sheets are present, the local environment of the different residues will vary more and give rise to a more dispersed spectrum (Kwan et al., 2011), typical of folded molecules.

These techniques are intrinsically time consuming since they require a large number of scans. New pulse sequences have been developed and optimized to decrease the experimental time. In this study, we used ^{15}N - ^1H SOFAST-HMQC (band-Selective Optimized-Flip-Angle Short Transient-HMQC) (Schanda et al., 2005).

Individual crosspeaks can be traced to a specific protein residue. Changing the environment of the protein will result in changes in chemical shift positions and/or broadening of the resonance peak (also noticed in the change of the peak intensity). By comparing a reference spectrum of the protein with a spectrum of interest (for instance when an interacting partner is added), intra or intermolecular interactions can be followed, as illustrated in Figure 6.2 B.

Here, USrc and SH3 assignments have been previously reported (Pérez et al., 2013) (Maffei et al., 2015). Therefore, the intramolecular interactions induced by the presence of the myristoyl tail can be studied by comparing the spectra of the myristoylated and non-myristoylated form. ^{15}N - ^1H spectra of MyrUSrc and MyrUSH3 constructs were acquired with ^{15}N - ^1H SOFAST-HMQC pulse sequences. Since c-Src resides at the membrane interface, the spectra were also acquired in presence of lipids. Lipid interactions of non-myristoylated constructs had been previously studied by adding high concentrations of DHPC:DMPG bicelles, thus for consistency reasons, we kept this lipid system in the non-

myristoylated forms. In case of myristoylated proteins we used liposomes composed of DOPC or DOPC:DOPG, for which the transition temperature is very low, ensuring that even at 4 °C , they remain in a fluid state to allow diffusion of the anchored protein in the bilayer. Figure 6.2 gives a scheme of the different measurements performed. Effect of the myristoylation was studied by comparing the spectra of the protein in absence or in presence of myristoyl. Effect of lipid binding was studied by comparing the spectra of the myristoylated protein in absence or in presence of liposomes.

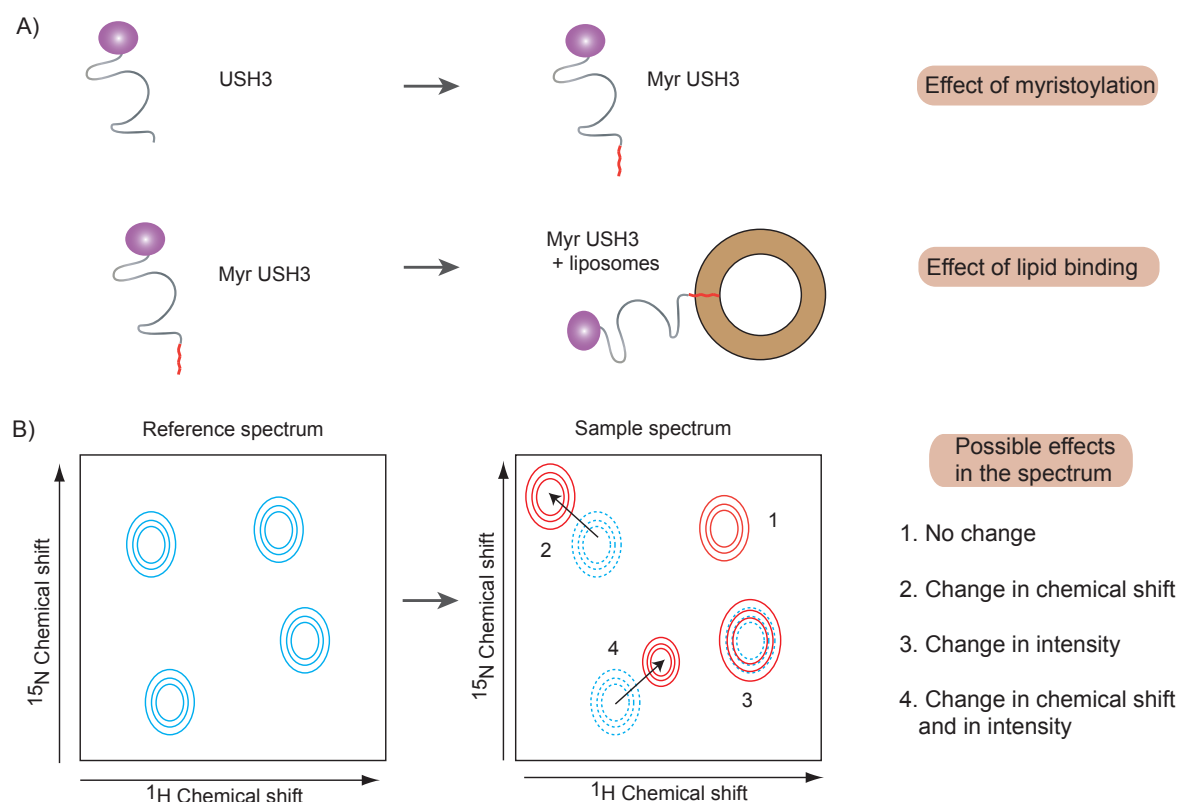


Figure 6.2: A) Main samples measured in this study: *USH3*, *MyrUSH3*, *MyrUSH3+liposomes* B) Possible effect on the chemical shifts of the protein residues involved in intra or intermolecular interactions: changes in chemical shift and/or increase or decrease in the intensity

Changes in the chemical shifts position or intensity could be observed in presence of the myristoyl tail, and upon liposome addition. These experiments shed light on the weak interactions features, either intramolecular or intermolecular, in presence of the myristoyl tail.

6.2. NMR study of MyrUSrc

6.2.1. MyrUSrc in solution

^{15}N labeled MyrUSrc was obtained by the in-vitro purification method. Figure 6.3 shows the SOFAST-HMQC spectra of MyrUSrc, compared with that of USrc. The ^1H - ^{15}N 2D spectrum of MyrUSrc was measured at two concentrations, 150 μM and 400 μM . The spectrum could be compared with that of USrc. ^1H - ^{15}N USrc spectrum had been previously assigned, however the signals from the first three residues were not visible. Glycine 2 is in its free amino acid form. The other two residues may be absent due to solvent exchange or because they are involved in some conformational exchange processes. In case of MyrUSrc, the three missing signals were visible. There were few chemical shifts changes between the non-myristoylated and the myristoylated form of USrc, however, the changes in intensity were more significant. Intensity changes in SOFAST-HMQC spectra can originate from two effects. A decrease in intensity, as in other NMR experiments, may reflect signal broadening caused by a shorter T_2 (related to an increase in correlation time or chemical exchange in an intermediate time-scale). An increase in intensity may be the result of shortening of T_1 , which can only originate from an increase in the local correlation time. SOFAST-HMQC experiments are recorded under steady state conditions due to the fast pulse rate. The intensity of the signal depends on the balance between saturation (caused by fast pulsing) and relaxation. Under identical experimental conditions, faster relaxation (T_1) will lead to stronger steady state signals.

Quantifying the changes in intensity of different samples implies that the spectra have to be normalized. The choice of the normalization signal is somewhat arbitrary. We chose residue A79, near the C-terminal extreme, opposite to the myristoylation site.

Figure 6.4 displays the differences in intensity between MyrUSrc (150 μM) and USrc. The increase in intensity of the signals in all residues up to the ULBR suggested a conformational restriction induced by the presence of the myristoyl tail. The restriction seemed to affect all the N-terminal amino acids until the ULBR, pointing out that the myristoyl tail may be interacting with this region; since the ULBR has a lipid binding propensity and possesses hydrophobic amino acids, its interaction with the myristoyl tail could be favored and minimize the contacts of the tail with water.

Figure 6.4 plots the differences in intensity between MyrUSrc measured at the two con-

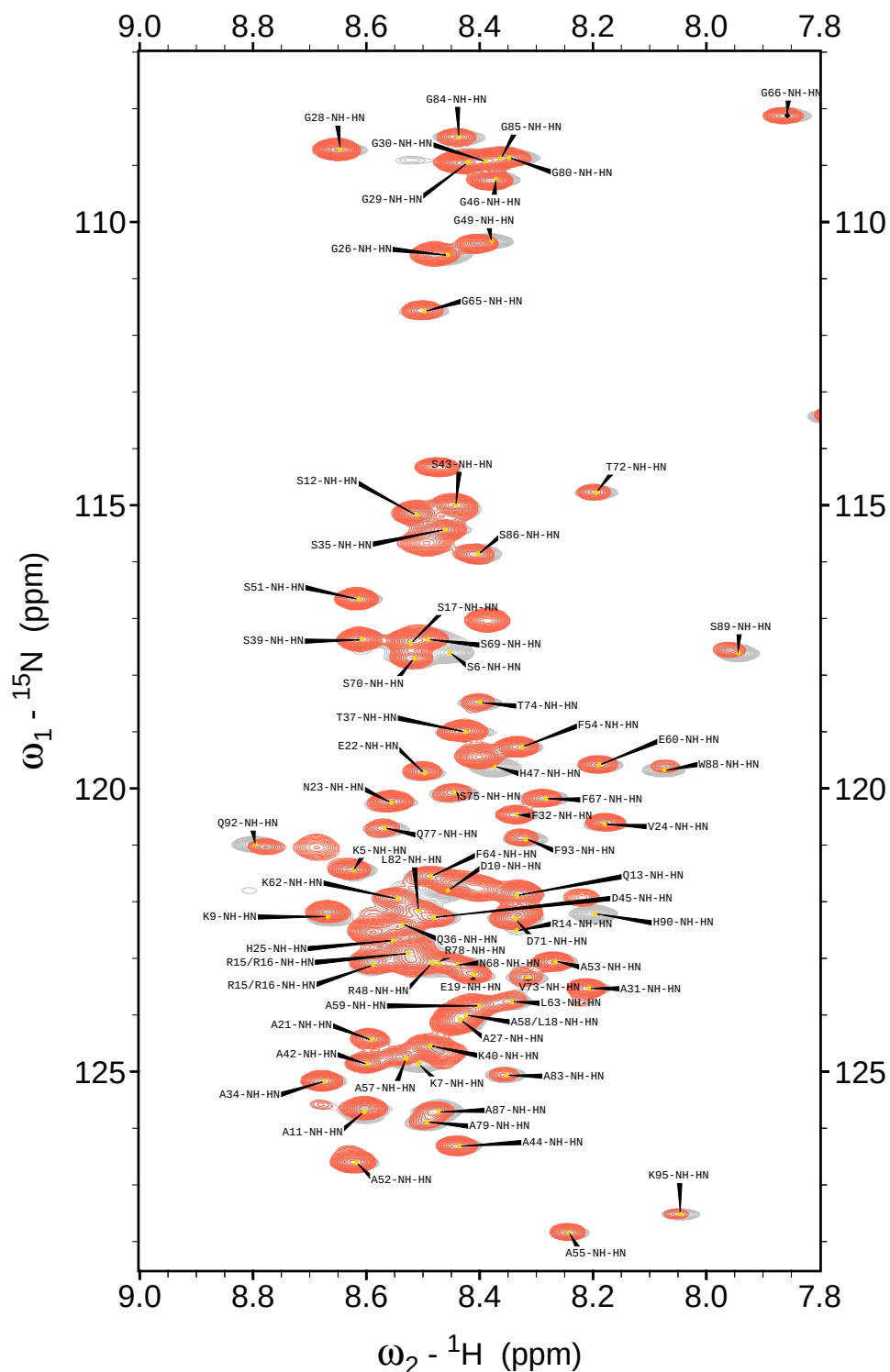


Figure 6.3: A) ^1H ^{15}N spectra of MyrUSrc (red), compared with that of USrc (grey)

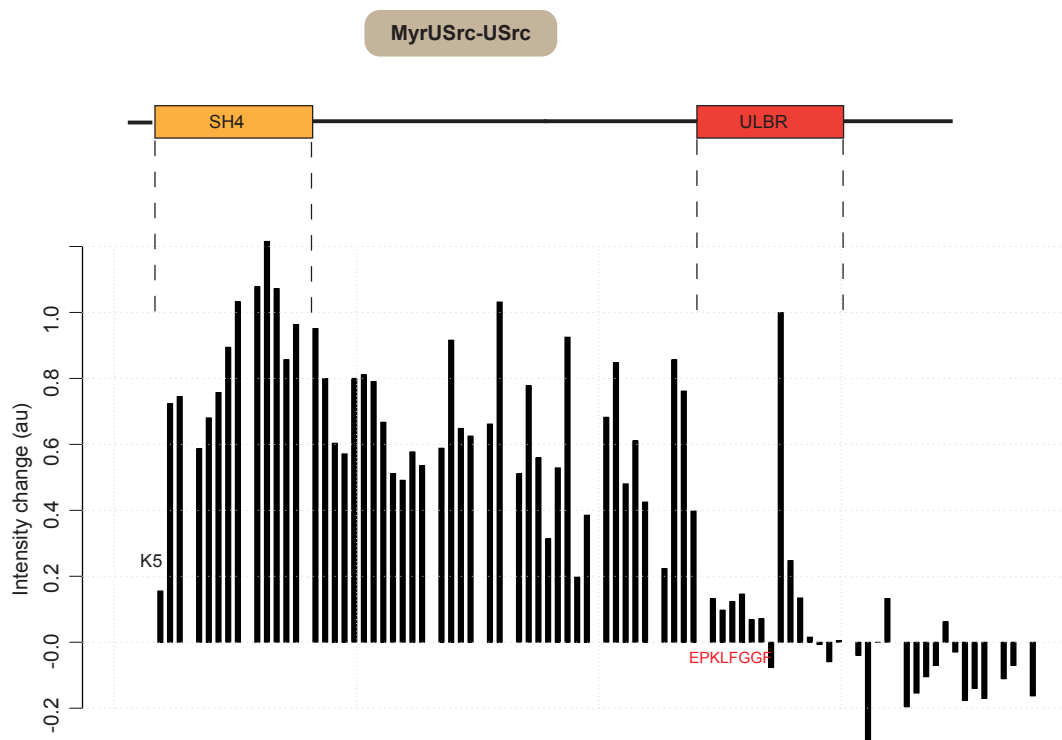


Figure 6.4: *Normalized Intensity values of MyrUSrc, 150 μ M, subtracted from Normalized Intensity values of USrc.*

centrations. Increasing the concentration provoked a general effect on the protein, since the subtracted intensities were all negative, indicating that some intermolecular interactions are occurring. The SH4 region was most affected, suggesting that the global effect on the whole protein was mediated by intermolecular interactions in the SH4 domain.

6.2.2. MyrUSrc bound to liposomes

DOPC liposomes Liposomes were added to MyrUSrc in a 67 fold excess (75 μ M of MyrUSrc, 5 mM of lipids). Binding of MyrUSrc to liposomes was first recorded in the case of zwitterionic liposomes composed of DOPC. The spectrum obtained in presence of neutral liposomes was compared with that of MyrUSrc in solution. Here again, changes in the intensity were more important than chemical shifts. Figure 6.6 represents the difference in intensity of MyrUSrc bound to DOPC liposomes and MyrUSrc in solution. Expectedly, two main regions were perturbed by the presence of neutral lipids: the SH4 region and the ULBR region. The SH4 is most probably perturbed because of the myristoyl insertion (in the non myristoylated form, the SH4 region was hardly perturbed by

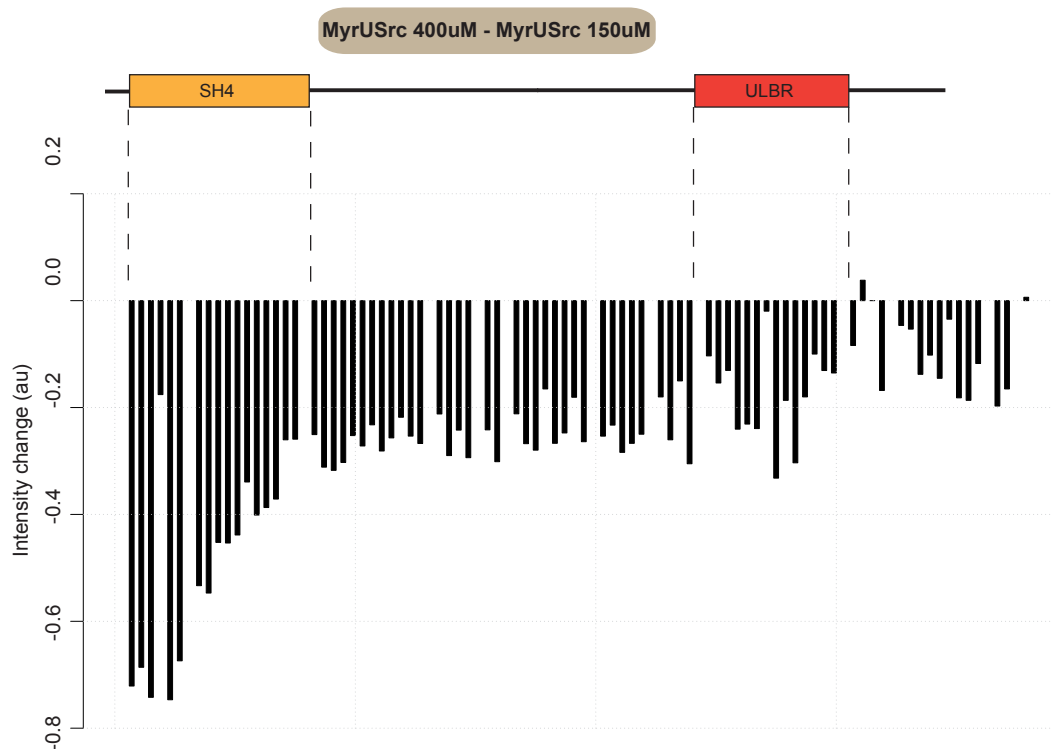


Figure 6.5: *Normalized Intensity values of MyrUSrc, 400 μ M, subtracted from Normalized Intensity values of MyrUSrc, 150 μ M.*

addition of DMPC:DHPC bicelles (Pérez et al., 2013)). As in the non-myristoylated form, the ULBR was also perturbed, confirming its affinity for lipids. Hydrophobic amino acids were mainly affected, such as L62, F63, F67. In this experiment, the concentration of lipids used was much lower than the one used in the USrc-bicelles experiments (Pérez et al., 2013), highlighting the fact that ULBR interaction was facilitated in presence of the primary myristoyl anchor point. A third region was found to be slightly influenced by the presence of liposomes, especially K40.

DOPC:DOPG liposomes The experiment was then repeated in presence of negatively charged liposomes composed of DOPC:DOPG 3:1, in the same lipid:protein ratio. Figure 6.7 compares MyrUSrc bound to DOPC:DOPG liposomes with MyrUSrc.

The same regions as for neutral liposomes were interacting, but as expected, the strengths of the binding of both the SH4 and the ULBR increased with the presence of negative charges in the liposomes. Chemical shift perturbation were higher in the SH4 domain in case of charged liposomes; the 6 charged residues, 3 lysines and 3 arginines interact electrostatically with the negatively charged lipids. In the case of the ULBR, the

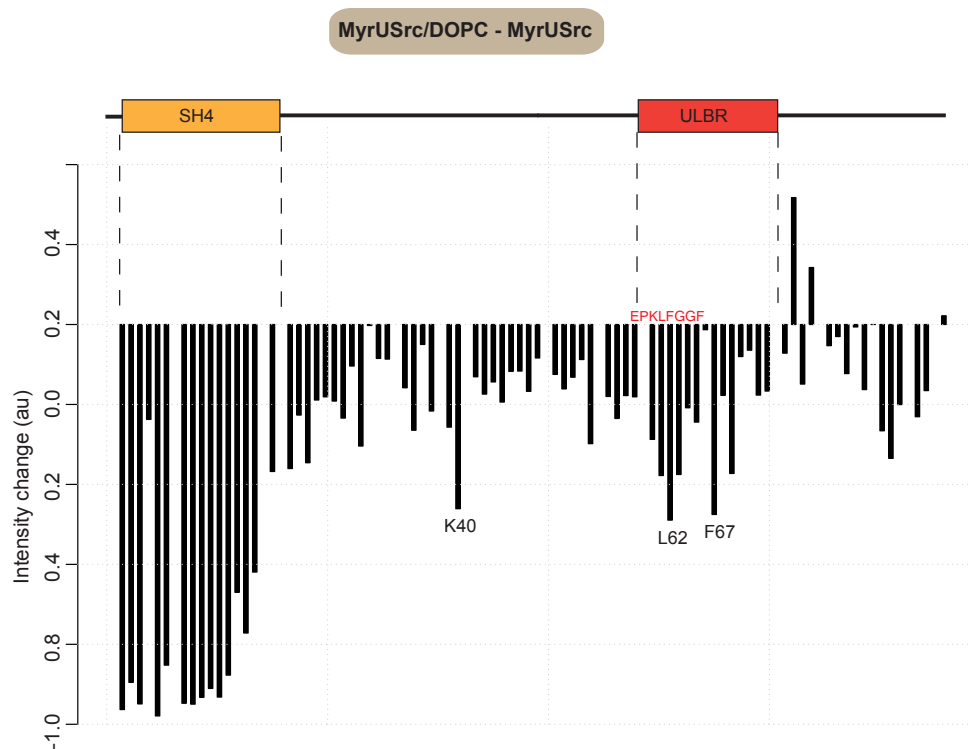


Figure 6.6: (Normalized Intensity values of MyrUSrc, bound to DOPC liposomes subtracted from Normalized Intensity values of MyrUSrc in solution).

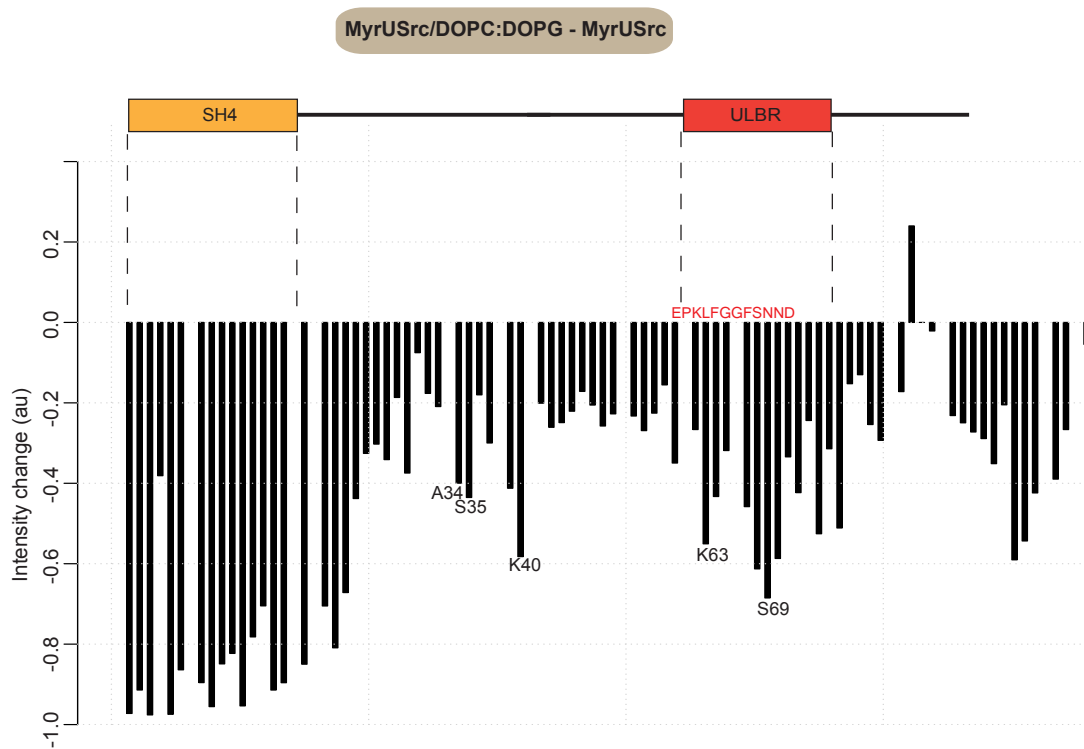


Figure 6.7: Normalized Intensity values of MyrUSrc bound to DOPC-DOPG liposomes subtracted from Normalized Intensity values of MyrUSrc in solution.

chemical shift perturbations are also stronger than in absence of charges. The additional electrostatic effect was also visible since positively charged or polar amino acids (K63 and S68 were more affected than F64 and F67).

The third region, from amino acids 34 to 40, is composed of mainly polar and positively charged amino acids, and also interacted stronger in case of DOPC:DOPG (in particular A34 and S35 appeared to be affected). The importance of this binding still needs to be investigated; it is interesting to note that two serine are present in this region, S35 and S39, just at the side of a lysine (K40).

Interestingly, while the residues perturbed by neutral lipids were in good agreement with the boundaries of the SH4 domain, in the case of charged liposomes the interaction extended up to at least residues 25. The streptag region also displays some interactions with lipids in MyrUSrc bound to neutral or charged liposomes.

DOPC:PI(3,4,5)P3 liposomes Experiments were also conducted with another liposome type, namely by supplementing short chains C8-PI(3,4,5)P3 to the DOPC liposomes and conserving the same protein lipid proportions, 75 μ M MyrUSrc, 5 mM lipids. Since C8-PI(3,4,5)P3 are soluble, they were incubated at room temperature to the DOPC liposomes, assuming that they would insert into their outer layer. Spectra of MyrUSrc bound to DOPC:PI(3,4,5)P3 liposomes were recorded, and a titration was performed in which the liposomes were doped with increasing concentrations of PI(3,4,5)P3 (40, 80, 160, and 330 μ M). It is not obvious to evaluate the net charge of PI(3,4,5)P3; Zheng Li et al. (2000) have estimated it to 6 positive charges at pH 7.

Figure 6.9 shows the difference in intensity of NMR signals between MyrUSrc bound to DOPC:PI(3,4,5)P3 liposomes and MyrUSrc in solution. The plot shows the comparison when using DOPC liposomes supplemented with 80 μ M PI(3,4,5)P3. It gives a total amount of negative charges in the same order of magnitude of that used in DOPC:DOPG 3:1 liposomes (DOPG has only one negative charge), however the local charge distribution will be very different in case of DOPG or PI(3,4,5)P3 liposomes. Figure 6.8 gives the chemical structure of PI(3,4,5)P3.

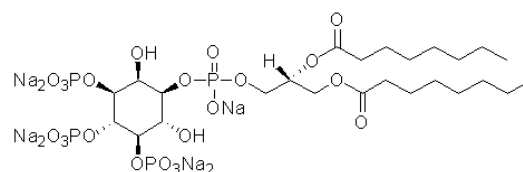


Figure 6.8: A) C8-PI(3,4,5)P3

In a similar way as with DOPC:DOPG liposomes, the SH4 region was affected by

myristoyl insertion and electrostatic attraction between the positively charged residues and the negatively charged lipids. The third region, from amino acids 34 to 40, also interacted in a very similar way that in case of DOPC:DOPG. However, the ULBR region displayed only a moderate interaction with liposomes, in which charged amino acids are not involved so much in the binding (A58 and 59, F64 and S69 were mostly affected). Surprisingly, the effect resembled more the one observed with DOPC than with DOPC:DOPG liposomes. The higher charge density in PI(3,4,5)P3 may favor the interaction with the highly charged SH4 domain. In addition, adequate separation of the charged amino acids within a polybasic peptide may facilitate binding of densely negatively charged lipids [Li et al. \(2014\)](#) [Hansen et al. \(2011\)](#) such as PI(3,4,5)P3. This may result in PIP(3,4,5)PI recruitment around this domain in a synergistic effect. PI(3,4,5)P3 clustering at the SH4 domain would deplete the rest of the bilayer from charged groups. This results point out the differential effect of the charge density in the bilayer; though both DMPG and PI(3,4,5)P3 are negatively charged, the binding of the ULBR to bilayers containing one or the other was different.

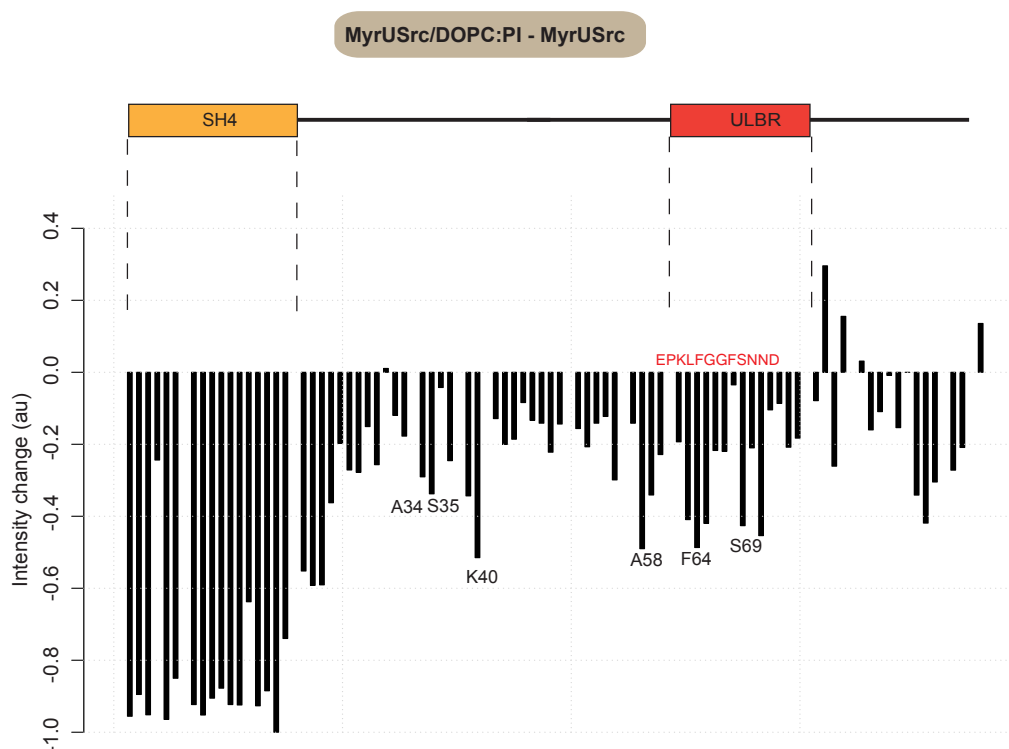


Figure 6.9: *Normalized intensity values of MyrUSrc bound to DOPC-PI(3,4,5)P3 liposomes subtracted from normalized intensity values of MyrUSrc in solution.*

Figure 6.10 shows the effect of MyrUSrc binding to DOPC liposomes when increasing concentrations of PI(3,4,5)P3 were used. The figure focuses in the two affected regions, the SH4 and ULBR domains, and shows (as in the previous plot) the differences in NMR signal intensities between the protein bound to liposomes and the protein in solution. In the SH4 region, an increase in the PI(3,4,5)P3 concentration resulted in increasingly perturbed amino acids, though the extent of perturbation was already very high at the lowest concentration used (40 μ M). Interestingly, as observed with the DOPC:DOPG liposomes, the perturbations extended up to residue 25 (A22 and E23); these residues were mostly sensing the increase in PIP(3,4,5)P3 concentration, probably indicating increasing electrostatic repulsion between E23 and the negatively charged PI(3,4,5)P3. In the ULBR region, the effect of increasing the PIP(3,4,5)P3 was very subtle; increasing concentrations of PI(3,4,5)P3 did not result systematically in increased perturbations of the residues in the ULBR.

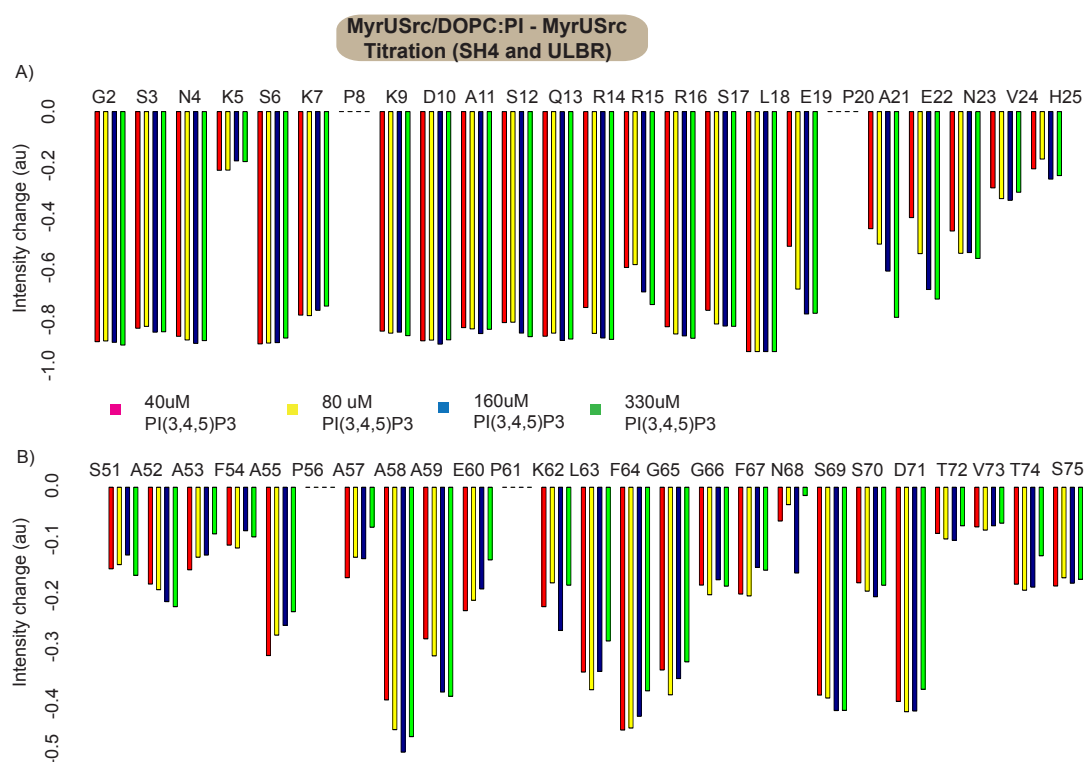


Figure 6.10: Normalized intensity values of MyrUSrc bound to DOPC:PI(3,4,5)P3 liposomes subtracted with normalized intensity values of MyrUSrc in solution; DOPC liposomes are supplemented with increasing concentrations of PI(3,4,5)P3 A) residues of the SH4 domain B) residues of the ULBR

These results point out the complexity of the modulation of ULBR binding to lipid bilayers of different types and concentrations.

Our USrc and MyrUSrc constructs possess a N-terminal Streptag for purification purposes, and these spectra also revealed that the Streptag had some affinity for lipids. This might favor the binding of USrc to lipids; but since the natural form contains the SH3 domain just after USrc (SH3 also has some affinity for lipids), it could also “represent” an additional lipid binding region present in the natural form. Nevertheless, optimization of the expression conditions of MyrUSH3 in minimum medium enabled to further study the MyrUSH3 construct by NMR.

6.3. NMR study of MyrUSH3

^{15}N -labeled MyrUSH3 and MyrUSH3A3 were obtained by the coexpression methods. The MyrUSH3 construct contained a HisTag situated after the folded SH3 domain. In this construct, the influence of the tag is expected to be much smaller than the StrepTag in the case of USrc. The non-myristoylated forms of USH3 did not contain the histidine tail, since they came from expression and purification of a construct with a N-terminal GST-HisTag. The two tags were enzymatically removed. This construct was not adequate to obtain proteins myristoylated at the N-terminal glycine. Thus, all reference spectra were measured again in order to serve as a reference. Spectra of the mutant forms in the ULBR region (USH3A3) and in the RT loop of the SH3 domain (USH3IHH) were also recorded, in order to study the effect of removing one of this region. USH3IHH could not be obtained in its myristoylated form due to fast sample degradation. Table 6.1 lists the proteins from which 2D-spectra were recorded. In the case of myristoylated constructs, spectra were recorded in absence or presence of DOPC:DOPG 3:1 liposomes (at 5 mM lipid concentration). In the case of non-myristoylated constructs, the spectra were recorded in absence or presence of DMPC:DHPC 0.8:1 bicelles.

Table 6.1: List of USH3 related constructs measured by NMR

Protein	Bilayer Model
USH3 A2G	
MyrUSH3 WT	DOPC:DOPG 3:1 liposomes
USH3A3 A2G	
MyrUSH3A3	DOPC:DOPG 3:1 liposomes
USH3IHH A2G	DMPC:DHPC 0.8:1 bicelles
SH3	

6.3.1. MyrUSH3 in solution

Figure 6.11 displays the combined ^1H - ^{15}N chemical shift differences between MyrUSH3 and USH3. Chemical shift perturbations (CSPs) were observed in the N-terminal region, mainly affecting serine 6 and lysine 7. Similar perturbations were also observed when comparing MyrUSrc *versus* USrc (Figure 6.12). Thus, they were most probably due to the local effect of myristoylation. Moreover, in USH3, additional changes were observed in the entire SH4 domain and also in the SH3 domain. The changes in K5 and S6 could not be monitored, since they were already affected by myristoylation. In the nSrc loop, no important chemical shift changes were observed, except for V114. The chemical shifts corresponding to V114 in MyrUSH3 are represented in Figure 6.15 and compared with the position of V114 in SH3 (a non interacting situation). In all constructs, V114 was interacting, since its chemical shift was displaced from its non-interacting position in SH3. In addition, in MyrUSH3, V114 showed two signals indicating a slow exchange between two conformations. The “closed” conformation of USH3 coming from the interactions between the SH4 and SH3 domains (Maffei et al., 2015) was thus probably retained in the myristoylated form, nevertheless, the splitting of V114 may indicate that the nSrc loop is affected by the presence of the myristoyl group bound to the SH4 domain.

The ULBR itself was not affected by the presence of the myristoyl chain, in contrast with what was observed for MyrUSrc in solution. The interaction of the SH4 domain and myristoyl group with the SH3 domain probably prevents or competes with the interaction of the myristoyl group with the ULBR.

The region most affected by the presence of the myristoyl was situated in the RT loop of the SH3 domain, as shown in Figure 6.11. Five amino acids in this region showed split signals (R98, T99, T101, D102 and L103), meaning that they were in slow exchange between two different states. At least one of them involved the myristoyl group. The most affected residues were R98, T99 and T101. This region was also found to be responsible for Unique/SH3 domains interactions (amino acids A55, E60 and K62 were affected in the ULBR, and R98 and D102 in the RT loop). The absence of perturbation in the ULBR suggested that the interaction was partially retained in the myristoylated form.

A similar comparison was conducted with MyrUSH3A3. Figure 6.13 A displays the CSP between MyrUSH3A3 and USH3A3 (the amino acids corresponding to the ULBR are absent of the plot). Here again, the entire SH4 domain was perturbed by the presence

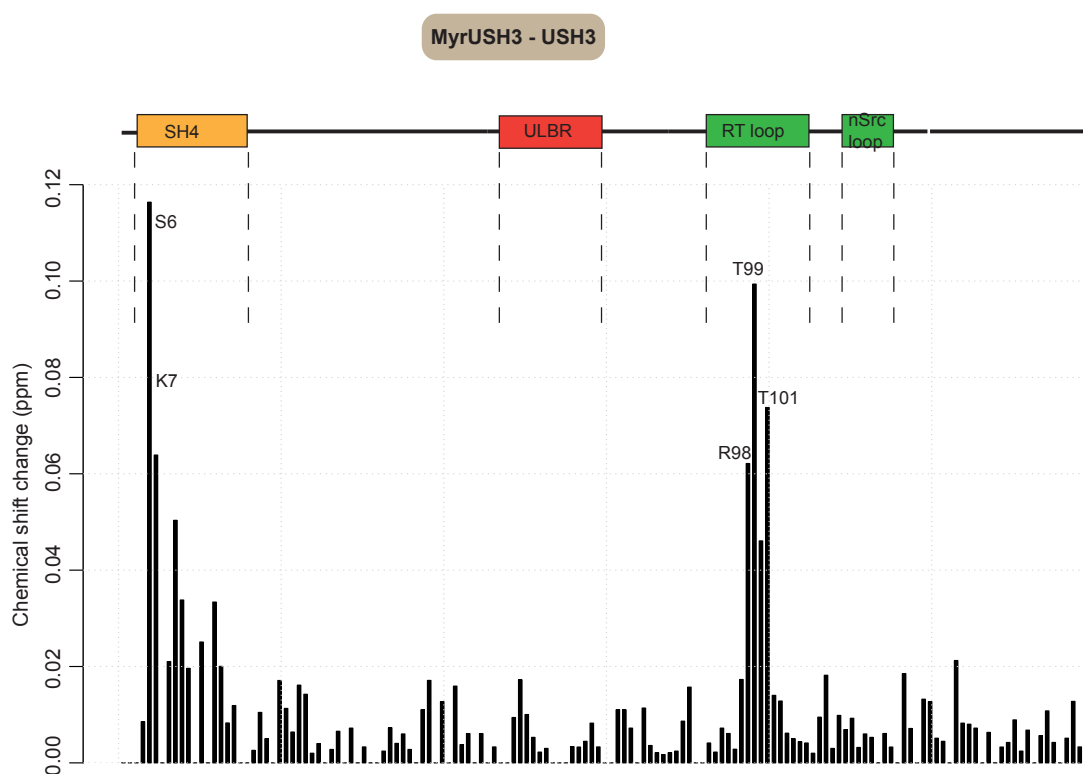


Figure 6.11: Chemical shifts changes between *MyrUSH3* and *USH3 A2G*, both in solution.

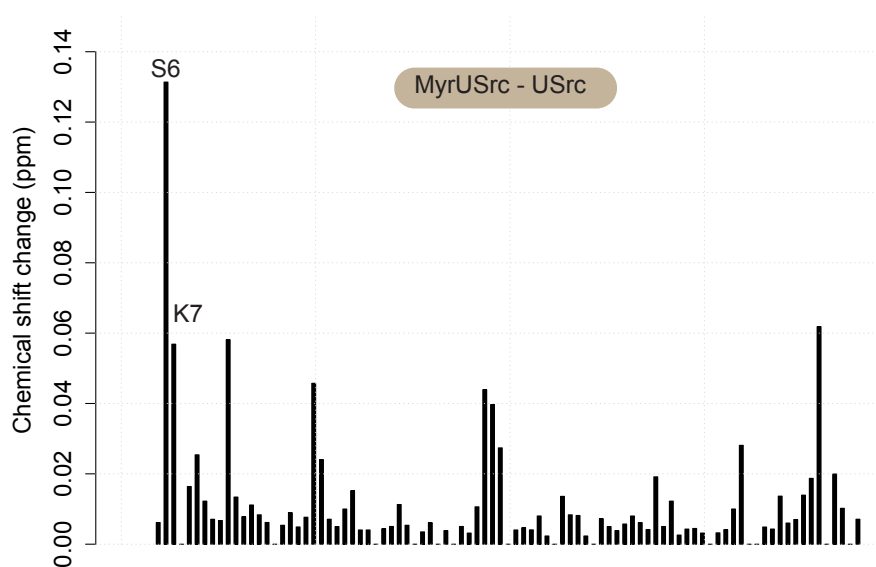


Figure 6.12: Chemical shift changes between *MyrUSrc* and *USrc*, both in solution.

of the myristoyl tail, with S6 and K7 most affected by the acyl chain addition. The other perturbed region was situated in the RT loop, as for the wild type, at residues R98, T99, T101 and D102. However, only one peak per residue was observed in this case, which means that the species having the RT loop interacting with the myristoyl group dominate in absence of the ULBR.

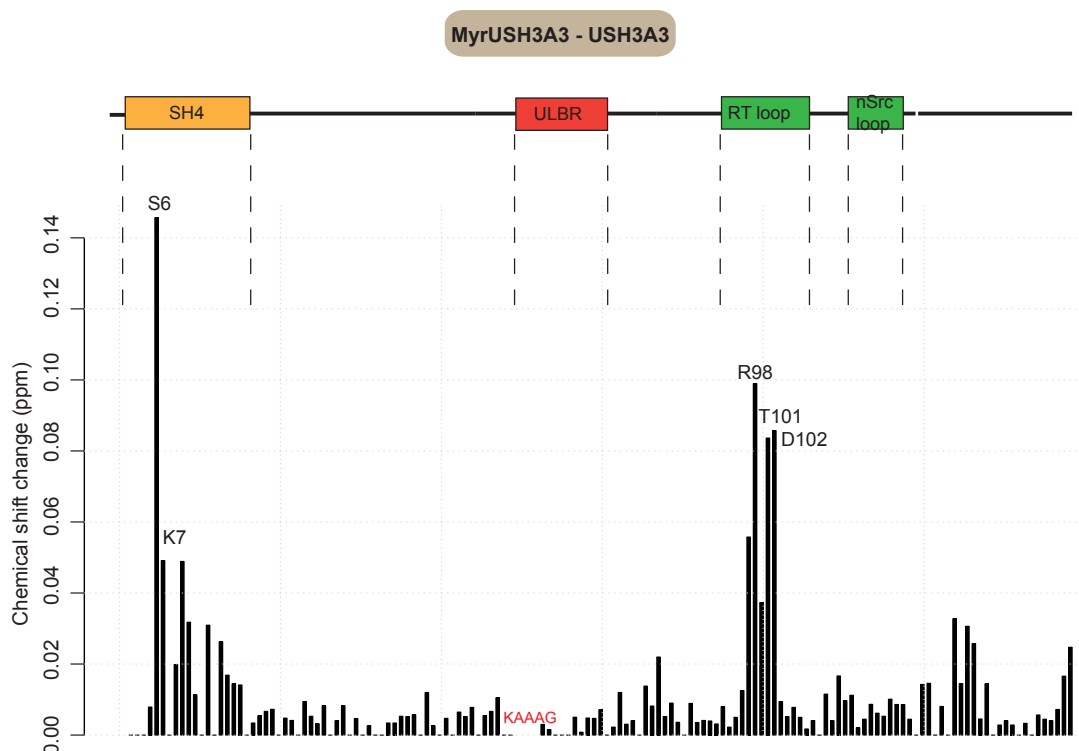


Figure 6.13: *Chemical shifts changes between MyrUSH3A3 and USH3A3 A2G, both in solution.*

6.3.2. MyrUSH3 bound to liposomes

Figure 6.14 shows the chemical shift changes between MyrUSH3 bound to liposomes and MyrUSH3 in solution.

As expected, the most affected region was the SH4 regions. Interestingly, arginines (R14, R15 and R16) were more affected than lysines (K5, K7 and K9). Surprisingly, the ULBR was only very slightly affected, indicating a very low interaction with liposomes in the conditions used. Since S6 and K5 were not substantially affected by the addition of liposomes, we can conclude that the SH3/SH4 interactions were retained in this case as well. The splitting observed in V114 almost disappeared (See Figure 6.15), probably

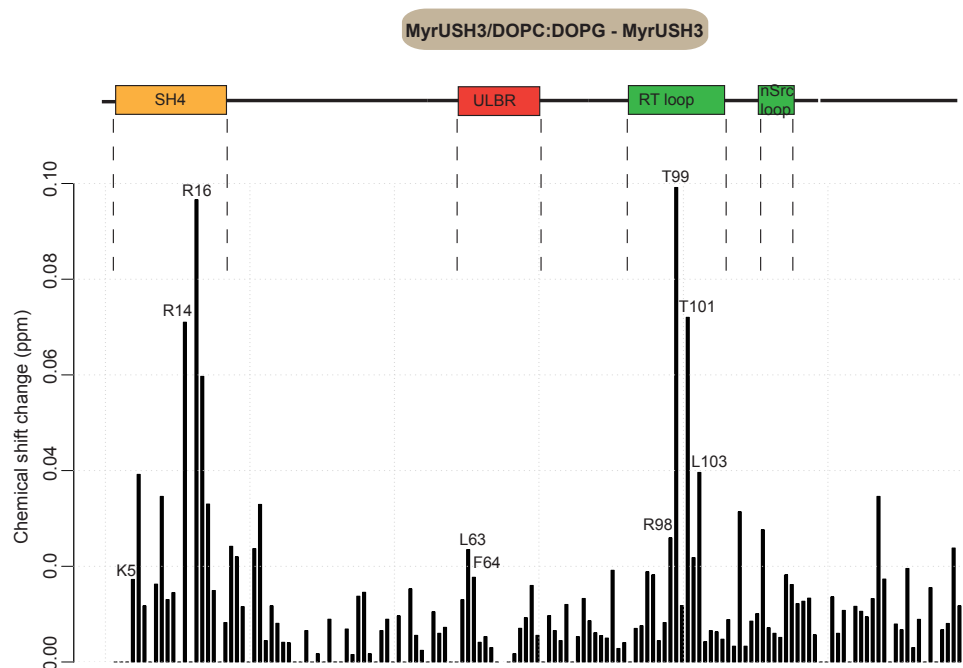


Figure 6.14: Chemical shifts changes between *MyrUSH3* bound to liposomes and *MyrUSH3* in solution.

indicating that the nSrc loop was affected in a much less extent by the myristoyl group, since the latter is inserted in the bilayer. Still, the position of V114 did not come back to the non-interacting position, therefore indicating that the “closed” USH3 configuration, involving the interaction of the SH4 domain with the nSrc loop of SH3 was also probably retained in the liposome bound form.

The residues in the RT loop are affected as well by the addition of liposomes. However, this is interpreted as a loss of the intramolecular interactions with the myristoyl group, rather than a new intermolecular interaction of the RT loop with the added lipids. The position of the RT loop signals in the presence of liposomes is identical to that observed in USH3 in absence of lipids. A detailed analysis of the CSPs in the RT loop is conducted in part 6.3.3

6.3.3. Study of the RT loop

IHH mutant A mutant in the RT loop was designed; amino acids RTE were replaced by IHH, a naturally occurring sequence in the SH3 domain of Hck – the RT loop in Hck has a higher flexibility than in c-Src (Arold et al., 1998). USH3IHH was compared with the wild type form. Changes in the SH3 region involved only the mutated residues. No

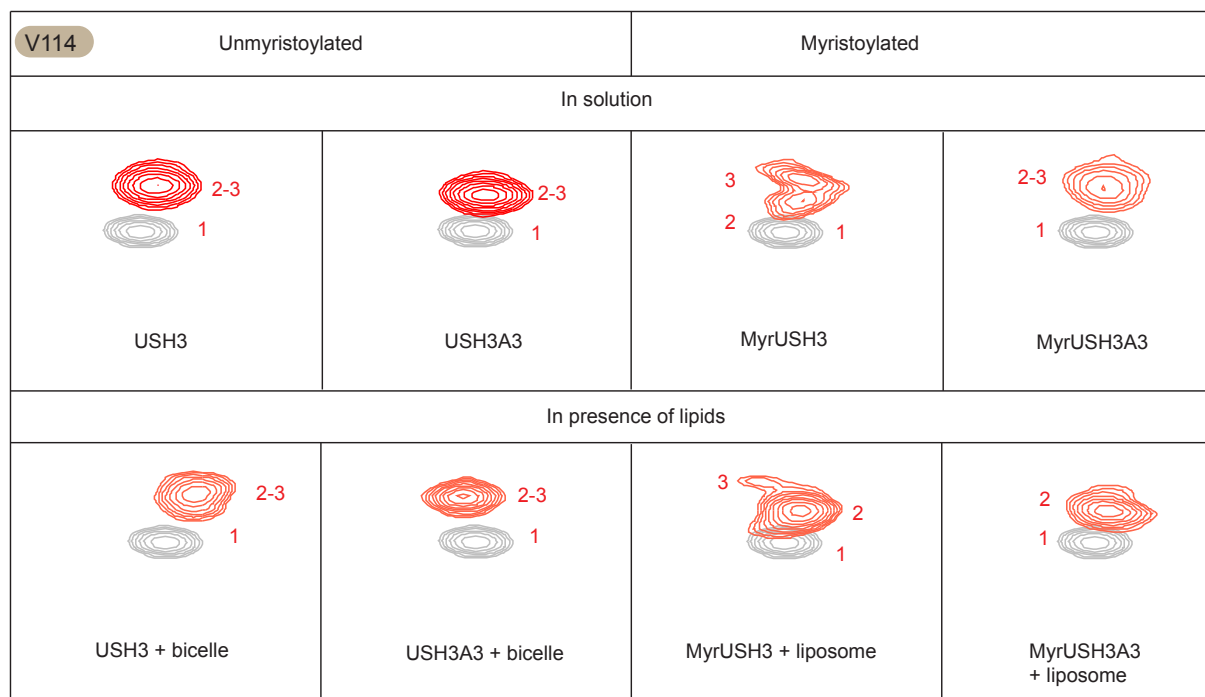


Figure 6.15: *Detail of the chemical shift corresponding to residue V114 in the different constructs; red: USH3 construct of interest, grey: SH3.*

chemical shift changes were observed in the Unique Domain, however changes in intensity in the Unique Domain were observed in certain residues, as shown in Figure 6.16.

In wild type USH3, residues A55, E60 and K62 were involved in the Unique/RT loop interaction (Maffei et al., 2015). Here we observed changes in intensity in related amino acids, A53, E60 and K62. Additional residues were affected, before (F32, S43) and after (F64). This results confirmed that upon mutations in the RT loop, amino acids in the ULBR were affected, as already observed indirectly in the wild type forms (Maffei et al., 2015). The myristoylated mutant would provide valuable additional information on the interplay between the myristoyl tail, the ULBR and the RT loop, but could not be produced due to fast degradation following myristoylation.

USH3IIHH was subsequently measured in presence of DMPG:DHPC 0.8:1 bicelles. Here again, we observed interactions between the SH4 or the ULBR domain and lipids (as in others non-myristoylated forms in similar conditions). No significant binding by the SH3 domain was found, consistent with the location of the SH3 lipid binding site of c-Src in the RT loop.

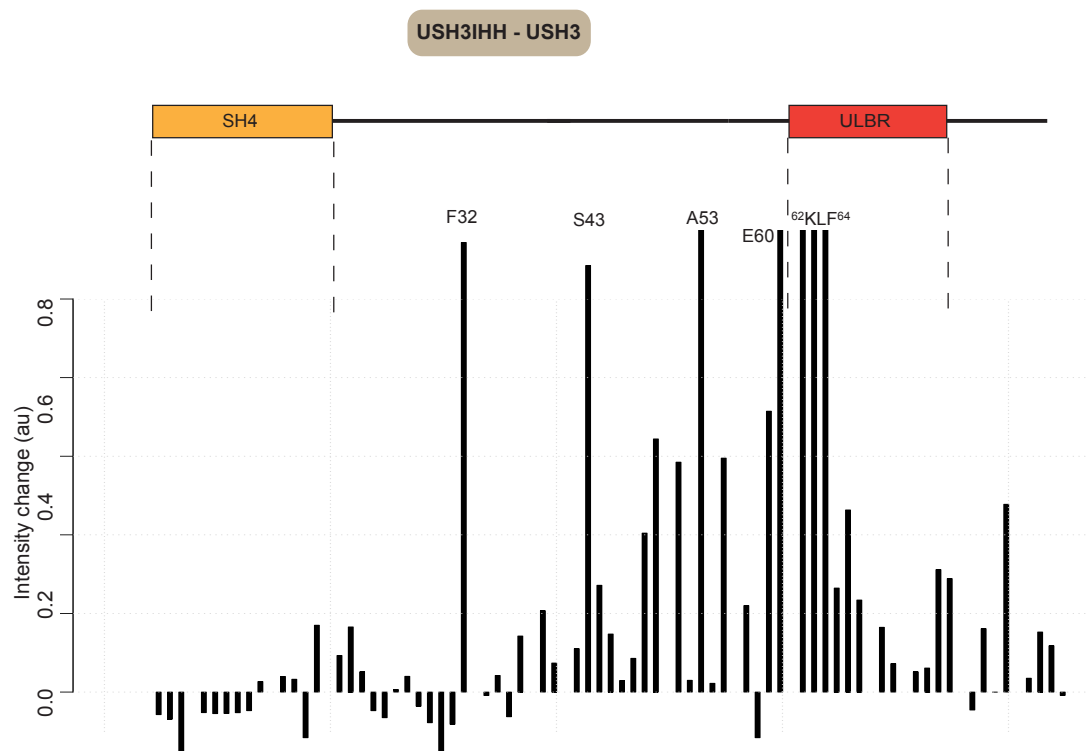


Figure 6.16: *Intensity changes between USH3IHH and USH3 A2G, both in solution; the spectra were recorded at 278 K*

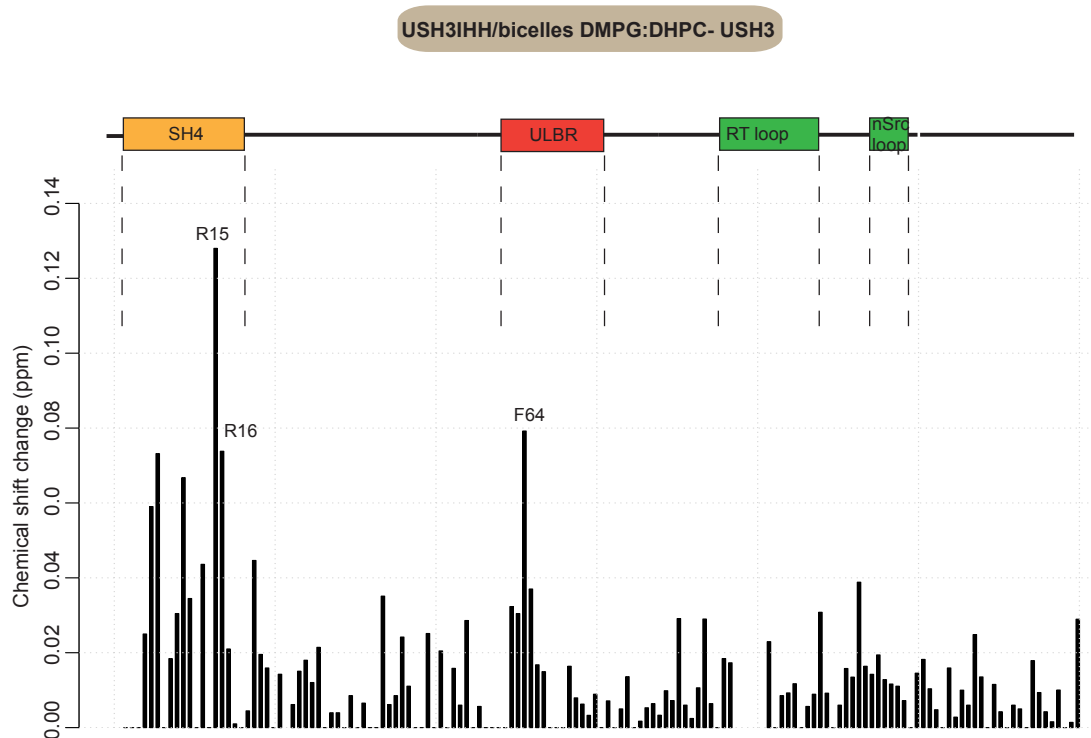


Figure 6.17: *Chemical shifts changes between USH3IHH+bicelles and USH3IHH)*

RT loop interactions As described earlier, the signals from the residues in the RT loop of the SH3 domain were split, only in the case of MyrUSH3 in solution. This phenomenon did not occur when the MyrUSH3A3 mutant was in solution, neither when liposomes were added to the wild type or the mutant forms. A study of the chemical shifts of these residues showed how their environment changes under various conditions.

To understand whether the observed CSPs were explained by interactions of the RT loop with other partners, the positions needed to be compared to a situation in which there were no interactions. The SH3 domain was actually the reference in which the RT loop is not interacting with anyone. Figure 6.18 to 6.22 gives the detailed position of the chemical shifts corresponding to the different amino acids in the RT loop of the different constructs. In all figures, the reference position of the SH3 spectrum is displayed in grey, and the chemical shifts of the other species are in red. We label as position 1 the location of the chemical shifts observed in the isolated SH3 domain, corresponding to a lack of interaction.

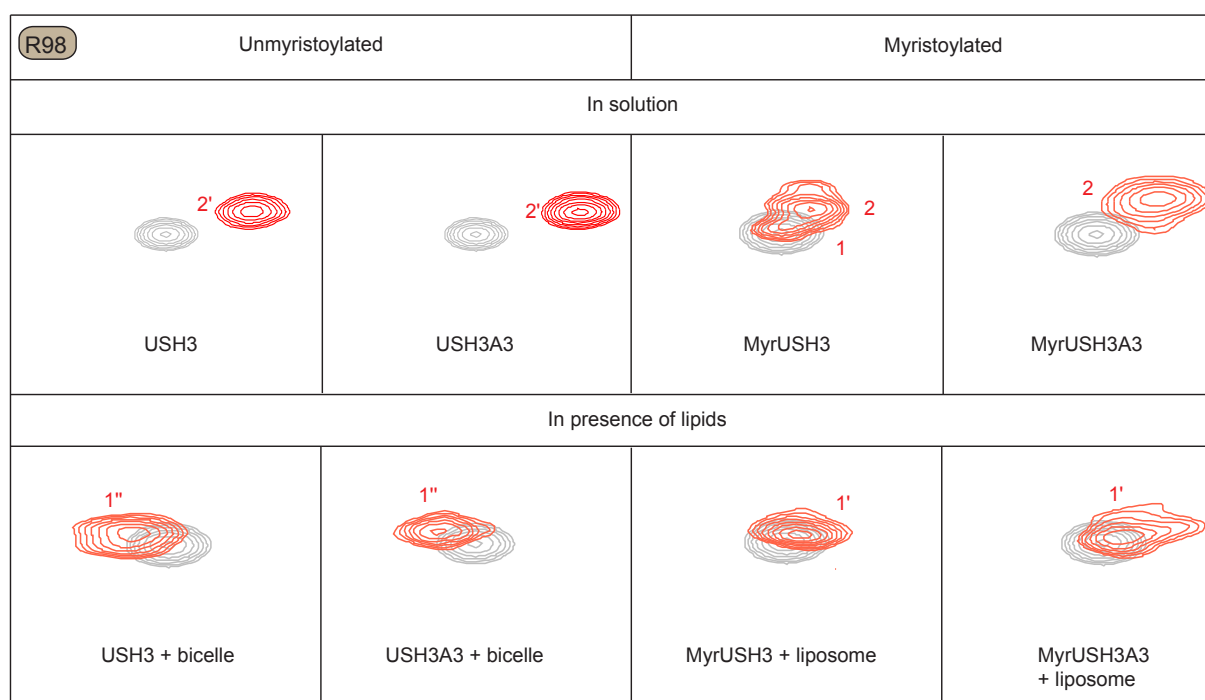


Figure 6.18: *Detail of the chemical shift corresponding to residue R98 in the different constructs; red: USH3 construct of interest, grey: SH3.*

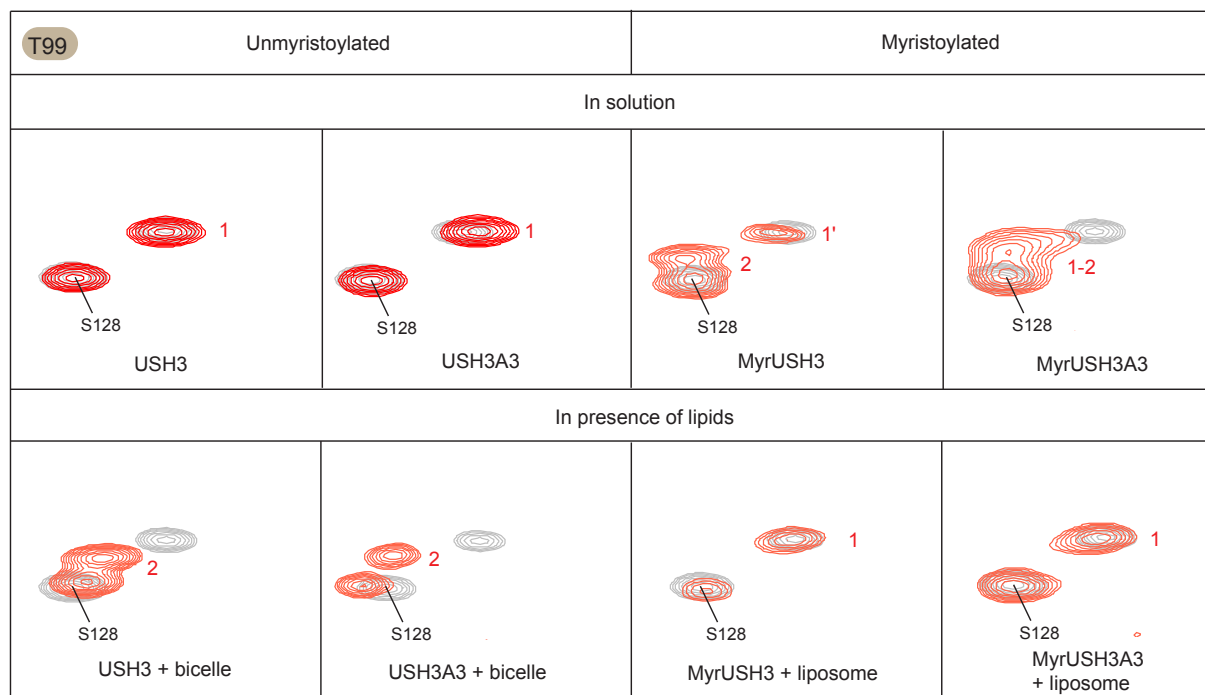


Figure 6.19: Detail of the chemical shift corresponding to residue T99 in the different constructs; red: USH3 construct of interest, grey: SH3.

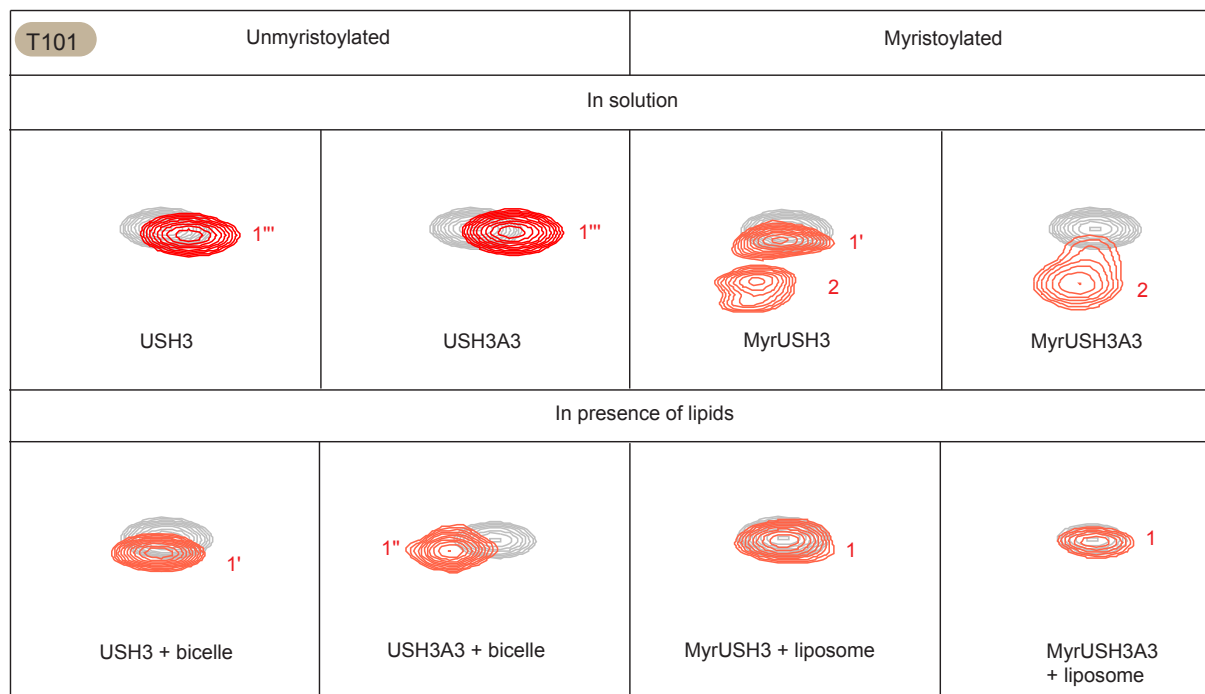


Figure 6.20: Detail of the chemical shift corresponding to residue T101 in the different constructs; red: USH3 construct of interest, grey: SH3.

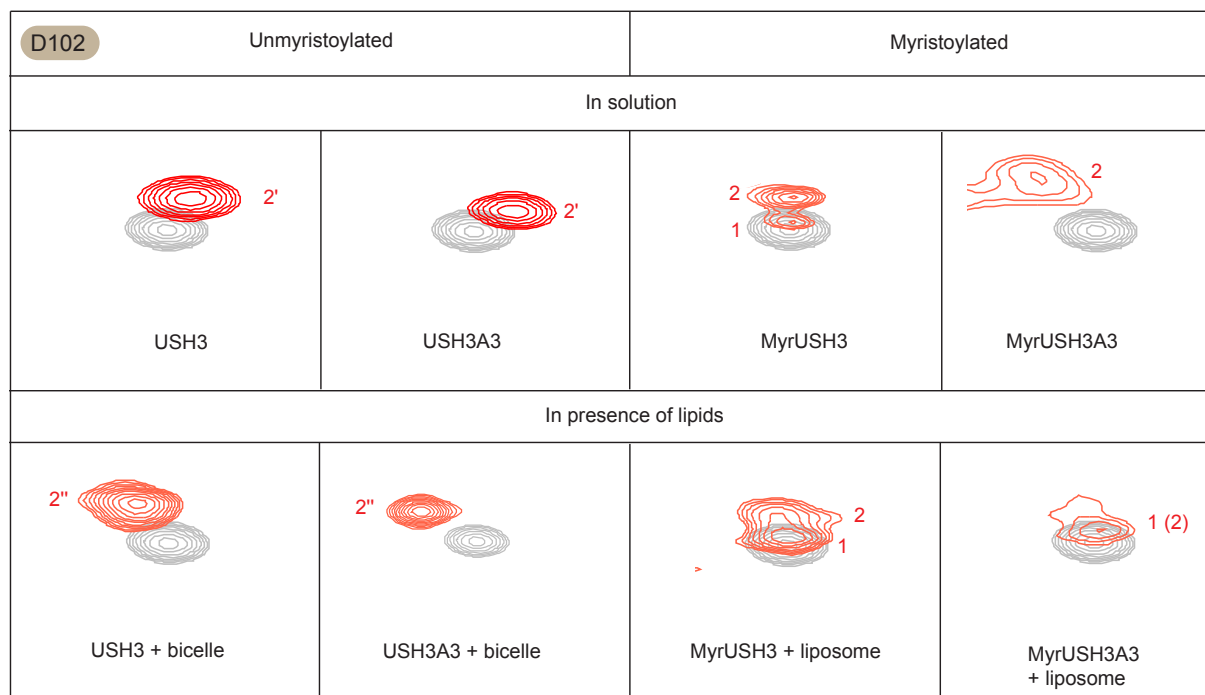


Figure 6.21: Detail of the chemical shift corresponding to residue D102 in the different constructs; red: USH3 construct of interest, grey: SH3.

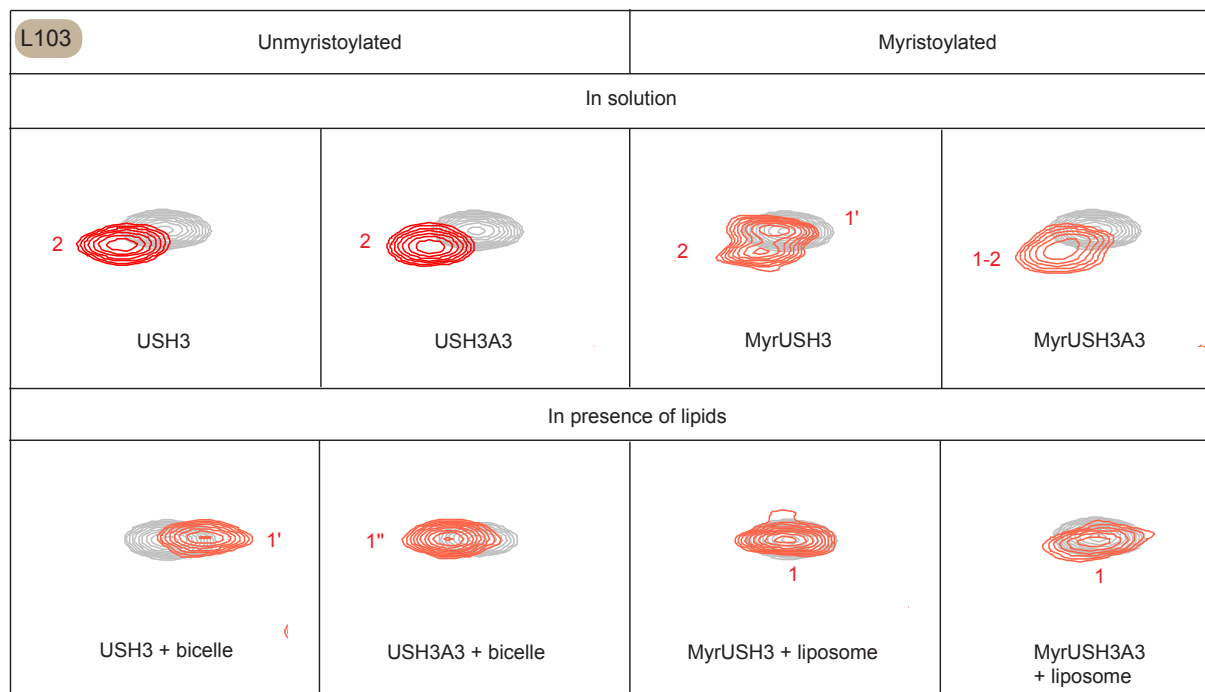


Figure 6.22: Detail of the chemical shift corresponding to residue L103 in the different constructs; red: USH3 construct of interest, grey: SH3.

The first observation was that in myristoylated forms bound to liposomes, signals from residues in the RT loop were always in position 1, thus they were not interacting. The capacity of the RT loop to interact with lipids observed in the non-myristoylated form (CSP of T99 and D102) was “lost” when the myristoyl group at the N-terminus of the molecule was inserted in the lipid bilayer. This is probably caused by the SH3 domain being forced in an orientation with respect to the lipid membrane that prevents the RT domain to interact with it.

Secondly, when MyrUSH3 was in solution, all amino acids of the RT loop were in slow exchange between position 1 and a second position (2). Position 2 was also found when the ULBR was removed, in the MyrUSH3A3 construct. This result suggests that the RT loop is interacting with both the myristoyl group and the Unique domain in the wild-type constructs, but that in the absence of the ULBR only the interaction with the myristoyl tail is retained. Indeed, in USH3, signals from residues D102 and L103 were found in a position close to position 2, corresponding to a situation in which the RT loop was exclusively interacting with the ULBR since the acyl chain was absent.

In order to provide an overview of the different positions of the residues in the RT loop, Table 6.23 summarizes the positions in the different USH3. The table contains the positions in absence or presence of the myristoyl group, of the ULBR or of lipids. V114 from the nSrc loop is also included.

Construct	Lipid	Myr	UL BR	Position					V114
				R98	T99	T101	D102	L103	
USH3 H6 WT	-	-	+	2'	1	1'''	2'	2'	2 – 3
USH3 H6 A3	-	-	-	2'	1	1'''	2'	2'	2 – 3
MyrUSH3 H6 WT	-	+	+	1+2	1'+2	1'+2	(1+) 2	1'+2	2+3
MyrUSH3 H6 A3	-	+	-	2	1 – 2	2(1')	2	1 – 2	2 – 3
USH3 WT H6 + bicelles	+	-	+	1''	2	1'	2'	1'	2 – 3
USH3 A3 + bicelles	+	-	-	1''	2	1''	2''	1''	2 – 3
MyrUSH3 H6 WT + liposomes	+	+	+	1 – 2	1	1	1+2	1	2(+3)
MyrUSH3 H6 A3 + liposomes	+	+	-	1 – 2	1	1	1(+2)	1	2
SH3				1	1	1	1	1	1

Figure 6.23: Summary of the positions of the different amino acids in the RT loop in the different constructs, and in V114 in the nSrc loop.

The interaction between the RT loop and the Unique domain is not directly abolished by the MyrUSH3AAA mutation in the ULBR. The interaction was observed in the non-myristoylated USH3AAA mutant. However, the differences observed between the wild type and mutant in the myristoylated USH3 constructs suggests that the interaction between the Unique and the SH3 domains is perturbed in the AAA mutant. As a consequence, the chemical shifts observed in the AAA mutant reflect simply the interaction of the RT loop with the myristoyl group.

This simple two-state switching model between alternative positions associated to the positions labeled 1 and 2 is clearly an oversimplification and the real situation is more complex. Additional chemical shift positions were observed (some residues adopt positions that significantly differed from positions 1 or 2). Often, the positions were close but not identical to positions 1 or 2; the positions slightly differing from the actual reference positions 1 or 2 are indicated by a “prime” or a “double prime” mark in the table. Also, not all residues within the RT loop are directly sensing the interaction as changes in chemical shift: T99 and T101 were in position 1 in the non-myristoylated forms of USH3.

6.4. Conclusion and Discussion

^1H - ^{15}N -2D spectra of MyrUSrc, MyrUSH3 and MyrUSH3A3 were recorded in solution and compared to those of the non myristoylated proteins. The presence of the myristoyl tail permitted to visualize the first three residues of the N-terminal; since they are not visible in the non- myristoylated construct, the effect of myristoylation on these residues can not be assessed. The N-terminal residues S6 and K7 were mostly affected by myristoylation. In absence and presence of the SH3, the changes observed in the myristoylated forms revealed that the myristoylated proteins remained soluble but adopted a different conformation in presence of the acyl chain. In MyrUSrc, the myristoyl group may interact with the slightly hydrophobic ULBR region. In case of MyrUSH3, it may interact with the RT loop. Many myristoylated proteins possess a hydrophobic pocket, and presence of a myristoyl binding pocket has been suggested for c-Src by [Cowan-Jacob et al. \(2005\)](#) – though the binding site was not found until now ([Patwardhan and Resh, 2010](#)). Our results indicate that the N-terminal myristoyl group interacts with the RT loop.

c-Src is mostly anchored to membranes in cells, however, the behavior of its soluble form gives insight on the propensities of the different regions of the USH3 to undergo intra or intermolecular interactions and on how the presence of the myristoyl tail influences them. In addition, as pointed out in the introduction, soluble pools of c-Src have been observed in cells, so the observed conformations may be biologically relevant. In summary:

- MyrUSrc has been measured at two concentrations and most of the differences were visible in the SH4 domain, indicating possibly intermolecular interactions through this domain. This result is compatible with the observed dimerization of several myristoyl constructs upon binding to lipid bilayers, as studied in the previous parts. MyrSH4 may be the promoter for intermolecular interactions.
- The “closed” conformation through the SH3/SH4 interaction is retained in presence of the myristoyl since no changes have been found between the myristoylated or non-myristoylated forms in the regions that are known to be reporting on this particular interaction. This result is also compatible with the experiments in which MyrUSH3 was separated from its degraded form using SEC at low pH which indicate that MyrUSH3 has probably an even more restricted conformation in presence of myristoyl.

- In the MyrUSH3 construct, the RT loop was found to interact with the myristoyl tail, as well as with the Unique domain; in the myristoylated form, the RT/Unique domain interaction is at least partially retained. In case of the MyrUSH3A3 mutant, the RT loop appears to interact almost only with the myristoyl group.
- The ULBR constituted the cleavage point observed when myristoylation-dependent degradation occurs. The forced conformation induced by the intramolecular interactions of the myristoyl tail with the ULBR (observed mainly in the absence of the SH3 domain), or with the RT loop when the SH3 domain is present, probably plays a role in the cleavage propensity. Interestingly, the USH3IHH mutant showed more extensive degradation than the wild type when it was myristoylated. Since in this mutant, the modified RT loop has lost its capacity to interact with the Unique domain, the interaction between the myristoyl group and the RT loop in wild-type c-Src may in fact decrease the degradation process. This raises the intriguing hypothesis that modulation of the interaction between the SH3 and Unique domains may affect the protein life-time under physiological conditions, and therefore, constitute a further Unique domain-related regulatory process in c-Src signaling.

Since these experiments gave data at the residue level, the weak intra or intermolecular interactions, mainly in the ULBR and the RT loop, could be studied also when N-terminal myristoylated proteins were anchored to lipid bilayers. In the experimental conditions used, enhanced binding of the secondary lipid binding region ULBR and RT loop was expected, because of the entropic gain of belonging to the same peptide; in fact, it only occurred in absence of the SH3 domain in our measurements.

- When MyrUSrc was bound to neutral liposomes, the SH4 was found to interact strongly with the liposomes upon myristoyl insertion and hydrophobic interactions of the ULBR to lipids were also observed. Anchoring of the myristoyl group to the lipid bilayer facilitated the secondary ULBR binding, since the amount of lipids needed to observe the interaction between ULBR and liposomes was much smaller than in case of the non myristoylated form (a molar excess of 67 was used in the experiments with the myristoylated form, while it was of 800 in case of the non-myristoylated form). When negative charges were introduced in the bilayer, electrostatic enhancement of SH4 binding to lipids was noticed at the two charge

density studied. ULBR binding to lipids was also reinforced with DOPC:DOPG liposomes. However, with an otherwise comparable charge content, a higher charge density did not favor electrostatic interactions in this region (as measured in case of DOPC:PI(3,4,5)P3). These lipids might be depleted from the membrane through MyrSH4 recruitment, as happens in other myristoylated protein: this phenomena has been described in MARCKS (Roskoski, 2004). Thus the charge density or the nature of the charged lipid may modulate the strength of the ULBR interaction to membranes. This finding can be related to one of our working hypothesis, in which the binding of ULBR to membranes could modulate the position of the catalytic domain of c-Src (Maffei, 2014), and thereby the enzyme's accessible substrates.

- In presence of USH3, the cooperative effect enhancing ULBR binding to lipids was not observed. When MyrUSH3 was bound to DOPC:DOPG liposomes, neither the ULBR nor the RT loop were found to interact with the lipids. The intramolecular interactions between the Unique and the SH3 domains probably directed the lipid binding domains away from the lipid bilayer. Nevertheless, it might be possible to recover these interactions in the presence of competing reactions. For instance, adding a polyproline peptide – which binds to the SH3 domain – might hamper the interaction between the RT loop and the ULBR, and permit interaction of the latter with lipids. The previous hypothesis of further regulation by lipid composition would hold. The SH3 domain or the ULBR might also display stronger affinities to other lipid types (this parameter was only very partially screened in this study). If it would be the case, new interactions between the SH3 or the ULBR domains might be forced and trigger protein spatial reorganization, and subsequently enzymatic activity. Dimerization concerns a minor protein population and is probably not observed in these data. Nevertheless, if dimerization would occur via the SH4 domain, it may compete with the SH4/SH3 interaction and induce conformational changes with respect to the monomeric form.

The results described in this chapter confirm some previous findings about long-range intramolecular interactions within the SH4-Unique-SH3 region of c-Src studied in the absence of the naturally occurring myristoyl group. Thus we confirm that these interactions,

and in particular the key interaction between SH4 and SH3 restraining the intrinsically disordered Unique domain in a loop, are preserved in the myristoylated forms.

In contrast, the additional lipid-binding sites present in the Unique and SH3 domains are clearly affected by the insertion of the myristoyl group.

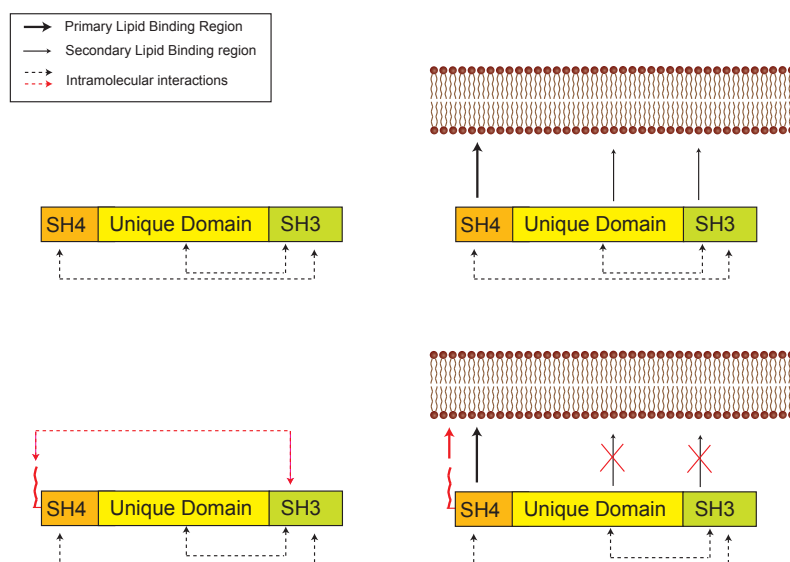


Figure 6.24: Scheme of the effect of the myristoyl on the intra- and intermolecular interactions in the USH3 domain

However, the presence of the myristoyl group enables additional interactions, beyond the known effect of lipid anchoring: the SH3 domain presents a binding site that is occupied by the myristoyl group when it is not inserted in the membrane. This finding suggests that c-Src may possess a myristoyl-switch, as found in other signaling membrane-associated proteins such as Abl. This putative myristoyl-switch could be relevant for c-Src trafficking processes.

Therefore, this work has demonstrated new potentially relevant interactions for c-Src function and, possibly, some general processes that are important to understand the functions of membrane-anchored intrinsically disordered proteins. As a consequence, a whole new field has just been opened.

6.5. Experimental Methods

Liposome preparation 1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC, and 1,2-dioleoyl - sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt), DOPG (both from TebuBio) were dissolved in chloroform, in a proportion of DOPC:DOPG 3:1. The solvent was evaporated in a rotavapor, and the lipid film was rehydrated with buffer (50mM NaP, pH 7) by vortexing the lipid film at ambient temperature, rendering a final total lipid concentration of 10 mM. Large unilamellar vesicles were prepared by mechanical extrusion using a 10-mL Thermobarrel extruder (LipexTM. Northern Lipids Inc.). The lipid suspension was extruded repeatedly (10 times) through a 100 nm polycarbonate filter at room temperature. To verify the appropriate size of the liposomes, the mean diameter was checked using Dynamic Light Scattering (Zetasizer Nanoseries S, Malvern instruments).

A third liposome type was prepared, in which Phosphatidylinositol 3,4,5-trisphosphate diC8, PI(3,4,5)P3, was supplemented to DOPC liposomes. The short chain C8-PI(3,4,5)P3 are soluble in water; they were resuspended in water and incubated half an hour with DOPC liposomes at ambient temperature, before using them immediately for the NMR experiments. The concentration of DOPC was kept at 5 mM and small volumes of concentrated PI(3,4,5)P3 solutions were added. Four liposome compositions were used by supplemented the DOPC liposomes with 40, 80, 160 and 330 μ M of PI(3,4,5)P3.

Bicelle preparation 1,2-dihexanoyl-sn-glycero-3-phosphocholine, DHPC (Avanti Polar Lipids) was dissolved in chloroform and 1,2-Dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DMPG) (TebuBio) in chloroform:methanol:H₂O 65:35:8. The lipids were dissolved in a DHPC:DMPG 1:0.8 proportion and the solvent was evaporated in a rotavapor; lipid film was rehydrated with warm buffer (50mM NaP, pH 7, 40 °C) and vortexing, which rendered a solution containing of 12.5% w/w of lipids. Bicelles were homogenized by repeating 10 freeze-thaw cycles (the solution was frozen in liquid nitrogen and warmed at 65 °C).

NMR sample preparation All samples were dialysed in the “NMR buffer” used throughout all NMR experiments, namely 50 mM NaP at pH 7.

¹⁵N MyrUSrc was obtained by the in-vitro myristoylation method and measured at two different concentrations, 150 and 400 μ M. ¹H ¹⁵N 2SOFAS-HMQC spectra were

recorded 50 mM NaP at pH 7, at 278 K. In the experiments with liposomes, the latter were added to a concentrated protein solution, rendering a final concentration of 75 μ M protein and 5 mM lipids.

^{15}N MyrUSH3 and ^{15}N MyrUSH3A3 were obtained by the coexpression method. Degradation free MyrUSH3 sample were obtained by the combination of Histrap chromatography with an imidazole gradient, and lyophilization/dyalysis cycles. Both proteins were measured at 150 μ M, at two temperatures, 278 K and 298 K. Residues in the disordered Unique domain are best seen at 278 K, but were also still sufficiently resolved at 298 K. Thus all spectra of the USH3 constructs presented in this part were acquired at 298 K. In the experiments with liposomes, the latter were added to a concentrated protein solution, rendering a final concentration of 150 μ M protein and 5 mM lipids.

A2G mutation in the USH3 or USH3A3 pETDuet1 plasmid enabled to express non acylated proteins. USH3 A2G and USH3A3 A2G were expressed and purified by standard expression and purification methods (Pérez et al., 2013) (Maffei et al., 2015). In the experiments with bicelles, the latter were added to a concentrated protein solution, rendering a final concentration of 150 μ M protein and 8 % w/v bicelles.

NMR spectra acquisition All experiments were performed in a Bruker 600 MHz Advance III spectrometer equipped with a TCI CryoProbe. All measurements were ^1H ^{15}N SOFAST-HMQC experiments.

NMR data treatment NMR spectra were processed using Bruker TopSpin 3.0 and NMRPipe (Delaglio et al., 1995) and analysed using the free-software Sparky (Goddard and Kneller).

Combined chemical shift differences were calculated using the following equation:

$$\Delta\delta = \sqrt{\Delta\delta_{\text{H}}^2 + \frac{\Delta\delta_{\text{N}}^2}{5}} \quad (\text{Equation 16})$$

Relative intensity differences were calculated using the following equation:

$$\Delta I = \frac{\frac{I_{\text{s,n}}}{I_{\text{s,0}}} - \frac{I_{\text{r,n}}}{I_{\text{r,0}}}}{\frac{I_{\text{r,n}}}{I_{\text{r,0}}}} \quad (\text{Equation 17})$$

where I is the peak height, n are the different residues along the sequence and 0 is the normalizer residue. s corresponds to the amino acids from the sample spectra and r to that of the reference spectra. The normalizing residue was chosen as a non affected residue (not affected by changes from the environment). The sample can be for instance the myristoylated construct, or the myristoylated construct in presence of liposomes. The reference can be for instance the non myristoylated construct, or the myristoylated construct in absence of liposomes.

Chapter 7

Conclusion

7.1. Concluding Remarks

Although the association of c-Src with membranes through the myristoylated SH4 domain was well known, the details of the interactions of other regions of c-Src with lipids remained obscure. Previous work had demonstrated that at least the Unique and SH3 domains contained lipid binding regions. However, no attempt had been made to place these interactions in the context of the myristoylated protein.

We have studied myristoylated constructs containing the SH4, unique and SH3 domains of c-Src. In this process, a general problem in the preparation of recombinant myristoylated protein associated with shorter acyl chains addition was solved, together with additional problems specific to the SH4-Unique domain of c-Src.

Biophysical studies have uncovered intermolecular protein-protein interactions of myristoylated constructs of c-Src causing clustering when they were binding to supported lipid bilayers. A myristoyl binding site has been identified in the SH3 domain of c-Src. Significant effects of myristoylation on intramolecular and lipid interactions within the Unique and SH3 domains have been demonstrated.

7.2. Conclusions

(I) Protocols for the efficient production of myristoylated proteins have been developed.

In particular:

- The incorporation of shorter acyl chains has been characterized as a general problem in the preparation of myristoylated proteins, and conditions minimizing their formation have been found.
- A well-defined myristoylation-induced cleavage site has been identified and characterized in the Unique Lipid Binding Region of the Unique Domain of c-Src. Conditions to obtain degradation-free samples for structural studies have been established.

(II) Formation of self-associated complexes of limited size was observed upon MyrUSH3 or MyrUGFP binding to supported lipid bilayers, by two independent methods. A short MyrSH4 peptide was also found to autoassociate in the lipid bilayer.

(III) A myristoyl binding site has been found in the SH3 domain in soluble MyrUSH3 constructs. Interaction of the myristoyl chain with lipids results in the loss of other lipid binding interactions in the Unique and SH3 domains, that were observed in the non myristoylated form.

(IV) The interaction between the SH4 and the SH3 domains that restricts the conformational space of the Unique domain is preserved in the myristoylated forms and in the presence of lipids.

Capítulo 8

Resumen de la tesis

8.1. Introducción

Membranas y proteínas periféricas La organización espontanea de lípidos en membranas, generando espacios cerrados, contribuyó a la aparición de la vida, principalmente porque permitió la segregación de reacciones químicas específicas. Las membranas de las células eucariotas y de sus orgánulos están compuestas de glicerofosfolípidos, esfingolípidos y colesterol, pero contienen también carbohidratos y proteínas en abundancia. Las membranas no son únicamente bidimensionales y tampoco son estáticas: pueden adoptar una variedad de formas y están en un estado fluídico, dejando que las moléculas que las constituyen se difundan en su bicapa. Sus dos capas son asimétricas. Lateralmente, se pueden encontrar nanodominios llamados balsas lipídicas, que se forman y se deshacen dinámicamente, y enriquecidos tanto en lípidos específicos (esfingolípidos, colesterol, fosfoinositoles) como en ciertas proteínas. Estos dominios permiten a su vez una segregación en dos dimensiones de reacciones químicas, por su capacidad de incluir o excluir moléculas ([Sonnino and Prinetti, 2013](#)). Son entidades cruciales en los fenómenos de señalización celular. Nuevas teorías completan la descripción de las membranas; una de ellas describe la existencia de proteínas rodeadas de lípidos específicos fuera de las balsas lipídicas. El inicio de la señalización podría ocurrir cuando pequeñas balsas o proteínas rodeadas de lípidos se unen, causando la activación de moléculas inactivas ([Simons and Toomre, 2000](#)) ([Ken~Jacobson et~al., 2007](#)). Las proteínas que residen en las membranas son de varios tipos, principalmente proteínas transmembranales o periféricas ([Makoto~Asashima et~al., 2010](#)). Dentro de las proteínas periféricas, nos enfocaremos en las proteínas que poseen

un grupo lipídico que contribuye a su interacción con la superficie de la membrana, y en particular en las proteínas miristoiladas (Martin et al., 2011), en las que el lípido unido es un grupo miristoilo.

La miristoilación es una modificación que tiene lugar en la glicina 2 de algunas proteínas, generalmente durante la síntesis proteínica, catalizada por la enzima N-miristoiltransferasa (NMT). El grupo mirístico resulta atado de manera covalente y la reacción es irreversible. El mirístico es la cadena acilada más corta que permite la unión de proteínas a membranas de manera estable, pero no irreversible (Taniguchia, 1999). Ciertas proteínas miristoiladas tienen en su cadena peptídica una zona hidrófoba en la que el grupo mirístico se puede “esconder” del agua, lo cual les confiere una solubilidad completa cuando no están unidas a membranas (Resh, 1999). Por su afinidad limitada para las membranas, la unión de proteínas miristoiladas a membranas esta en general reforzada por otros elementos de unión a lípidos (Buss and Sefton, 1985).

Dichos elementos pueden ser otras cadenas aciladas: un grupo palmitoilo está reversiblemente añadido en una cisteína y con su cadena más larga, asegura una unión fuerte de la proteína a la membrana. Una alternativa es que la proteína posea un motivo polibásico en su secuencia: los aminoácidos cargados positivamente interactúan electrostáticamente con los lípidos cargados negativamente en la membrana, aumentando la afinidad de la

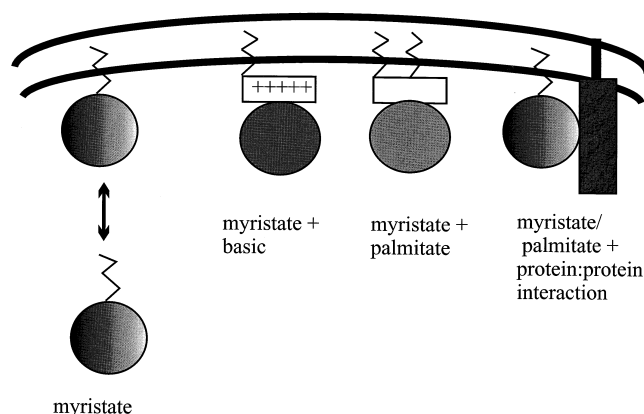


Figura 8.1: Unión de proteínas miristoiladas a membranas, reforzadas por otros señales

proteína por la membrana en un efecto cooperativo. Llamaremos esta secuencia un dominio positivamente cargado, PCD¹ (Tournaviti et al., 2009). La interacción electrostática puede ser modulada por la fosforilación en serinas o treoninas presentes en el PCD, que disminuye la carga total de la proteína, por lo tanto su afinidad para lípidos (Arbuzova et al., 1998). El papel del grupo mirístico, en conjunción con otros motivos de unión a lípidos, es principalmente de anclar la proteína a la membrana, pero también influye sobre

¹Positively Charged Domain

su localización. En particular, una proteína que posee las dos cadenas aciladas de mirístico y de palmítico se hallará generalmente dentro de las balsas lipídicas (Resh, 1999).

Proteínas desordenadas miristoiladas El paradigma que vincula la estructura tridimensional de las proteínas y sus funciones ha estado evolucionando en los años recientes. Muchas proteínas no tienen estructura tridimensional, pero sí son funcionales. Estas proteínas llamadas intrínsecamente desordenadas lo pueden ser enteramente, o pueden contener regiones plegadas y regiones desordenadas (Dunker and Obradovic, 2014). Las proteínas desordenadas tienen características específicas: tienen generalmente una carga neta alta, son poco hidrófobas, y su secuencia de aminoácidos es de baja complejidad (Uversky et al., 2000). Estas características permiten predecir por métodos estadísticos el carácter desordenado de una secuencia de aminoácidos, cálculos que han demostrado la abundancia significativa de tales proteínas (Radivojac, 2007). Con su carácter desordenado, estas proteínas presentan propiedades únicas: pueden adoptar de manera dinámica una multitud de conformaciones (desde completamente desordenadas hasta parcialmente y a veces completamente plegadas). En consecuencia, pueden interactuar con una multitud de sustratos; estas interacciones son generalmente altamente específicas pero fáciles de romper. Las interacciones pueden ser moduladas simplemente porque la proteína intercambia espacialmente entre muchas conformaciones, o porque un factor exterior ocasiona un cambio de conformación. La formación de una hélice α tras la unión a membrana de un péptido desordenado es un ejemplo de tal efecto. Por las propiedades que le confiere su plasticidad, las proteínas desordenadas tienen un papel muy importante en las cadenas de señalización celular (Dunker and Obradovic, 2001), y son generalmente altamente reguladas (Uversky et al., 2014). Estas proteínas son también fácilmente degradables y altamente susceptibles a modificaciones postraduccionales (Jakob et al., 2014). Una de ellas es la miristoilación.

La presencia del grupo miristoilo confiere la capacidad de unión a lípidos. El hecho que la región adyacente esté desordenado tiene un efecto sinérgico:

- La presencia de un dominio desordenado puede disminuir el impedimento estérico que ocasionaría la presencia de un dominio plegado muy cerca a la membrana (entonces el dominio desordenado facilita la unión a la membrana) (Pool and Thompson, 1998).

- Puede favorecer modificaciones postraduccionales adicionales en el dominio desordenado, más accesible; la palmitoilación (Resh, 1999) o la fosforilación (Tournaviti et al., 2009) son probablemente facilitadas en estos casos, y ayudan a la regulación de la proteína con estas modificaciones reversibles.
- La posible formación de helices α tras el encuentro del dominio desordenado con la membrana permite crear un dominio adicional de unión a lípidos y aumentar el anclaje de la proteína a la membrana (Tholey et al., 2001).
- La interacción del grupo miristoilo con la propia proteína puede representar una alternativa a su inetracción con lípidos. Los sitios de unión de grupos miristoilo conocidos se encuentran en dominios globulares. La interacción del grupo miristoilo puee afectar alostericamente la actividad de la enzima (Hantschel et al., 2003).

Tanto las proteínas miristoiladas (Traverso et al., 2013) como las desordenadas (Radivojac, 2007) son abundantes y la asociación entre miristoilación y desorden es probablemente un aspecto que merece considerable atención. Ambas características juegan un papel importante en la regulación de las proteínas que las poseen, proteínas a menudo implicadas en la señalización celular. Las proteínas que son a la vez desordenadas y miristoiladas podrían jugar un papel no despreciable en las cascadas de transducción de señal.

c-Src

c-Src y la familia tirosina quinasa c-Src es el miembro que da nombre a una importante familia de tirosina quinasas, las enzimas que transfieren un grupo fosfato a un residuo de tirosina de otras proteínas (Parsons and Parsons, 2004). Este proceso constituye una etapa muy importante dentro de la transducción de señales en las células, y de la regulación de la actividad enzimática. Las tirosinas quinasas de la familia Src están implicadas en varias rutas de señalización, y la desregulación de c-Src afecta a la migración, la proliferación y la supervivencia de las células (Ingleby, 2008). La actividad de c-Src está finamente controlada y su desregulación promueve cánceres (Irby, 2000). c-Src es una proteína miristoilada (Buss et al., 1994), lo cual confina su actividad catalítica a la interfase de la membrana, lo cual es esencial para su buen funcionamiento (Patwardhan and Resh, 2010). Los otros miembros de la familia Src poseen casi todos una cadena de

ácido palmítico adicional unida a la cisteína 3 (Buss and Sefton, 1985), pero c-Src se distingue por tener un dominio polibásico justo después de su sitio de miristoilación Sigal et al. (1994). La región N-terminal de c-Src y de los otros miembros de la familia está desordenada y poco conservada dentro de la familia, lo cual le ha dado su nombre, el Dominio Único. Debido a su carácter desordenado, este dominio está ausente de las estructuras de c-Src obtenidas por difracción de rayos X de cristales – que han permitido conocer la estructura e interacciones de los otros dominios que forman la parte globular de la proteína. La estructura de c-Src fue resuelta en 1997 (Xu et al., 1997). A continuación del dominio Único vienen los dominios plegados SH3, SH2 (dominios de interacción con ligandos) y SH1 (el dominio catalítico). Los dominios SH2 y SH1 están conectados por otro dominio semidesordenado que contiene un motivo de poliprolina. En la región C-terminal se encuentra una cola flexible que participa en la regulación negativa de la actividad de c-Src. El mecanismo de autoinhibición está controlado por dos tirosinas, una en la cola C-terminal, Y537, y otra en un bucle del dominio SH1, Y416. Cuando Y537 está fosforilada, se une al SH2 mientras el SH3 interacciona con el motivo de poliprolina del conector SH2-SH1, manteniendo c-Src en una conformación cerrada. Si Y537 es defosforilada, la proteína se abre y vuelve a ser enzimáticamente activa. La autofosforilación de Y416 permite la actividad enzimática máxima. Los miembros miristoilados y palmitoilados de la familia están situados en las balsas lipídicas, pero c-Src se encuentra dentro o fuera de ellas. Cuando no está activada, c-Src se halla en la zona perinuclear Kim et al. (2009), y viaja hasta la membrana plasmática (Courtneidge, 1980) donde llega a su estado de activación máxima (Yeatman, 2004). c-Src ha sido visualizada también en endosomas, que participan en la entrega de c-Src a la membrana plasmática. Además, c-Src ha sido observada en estado soluble en varios estudios (Buss et al., 1994) (David-Pfeuty et al., 1993) (Donepudi and Resh, 2008).

Los Dominios Únicos y SH3 (USH3) La figura 8.2-A y -B ilustra las consideraciones en este párrafo. La unión a membranas de c-Src tiene lugar principalmente por la inserción de la cola de miristoilo en la bicapa y por la interacción electrostática con lípidos negativos del dominio polibásico al extremo N-terminal de c-Src, el dominio SH4 (Buser et al., 1994). El dominio Único posee también una zona de unión a lípidos (denominada ULBR, “Unique Lipid Binding Region”) (Pérez et al., 2013). Varios residuos de serina o tirosina del SH4 y del Dominio Único pueden ser fosforilados en condiciones fisiológi-

cas (Amata et al., 2013), posiblemente modulando la carga y la fuerza de unión a lípidos de las zonas de interacción cercanas. El papel de la ULBR se entiende hasta ahora como un segundo nivel de regulación de la enzima y no participa en la unión fuerte de c-Src a membranas. Las mutaciones en la ULBR de c-Src entero en células de ranas y humanas resultaron en fenotipos muy perturbados y distintos de la forma no mutada, lo cual apunta a la importancia funcional del ULBR. El dominio SH3 viene después del Dominio Único y posee también sitios de unión a lípidos en dos zonas, el bucle RT y el bucle n-Src. La unión al dominio SH3 de un ligando con un motivo de poliprolina compite alostéricamente con la unión a lípidos del bucle RT (Pérez et al., 2013).

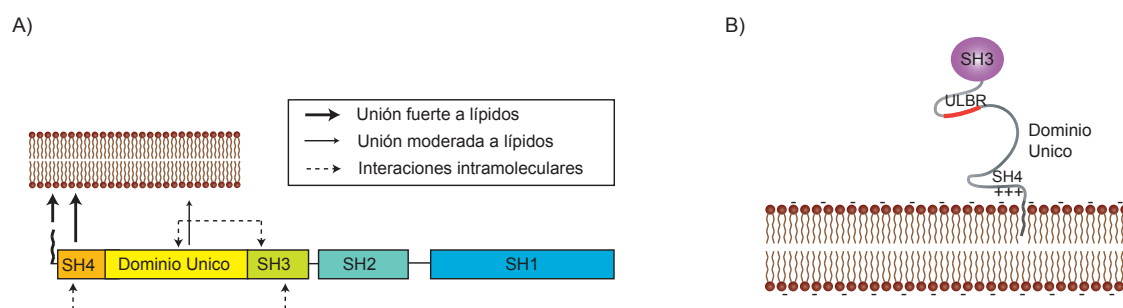


Figura 8.2: A) Estructura de la MyrUSH3; las interacciones intramoleculares o con lípidos están indicadas. B) Esquema de MyrUSH3 unido a una bicapa de lípidos.

Estudios *in-vitro* de la forma no miristoilada del USH3 revelaron interacciones intramoleculares entre la ULBR y el bucle RT, y entre el SH4 y el bucle nSrc. Estos resultados muestran que las interacciones intramoleculares limitan el espacio conformacional explo-
 rable por el Dominio Único, a pesar de ser desordenado, y que el USH3 mantiene, en las condiciones observadas, una conformación relativamente cerrada (Maffei et al., 2015).

8.2. Objetivos

Establecer protocolos experimentales para la producción de proteínas miristoiladas en cantidad adecuadas para su caracterización con técnicas biofísicas

- I Optimizar métodos de expresión y purificación de proteínas miristoiladas usando estrategias de coexpresión con NMT y eliminar posibles fuentes de contaminación.
- II Escalar métodos de miristoilación *in-vitro*

Caracterizar la unión de construcciones miristoiladas conteniendo el Dominio Único de c-Src con bicapas lipídicas.

- I Estudiar su cinética de unión a bicapas lipídicas
- II Estudiar su posible asociación cuando se une a bicapas lipídicas
- III Estudiar las interacciones de la ULBR y del bucle RT con lípidos en presencia de la cola miristoilada insertada en la bicapa.

8.3. Miristoilación

En la reacción de miristoilación intervienen tres especies: la enzima NMT, el sustrato y la especie donadora de ácido mirístico (myristoil-Coenzima A, MyrCoA) (Towler and Glaser, 1986). El sustrato es una proteína que, tras la eliminación de la metionina 1, posee una glicina en posición 2, y otros aminoácidos específicos en las posiciones siguientes, que permiten el reconocimiento por la enzima. MyrCoA se une primero a la NMT, quien después de un cambio conformacional puede acomodar la proteína en un segundo sitio, y catalizar la transferencia de la cadena acilada desde el CoA hasta la proteína (Farazi et al., 2001). La reacción está resumida en la Figura 8.3.

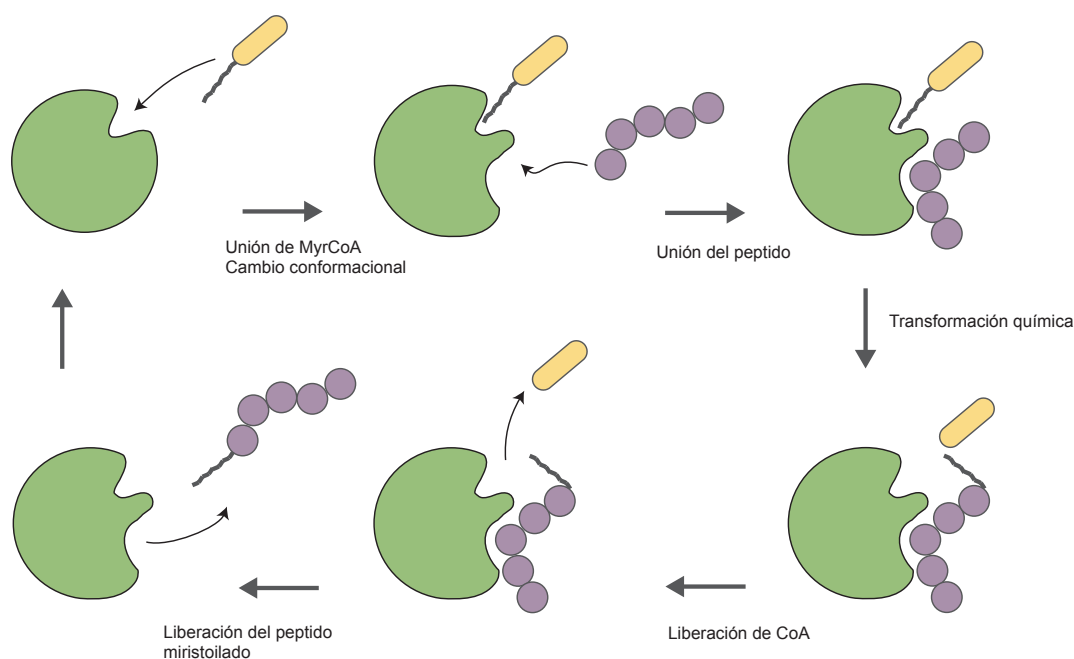


Figura 8.3: Esquema de la reacción de miristoilación

Se han utilizado dos métodos para obtener proteínas recombinantes miristoiladas:

- La expresión simultánea de la NMT y de la proteína en bacterias de *E.coli* (Gluck et al., 2010) (las bacterias no producen normalmente la NMT) - llamaremos este procedimiento coexpresión.
- Producir los tres productos por separado (la NMT, la proteína y MyrCoA) y mezclarlos para realizar la reacción de miristoilación (Padovani et al., 2013).

Myristoilación de la USH3 La estrategia de coexpresión se utilizó para producir la USH3 miristoilada (la MyrUSH3). En el protocolo clásico, después de la expresión de la proteína en *E.coli*, las células se sonicaron y centrifugaron, y la proteína se recuperó del sobrenadante. En el caso de la USH3, la presencia de una cola de histidinas en el extremo C-terminal permitió la purificación de la USH3 con una resina de níquel. Las proteínas fueron finalmente separadas por cromatografía de exclusión molecular (SEC²). Con este método, la proteína miristoilada se obtuvo pura juntamente con otras especies visibles por SEC (Figura 8.4 A, curva negra y B): una forma laurilada de USH3, la LaurUSH3, en la que la cadena acilada tenía dos átomos de carbono menos que la forma miristoilada y una forma degradada. La proteína MyrUSH3 deseada apareció formando un complejo con la NMT. Este complejo podría utilizarse para obtener la proteína MyrUSH3, separándola de la enzima, pero los métodos que lo permitirían funcionaron sólo parcialmente, y con poco rendimiento.

Se desarrolló otro método de purificación: en lugar de usar el producto soluble de la sonicación de las bacterias, se recuperó el sedimento celular, del que se extrajo la proteína MyrUSH3 con un detergente (Triton X) y después de una nueva centrifugación, el producto solubilizado se purificó como fue descrito anteriormente. Este método permitió deshacerse de la NMT (Figura 8.4-A, curva roja y -C), pero no eliminó totalmente la proteína laurilada y las formas degradadas.

- Las proteínas lauriladas se originan por la falta de especificidad de la enzima NMT que puede reconocer la LaurCoA (Towler and Glaser, 1986) (Yizhou~Liu et~al., 2009). La degradación en LaurCoA es la primera especie formada en el proceso de β -oxidación de la MyrCoA (una ruta celular de producción de energía). Para minimizar su producción, se añadió ácido palmítico, que aumenta la cantidad de la MyrCoA por la degradación de la PalmCoA mientras que el propio PalmCoA no es incorporado por la enzima NMT. Además, se usó un tiempo de expresión corto para evitar que las células recurrieran a la β -oxidación. En caso de crecimiento en medio mínimo, se usó adicionalmente el método llamado método de Marley Marley et~al. (2001), en el que las bacterias se hacen crecer en medio rico, y a continuación se transfieren a un medio mínimo para la inducción. En estas condiciones, se minimizó la formación de formas lauriladas, pero no se consiguió evitar la presencia de productos de degradación.

²Size exclusion chromatography

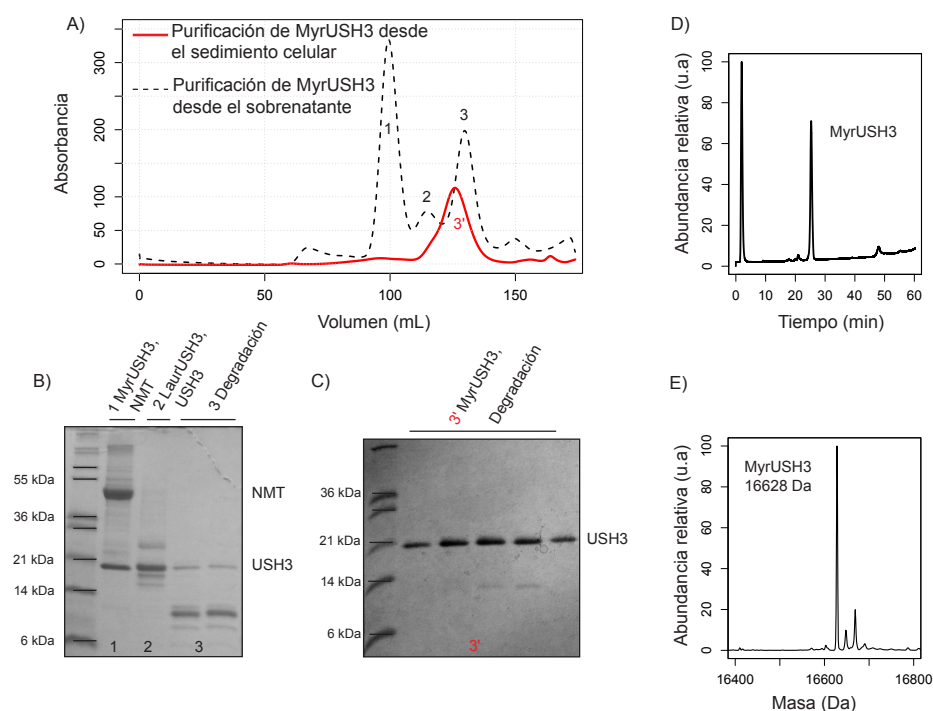


Figura 8.4: A) Cromatografía por exclusión molecular después de la purificación de MyrUSH3 con los dos métodos. Electroforesis en gel de las fracciones obtenidas en la SEC B) purificación desde el sobrenadante y C) purificación desde el sedimento celular E) Cromatografía líquida de alta resolución y F) Espectrometría de masas de las fracciones obtenidas en la SEC por purificación desde el sedimento celular.

- La forma degradada obtenida es un producto de degradación de MyrUSH3, que tiene la cola de histidinas pero no el grupo miristoilo. El sitio de corte se encontró mediante un estudio de análisis de masa: la ruptura tiene lugar en la zona del ULBR, después de la L63. Se expresó y purificó un mutante en el que los aminoácidos ⁶⁴LFG⁶⁶ se remplazaron por tres alaninas ⁶⁴AAA⁶⁶. Este mutante no se degradó. Desafortunadamente, el uso de este mutante no es apropiado en el estudio de las propiedades de la ULBR.
- La separación del producto de degradación se logró optimizando las condiciones de purificación por afinidad mediante una columna HisTrap. Después de unir el producto solubilizado a la columna HisTrap, la elución se inició con una concentración baja de imidazol. El producto de degradación se eluyó muy fácilmente en estas condiciones, mientras la MyrUSH3 tenía una afinidad más grande con la resina de níquel y permaneció mayoritariamente unida a la columna. Una vez se haya eluido todo el producto de degradación, MyrUSH3 se eluyó en forma pura subiendo la concentración de imidazol. Con este método, la MyrUSH3 se pudo obtener puro, sin producto degradado (Figura 8.4-D y -E).

Myristoilación del Dominio Único de c-Src El método de coexpresión del Dominio Único de c-Src (USrc³) con la NMT en las bacterias no permitió obtener USrc miristoilado (MyrUSrc). Los tres productos, la NMT, la MyrCoA y la USrc se obtuvieron por separado. El ácido mirístico se activó químicamente con carbonildiimidazol y se hizo reaccionar con CoA según el método de Gaffarogullari et al. (2011). USrc se expresó y se purificó en *E.coli* con el método clásico (Pérez et al., 2013). La NMT se recuperó del complejo con MyrUSH3 obtenido como fue descrito en parte 8.3: se unió el complejo a la resina de níquel, y se eluyó específicamente la NMT con una solución conteniendo MyrCoA. El protocolo de miristoilación consistió en mezclar la NMT con una solución conteniendo un exceso de MyrCoA, y añadir USrc después de 20 min, como fue ilustrado en el esquema de la Figura 8.5-A. La reacción se dejó 2 h a 37 °C, y a continuación a 4 °C durante la noche. El éxito de la reacción se comprobó con un ensayo de sedimentación de vesículas: después de mezclar el producto de la reacción con vesículas y de centrifugarlo, se separaron el sobrenatante y la fase lipídica. Una electrofóresis en gel de las dos fases permitió identificar

³Unique Domain of Src

si la proteína se encontraba unida a liposomas, lo cual fue el caso del producto de la reacción de miristoilación, pero no del control con USrc (Figura 8.5 B). Este producto de reacción se purificó mediante el uso de cromatografía líquida de alta resolución (HPLC), en la que se separaron los productos – la MyrCoA, la USrc, la MyrUSrc y la NMT (Figure 8.5-C). Las fracciones que contenían MyrUSrc se colectaron, se liofilizarono y MyrUSrc se resuspendió en una solución tampón.

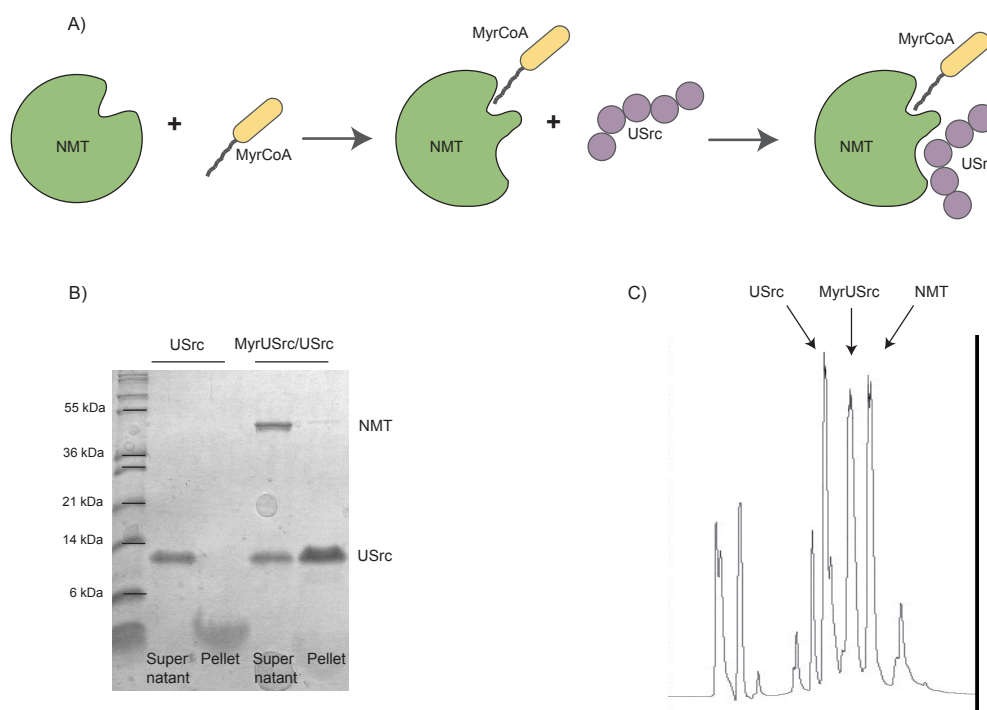


Figura 8.5: A) Esquema de la reacción in-vitro B) Electroforesis en gel de las especies de las USrc y MyrUSrc antes y después de la reacción de miristoilación, tras haber realizado un LSA con ellas. C) HPLC del producto de miristoilación de USrc.

8.4. Unión de la MyrUSH3 a los lípidos

La unión de MyrUSH3 a liposomas se estudió por resonancia de plasmones superficiales (SPR⁴). Esta técnica permite obtener la cinética de la unión entre el ligando inmovilizado en la superficie de la cámara y un analito (Bakhtiar, 2013). El ligando inmovilizado está constituido por liposomas en nuestro experimento, y el analito es una solución de MyrUSH3 a una concentración definida. Los liposomas se inmovilizaron en la superficie, retenidos por un lípido (fitoesfingosina) covalentemente unido a esta (Besenicar et al., 2006). La inyección de la proteína sobre los liposomas dió lugar a unas curvas de unión que mostraban una asociación relativamente rápida, y una disociación muy rápida, que indican una constante de asociación relativamente baja. Esta unión reversible se estudió de una manera clásica, como fue descrito en la primera parte de este párrafo. Sin embargo, la disociación de MyrUSH3 no era completa y se observó una señal residual que podría corresponder a la presencia de proteínas no disociadas. Para confirmar esta observación, se inyectó un anticuerpo secundario que reconoce al dominio SH4, el antiSH4. La respuesta positiva de SPR confirmó la presencia de una población de proteína que se disoció muy lentamente de los liposomas, que llamaremos “especie persistentemente unida” (PB⁵). Esta especie se caracterizó en un protocolo específico, recurriendo al antiSH4, como fue descrito en la segunda parte de este párrafo. Un comportamiento similar se observó tras la inyección del péptido MyrSH4, sugiriendo que los determinantes de la formación de las especies PB se localizan en el dominio SH4 miristoilado; aún así, la caracterización cinética se realizó con la proteína MyrUSH3, que contiene los dominios adicionales de unión a lípidos (la ULBR y el bucle RT).

Análisis cinético de la unión reversible de la MyrUSH3 a los liposomas La proteína se inyectó a varias concentraciones (los ciclos de inmovilización de liposomas e inyección de proteínas se pueden repetir gracias a la regeneración completa obtenida con la aplicación de un detergente, CHAPS⁶), y su asociación y disociación a liposomas se monitorizó. Las curvas de asociación y disociación pudieron ser ajustadas mediante un modelo simple de interacción 1:1, en el que la proteína P interacciona con los lípidos L para formar el complejo PL, con unas constantes de reacción k_a y k_d , equivalente a una

⁴surface plasmon resonance

⁵persistently bound

⁶3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate

constante de equilibrio $K = \frac{k_a}{k_d}$:



Estas constantes se midieron en dos casos: con lípidos neutros o lípidos conteniendo cargas negativas. La constante de equilibrio calculada se acerca a la descrita en la literatura para el péptido SH4 miristoilado (MyrSH4) (Buser et al., 1994), mostrando que este péptido es el responsable principal de la unión de c-Src a lípidos. La afinidad calculada es más fuerte en el caso de liposomas cargados negativamente, lo cual se esperaba debido a la interacción electrostática adicional que aparece con el SH4. La tabla 8.1 resume los resultados de los ajustes.

Cuadro 8.1: *Parametros de cinética resultantes del ajuste de los datos de unión directa de MyrUSH3 a liposomas neutrales o cargados negativamente, usando un modelo 1:1.*

	$k_a(\text{M}^{-1} \cdot \text{s}^{-1})$	$k_d(\text{s}^{-1})$	$K_D(\text{M}^{-1})$
DMPC	$4.54 \cdot 10^3 \pm 2.18 \cdot 10^3$	$2.81 \cdot 10^{-1} \pm 1.73 \cdot 10^{-1}$	$1.54 \cdot 10^4 \pm 7.01 \cdot 10^3$
DMPC:DMPG 2:1	$2.46 \cdot 10^4 \pm 1.31 \cdot 10^4$	$1.29 \cdot 10^{-1} \pm 8.63 \cdot 10^{-2}$	$1.9 \cdot 10^5 \pm 6.52 \cdot 10^4$

Análisis cinético de la unión irreversible de la MyrUSH3 a los liposomas El antiSH4 se usó como método de detección de las especies PB para estudiar por separado su cinética de unión a lípidos, dado que la respuesta del anticuerpo refleja la cantidad de especies PB presentes en la superficie. La proteína MyrUSH3 se inyectó con distintos tiempos de asociación, y después de un tiempo de disociación constante se realizó la detección secundaria de las especies PB con anticuerpo antiSH4. Este protocolo permitió estudiar la cinética de unión de las especies PB a liposomas. Por otra parte, MyrUSH3 se inyectó con tiempos constantes de asociación, pero la detección con antiSH4 se realizó a tiempos variables de disociación (Figura 8.6 A y B dan un ejemplo de tal protocolo con 60 s de asociación de MyrUSH3 y con una inyección del anticuerpo después de 350 s) . Este protocolo permitió estudiar la cinética de desunión de las especies PB a liposomas. Las curvas de respuestas a un tiempo dado del anticuerpo en función de los tiempos de asociación o disociación se trazaron, como está ilustrado en la figura 8.6-C a -F, mostrando primeramente que la disociación de las especies PB era despreciable, tanto en liposomas neutros como en los cargados negativamente. Incluyendo esta observación, se propuso un modelo cinético que permitió ajustar estas curvas para obtener la constante de asociación

k'_{on} . El modelo que explica los datos experimentales contiene una ecuación de segundo orden que sugiere que las especies PB son, como mínimo, dímeros, llamados PB_2 . La disociación muy lenta se explicaría por la presencia de especies unidas por dos cadenas de miristoiles en lugar de una, permitiendo una unión mucho más estable a lípidos, incluso en el caso de lípidos sin cargas negativas. El modelo se desarrolló a partir de las reacciones cinéticas (con k_{on} la constante de asociación de la formación de PB_2):



La ecuación que describe la unión de las especies PB_2 a lípidos obtenida a partir de estas reacciones es de la forma:

$$R = C_1 \cdot (1 - \exp(-k'_{\text{on}} \cdot t)) \quad (\text{ecuación 11s})$$

donde R representa la respuesta de SPR del antiSH4 y C_1 depende de la concentración de la proteína $[P]$. Las curvas de la figura 8.6-C y -D se ajustaron con esta ecuación y permitieron obtener los valores de k_{on} (resumidas en 8.2).

Cuadro 8.2: Ajustes de las curvas de las Figuras 8.6 C y D con (equation 11)

Concentration (M)		10^{-6}	$2 \cdot 10^{-6}$	$4 \cdot 10^{-6}$	10^{-5}		$2 \cdot 10^{-5}$
DMPC	$C_1(\text{RU})$	175.1 ± 26.9	272.1 ± 32.5	383.4 ± 32.8	512.3 ± 31	520.2 ± 23.3	697.3 ± 47
	$k'_{\text{on}}(\text{s}^{-1})$	0.01462 ± 0.004	0.02269 ± 0.006	0.06505 ± 0.022	0.1688 ± 0.077	0.1480 ± 0.046	0.2256 ± 0.155
DMPC:DMPG 2:1	$C_1(\text{RU})$	512.3 ± 75.6	889.3 ± 83.3	1223 ± 48.2	1484.1 ± 65	1419.8 ± 27.7	1678 ± 36.1
	$k'_{\text{on}}(\text{s}^{-1})$	0.00808 ± 0.002	0.00877 ± 0.001	0.01829 ± 0.002	0.04481 ± 0.009	0.05 ± 0.005	0.1050 ± 0.020

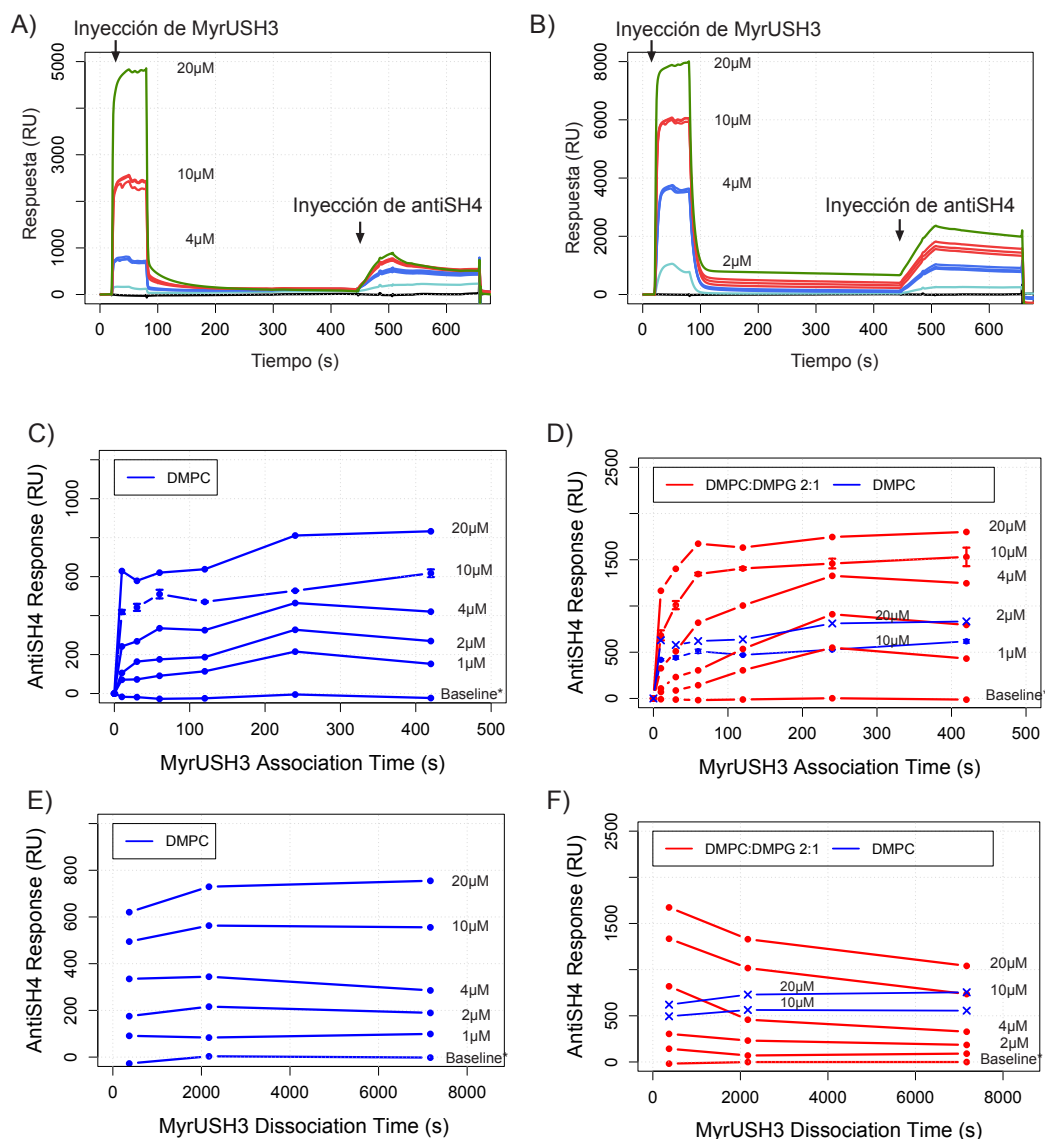


Figura 8.6: Asociación y disociación de MyrUSH3 a liposomas y detección secundaria con antiSH4 A) sobre liposomas neutros B) sobre liposomas cargados. Gráficas de la respuesta del anticuerpo en función del tiempo de asociación de MyrUSH3 C) en liposomas neutros y D) en liposomas cargados negativamente. Gráficas de la respuesta del anticuerpo en función del tiempo de disociación de MyrUSH3 E) en liposomas neutrales y F) en liposomas cargados negativamente.

8.5. Unión de la MyrUGFP a lípidos

Con el fin de obtener una variante de MyrUSrc susceptible de ser observada por fluorescencia, se diseñó la proteína MyrUGFP, que contiene una proteína verde fluorescente (GFP⁷) en lugar del dominio SH3. Para verificar su capacidad de interacción con lípidos, se comparó la inserción de MyrUGFP y de MyrSH4 en monocapas lipídicas cargadas negativamente. A continuación, se realizó un estudio de microscopía de fluorescencia de las especies PB de MyrUGFP unidas a liposomas, al nivel de las moléculas individuales (“single molecule experiments”).

Estudio de inserción en monocapas de MyrUGFP y MyrSH4 En un experimento de monocapas, se depositan lípidos en una interfase agua-aire. Se forma una monocapa, con las colas lipídicas apuntando al aire, y la cabezas polares tocando el agua. La presión generada por la presencia de lípidos en la interfase se puede medir con una balanza de Wilhemy [Maget-Dan \(1999\)](#). Una pequeña placa de papel o de metal se inmersa parcialmente en el agua y esta conectada a un tensiómetro, que mide la presión superficial. Si una proteína se inyecta en la subfase acuosa y se une a los lípidos de la monocapa, se detectará un cambio de presión. A medida que aumenta la presión inicial de los lípidos, disminuye la capacidad de inserción de las proteínas y el cambio de presión será menor. El MyrSH4 y la MyrUGFP se inyectaron a una concentración de proteína constante, pero con distintos valores de presión lipídica iniciales. Se derivó en ambos casos el cambio de presión debido a la inserción de la proteína en función de la presión inicial, y por regresión lineal se dedujo el valor de la presión de exclusión ([Calvez et al., 2009](#)) – la presión máxima a la que se pueden insertar MyrSH4 o MyrUGFP. En este experimento, la presión de exclusión de MyrSH4 era 50 mN/m, mientras la de la MyrUGFP era de 34 mN/m, valor mucho más representativo de valores obtenidos con otras proteínas. La presión de una monocapa con la misma capacidad de exclusión de las membranas celulares en equilibrio se ha estimado que es entre 30 y 35 mN/m. Este resultado confirma que MyrUGFP se puede insertar en membranas. El valor de exclusión de MyrUGFP, y posiblemente otras construcciones que contienen el dominio Único además del extremo SH4 miristoilado, sugiere que estas formas pueden unirse y disociarse de las membranas causando una mínima perturbación en el sistema lipídico. La extrema capacidad de penetración del péptido SH4 miristoilado

⁷Green Fluorescent Protein

parece ser modulada por la regiones vecinas de la proteína natural. Es importante, por tanto, estudiar las construcciones más largas, más cercanas a la realidad biológica.

Estudio de microscopía de fluorescencia de la MyrUGFP unido a los liposomas al nivel “single molecule” Para aclarar la naturaleza de las especies PB encontradas en el experimento de SPR, se estudió la unión de MyrUGFP a liposomas, mediante su observación con microscopía confocal, usando la técnica de fotoblanqueo de moléculas individuales (SMP⁸) (Garcia-Parajo et al., 2001).

Se usaron liposomas cargados negativamente que están en fase gel a temperatura ambiente, con el fin de limitar la difusión de proteínas una vez insertadas. Los liposomas se depositaron sobre unos cubreobjetos de vidrio hidrófilos. El exceso de liposomas se limpió con una solución tampón y una solución conteniendo la MyrUGFP se depositó sobre los liposomas adsorbidos en fase fluida (45 °C). El exceso de proteínas se limpió y el cubreobjetos se montó en una cámara permitiendo dejar la solución tampón encima de la preparación y observar la muestra con un microscopio confocal. La figura 8.7-A muestra tal imagen, en la que los puntos amarillos correspondían a la proteína de MyrUGFP. Los liposomas contenían una sonda fluorescente permitiendo visualizarlos también, en rojo en la imagen. La concentración de la proteína se ajustó para obtener imágenes separadas de moléculas individuales o pequeños grupos de moléculas, es decir puntos luminosos discretos, como está ilustrado en la figura 8.7-B . Sin embargo, estos puntos luminosos podrían corresponder a una o más proteínas asociadas, debido al límite de difracción intrínseco a la técnica. Para contar el número de moléculas presentes en cada punto luminoso, se fotoblanquearon cada uno de los puntos luminosos (apuntando precisamente un laser con alta intensidad sobre cada uno de ellos) y se gravó la señal de fluorescencia con el tiempo. Después de un cierto tiempo de exposición, las moléculas se fotoblanquearon: la pérdida de fluorescencia de cada molécula individual aparece como un escalón en la gráfica de fluorescencia versus tiempo. Si hay solo un escalón en esta gráfica, significa que el punto luminoso estaba constituido de una sola molécula. Si hay varios escalones, entonces habían varias moléculas en el punto, y el número de moléculas presentes corresponde al número de escalones. La figura 8.7 representa ejemplos de datos experimentales que corresponden a tales casos, con puntos luminosos conteniendo figura 8.7-C 1 molécula -D 2 moléculas o

⁸Single Molecule Photobleaching

-E tres moléculas.

Se prepararon muestras de MyrUGFP unida a lípidos a varias concentraciones, y se realizó el experimento de SMP. Se generaron estadísticas contando el número de moléculas en todos los puntos discretos estudiados. A todas las concentraciones usadas (250 pM hasta 750 pM), las estadísticas revelaron un número importante de dímeros, como se puede observar en la figura 8.7-F. Los datos experimentales se compararon con un control en ausencia de lípidos, en el se observó un número más grande de monómeros (figura 8.7-G). Para descartar que los dímeros observados eran reales y no simplemente el resultado de una distribución estadística de monómeros a distancias menores de la capacidad de resolución óptica del sistema, se realizaron simulaciones utilizando las densidades experimentales. La distribución de especies predichas para un modelo sin interacciones específicas coincidió con las observaciones realizadas en controles sin lípidos (figura 8.7-H). Por consiguiente, el predominio de especies diméricas en presencia de lípidos es un resultado significativo que indica que MyrUGFP se autoasocia en presencia de lípidos.

En otro experimento, se mezcló una proteína no fluorescente, MyrUSH3, con MyrUGFP, antes de la incubación, a concentración 1:1 (200 pM). Las estadísticas obtenidas revelaron una disminución del número de dímeros y un aumento de la de monómeros fluorescentes (respecto a los datos experimentales de la MyrUGFP unido a los liposomas), pero con una cantidad de monómeros todavía menor que la del control en ausencia de lípidos. Este experimento demuestra que la presencia de MyrUSH3 afecta la formación de dímeros de MyrUGFP. Dado que ambas proteínas comparten el grupo SH4 miristoilado y el dominio Único, el efecto de MyrUSH3 puede interpretarse como una evidencia adicional de la participación de esta región en la autoasociación de MyrUGFP o MyrUSH3 (anteriormente observada en los experimentos de SPR). La disminución de dímeros de MyrUGFP en presencia de MyrUSH3 sugiere un efecto de competición dando lugar a heterodímeros formados por MyrUGFP y MyrUSH3 que en el experimento de SMP darían lugar a un único evento de blanqueo y serían, por tanto, contabilizados como monómeros.

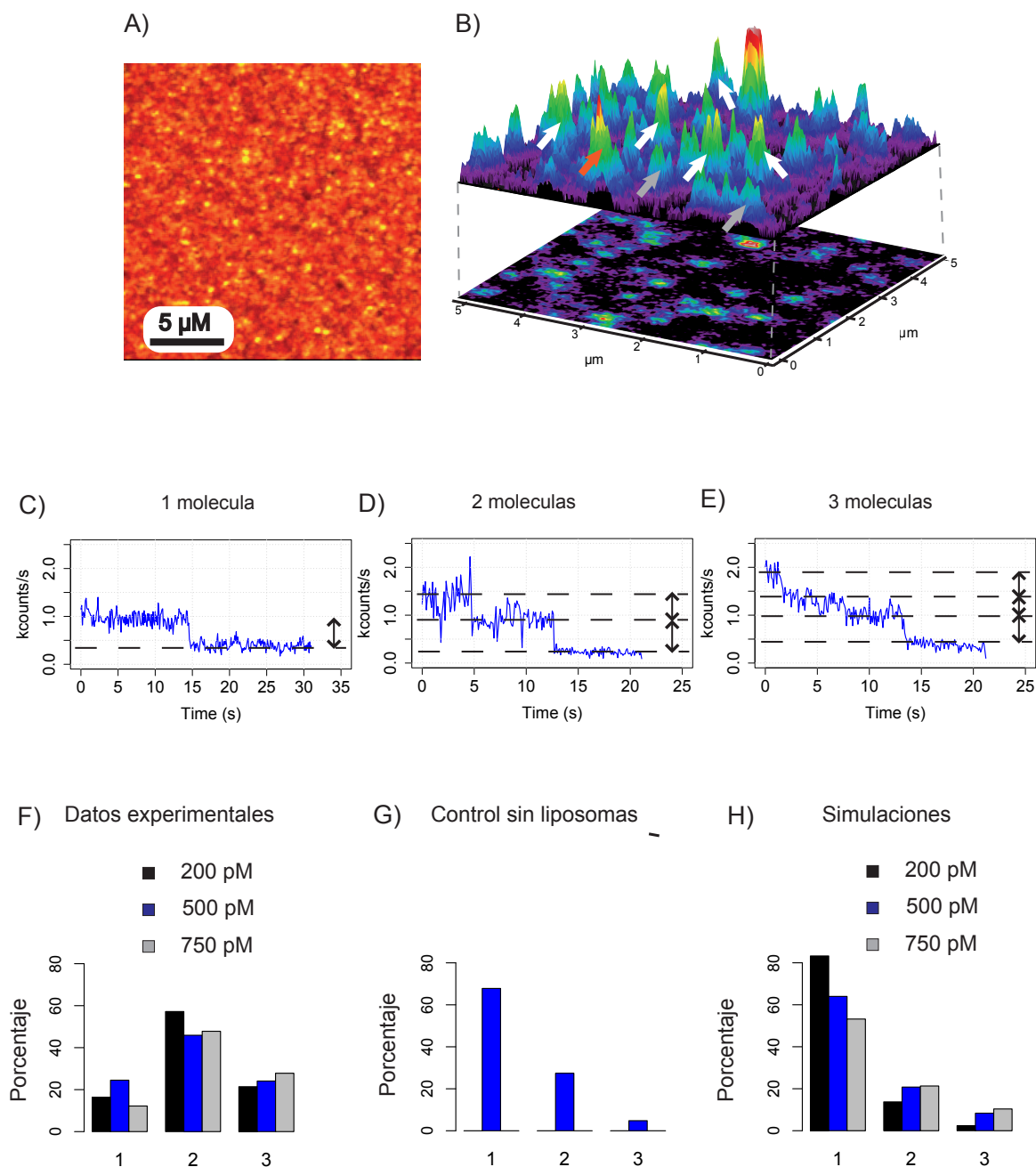


Figura 8.7: A) Imagen con doble color de MyrUGFP (amarillo) unida a liposomas (rojo). B) Imagen en tres dimensiones de MyrUGFP unido a liposomas al nivel de moléculas individuales. Fluorescencia en tiempo real de un punto discreto, limitado por la difracción, conteniendo C) una molécula D) dos moléculas E) tres moléculas. Estadísticas del número de eventos de blanqueo en experimentos de SMP de MyrUGFP F) unido a liposomas G) sobre vidrio sin liposomas. H) Simulaciones.

8.6. Estudio de Resonancia Magnética Nuclear de MyrUSrc y MyrUSH3

Las proteínas MyrUSrc y MyrUSH3 se obtuvieron con los métodos detallados en los párrafos 8.3 y 8.3 con marcado ^1H ^{15}N para el estudio de Resonancia Magnética Nuclear (RMN). Se adquirieron espectros ^1H ^{15}N SOFAST-HMQC (Schandaa et~al., 2005) de MyrUSrc y de MyrUSH3 en ausencia o presencia de lípidos. El espectro de MyrUSrc se midió a 280 K, mientras el de MyrUSH3 se observó a 298 K. Los distintos espectros adquiridos están resumidos en la tabla 8.3.

Cuadro 8.3: *Lista de constucciones con USH3 medidas por RMN*

Protein	Bilayer Model
USH3 A2G	
MyrUSH3 WT	DOPC:DOPG 3:1 liposomes
USH3A3 A2G	
MyrUSH3A3	DOPC:DOPG 3:1 liposomes
USH3IHH A2G	DMPC:DHPC 0.8:1 bicelles
SH3	

MyrUSrc El espectro de MyrUSrc en solución se comparó con el espectro de USrc no miristoilado. Los desplazamientos químicos de ambas especies eran similares, sin embargo se observaron cambios de intensidad significativos entre las dos especies. Se observó un aumento de la intensidad de las señales de MyrUSrc respecto a la de USrc en toda la zona N-terminal hasta la ULBR, lo cual sugiere una zona de restricción conformacional en esta parte. La coincidencia del extremo de la zona inmovilizada por la presencia de una cadena lipídica con la región de unión a lípidos del dominio Único sugiere que el grupo miristoilo pueda interaccionar, al menos de forma transitoria, con la ULBR. A continuación, se añadieron liposomas a la MyrUSrc y se comparó este nuevo espectro con el de la MyrUSrc en solución. De nuevo se observaron cambios de intensidad. En este caso se afectaron dos zonas, el dominio SH4 y la ULBR. El SH4 está probablemente perturbado por la inserción del grupo myristoilo en la bicapa, y la región correspondiente a la ULBR por su interacción con los liposomas *via* sus aminoácidos hidrófobos. Las mismas interacciones se observaron,

pero aumentadas, cuando los liposomas contenían cargas negativas. Esto indica que las interacciones de ambas regiones incluyen un componente de interacción electrostática adicional entre los aminoácidos cargados positivamente (las argininas en el dominio SH4 y las lisinas en la ULBR) y las cargas negativas de los liposomas. Estos resultados confirman las observaciones previamente obtenidas en ausencia del grupo miristoilo.

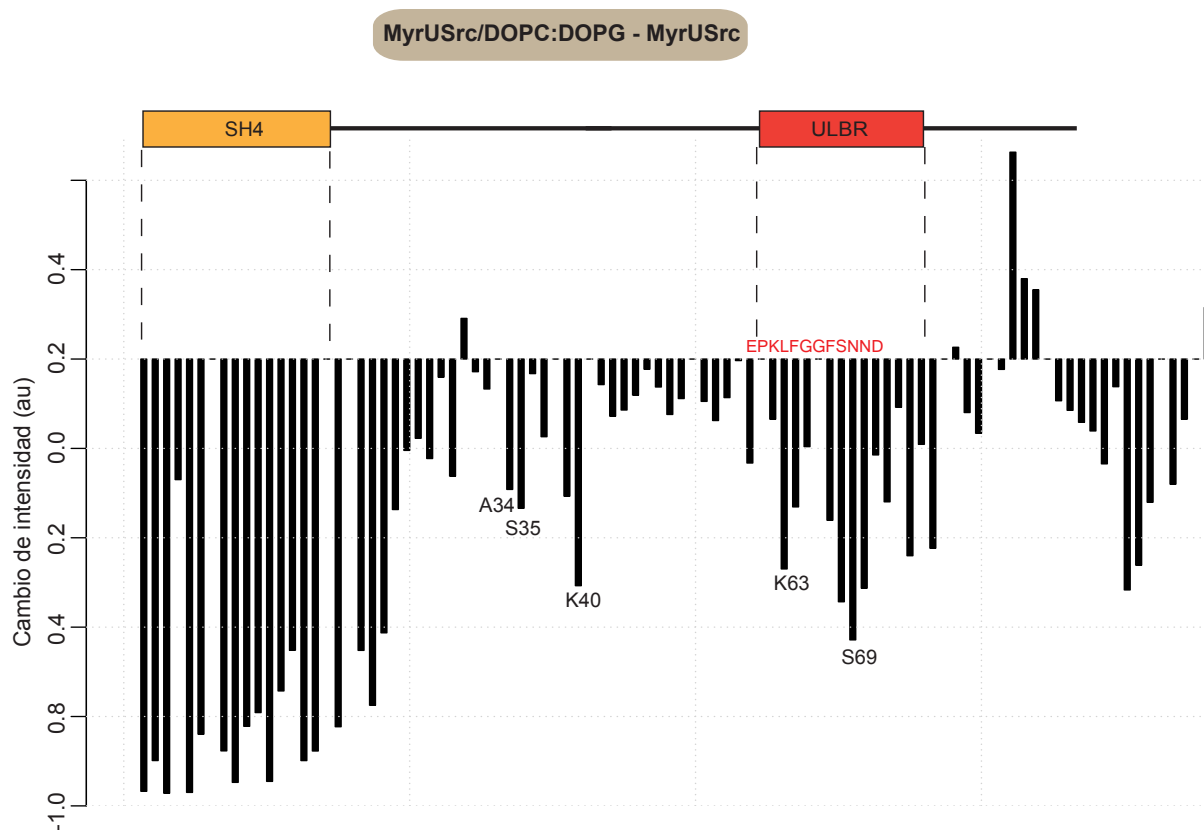


Figura 8.8: *Cambios de intensidad entre el espectro de la MyrUSrc con liposomas cargados negativamente y el de la MyrUSrc en solución.*

MyrUSH3 El espectro ^1H ^{15}N de MyrUSH3 en solución se comparó con el espectro de USH3 no miristoilado. En este caso, se observaron importantes cambios de desplazamientos químicos en varias zonas. La presencia del grupo miristoilo afectó a los desplazamientos químicos en el SH4 (S6 y K7), pero no se notaron otros cambios en la zona del SH4, tampoco en el bucle n-Src. Esto indica que la interacción entre los dominios SH4 y SH3 se mantiene en presencia del grupo miristoilo; la especie USH3 miristoilada conserva una conformación bastante cerrada debido a estas interacciones intramoleculares. Sin embargo, se observaron perturbaciones substanciales en los residuos del bucle RT, en el que

se observó un desdoblamiento en los picos de varios residuos. Este desdoblamiento indica que esta zona, el bucle RT, está intercambiando lentamente entre dos conformaciones distintas; las dos posiciones de los residuos desdoblados se llamaron posiciones 1 y 2. El mutante en la ULBR, MyrUSH3A3, se midió igualmente en solución, y se comparó con el espectro de USH3A3. Como en el caso de la forma no mutada, se observaron cambios en los desplazamientos químicos en el bucle RT, pero sin desdoblamiento, y casi todos los residuos de esta zona se hallaron en la posición 2 previamente observada. Sabiendo que en la forma no miristoilada se observaron interacciones entre la ULBR y el bucle RT, estos resultados podrían indicar que la presencia de la cola de miristoil perturba esta interacción ULBR/bucle RT. Es probable que el bucle RT interactúe con la ULBR o con el miristoil. En ausencia de la ULBR en el mutante MyrUSH3A3, el bucle RT interactúa únicamente con la cola acilada.

Se midió el espectro ^1H ^{15}N de la proteína MyrUSH3 en presencia de liposomas cargados negativamente, y el espectro se comparó con el de MyrUSH3.

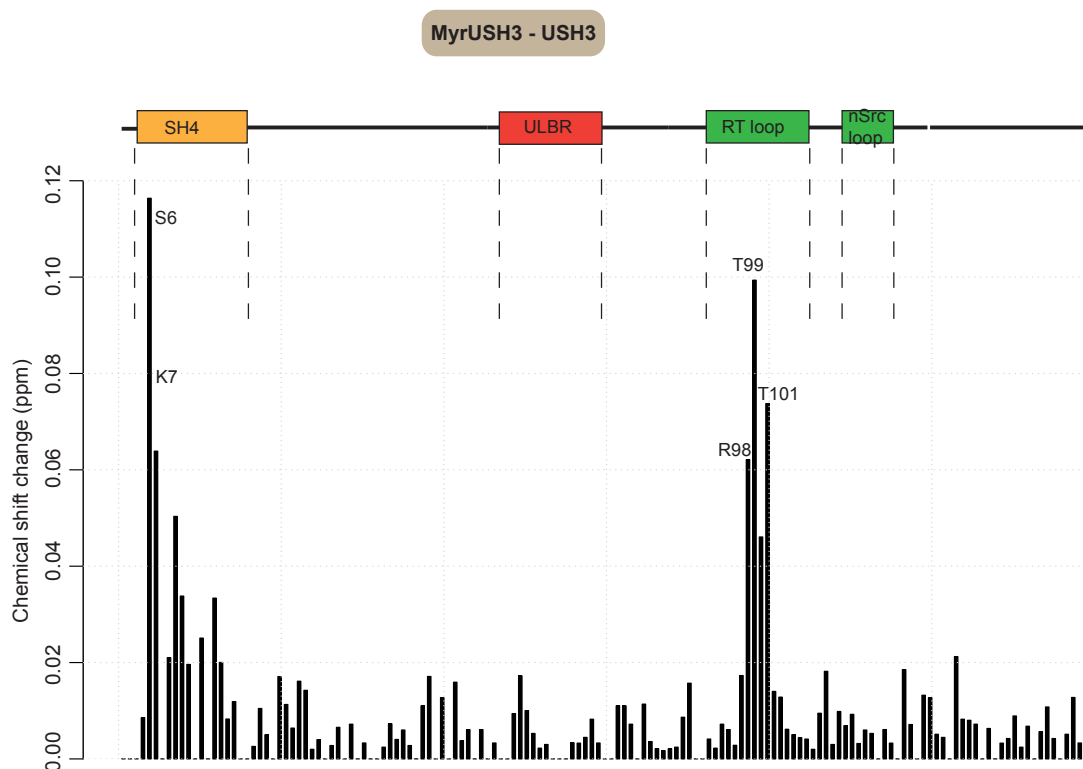


Figura 8.9: Cambios de desplazamientos químicos entre los espectros de MyrUSH3 y de USH3

Como en el caso de la MyrUSrc, aparecieron cambios significativos en la zona del dominio SH4, debido a la inserción del grupo miristoilo en la bicapa, y a la interacción

electrostática. En el bucle RT, se observaron desplazamientos químicos respecto a MyrUSH3, pero los desdoblamientos en los picos desaparecieron. El espectro del dominio SH3 en MyrUSH3 en presencia de liposomas se comparó con el del dominio SH3 aislado. Los desplazamientos químicos de los residuos del bucle RT eran muy parecidos en ambas moléculas sugiriendo que en presencia de liposomas, el bucle RT no interacciona con nadie (ni con ULBR, ni con liposomas). Este resultado apunta a que cuando MyrUSH3 está unido a liposomas, la interacción de MyrSH4 con lípidos y, simultáneamente SH4 con el bucle nSrc del dominio SH3, da lugar a una orientación del dominio SH3 respecto a la superficie de la membrana, que impide la interacción del bucle RT con lípidos. De la misma manera, se observaron muy pocos cambios en la zona de la ULBR entre los espectros de MyrUSH3 y MyrUSH3 en presencia de liposomas, al contrario de lo que se observó en ausencia del dominio SH3. Esto indica que el dominio SH3 en las formas miristoiladas impide el acercamiento de la ULBR a la superficie de la membrana. La persistencia de la interacción SH4/SH3 y la restricción en la orientación del dominio SH3 en la superficie de la membrana posiblemente también sean responsables de las restricciones observadas para el acercamiento de la ULBR a lípidos en la forma miristoilada MyrUSH3. No se observaron cambios en el bucle n-Src, sugiriendo que la conformación cerrada de USH3 se conserva en MyrUSH3 unida a liposomas.

8.7. Conclusiones

- (I) Se han desarrollado protocolos que permiten la producción de proteínas miristoiladas. Dichos protocolos se han optimizado para evitar los problemas identificados en estos procesos:
- La incorporación de cadenas aciladas más cortas ha sido identificado como un problema general en la preparación de proteínas miristoiladas y se optimizaron las condiciones, permitiendo minimizar la formación de tales cadenas.
 - La degradación inducida únicamente en presencia del grupo miristoil ha sido caracterizada y el sitio de corte exacto ha sido identificado. Se han desarrollado condiciones para obtener muestras sin degradación.
- (II) Se ha demostrado la autoasociación de las proteínas MyrUSH3 y MyrUGFP para formar complejos de pequeño tamaño tras su unión a bicapas lipídicas. Esta asociación se observó por dos métodos independientes. El péptido MyrSH4 forma complejos similares cuando se une a bicapas lipídicas.
- (III) Se ha identificado un sitio de unión del grupo miristoil en el bucle RT del dominio SH3 de la construcción MyrUSH3. La inserción del grupo miristoil en la bicapa lipídica compite con la interacción con el grupo SH3 y afecta la unión a lípidos de la región ULBR y del bucle RT que había sido observada en ausencia de miristoil. La interacción entre los dominios SH4 y SH3 que restringe el espacio conformacional del dominio Único se mantiene en las formas miristoiladas y en presencia de lípidos.

Chapter 9

Appendix

9.1. List of constructs

The different constructs used in this thesis is listed in this appendix. The complete sequence of the construct is indicated, including the C-terminal Tag sequence (SAWSH-PQFEK in the case of the StrepTag, HHHHHH in the case of the HisTag) and mutations differing from the wild type sequence are indicated in bold. The vector in which they are cloned is mentioned; the petDuet always contains the sequence of the protein and the sequence of the NMT; the pEt14b only contains the sequence of the protein). The molecular weight is indicated, with or without the processed methionine (MS analysis mostly encounter masses corresponding to proteins in which the methionine has been processed). Myristoylation occurs only if a Glycine is present in position 2; the masses of the myristoylated constructs are indicated in this case. For each construct, the isoelectric point (pI) and the extinction coefficient at 280 nm, ϵ , are indicated.

#	Name	Sequence	Tag	Vector	Restriction enzymes	MW (Da)	MW sin M1 (Da)	MW Myristoylated (Da)	pI	ϵ (280nm)
1	USrc WT	MGSNKS KPK DASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGSAWSHPQFEK	StrepTag	pet14b*	NdEI, NcoI	9612	9481	N/A	9,99	5500
2	USrc A3	MGSNKS KPK DASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPK AA AGFNSSDVTSPQRA GPLAGGSAWSHPQFEK	StrepTag	pET14b	NdEI, NcoI	9508	9377	9587	9,99	5500
3	USrc (NMT)	MGSNKS KPK DASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGSAWSHPQFEK	StrepTag	petDuet*	NdEI, XhoI	9612	9481	9691	9,99	5500
4	USrc A3 (NMT)	MGSNKS KPK DASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPK AA AGFNSSDVTSPQRA GPLAGGSAWSHPQFEK	StrepTag	petDuet	NdEI, XhoI	9508	9377	9587	9,99	5500
5	USrc A2G (NMT)	MASNKS KPK DASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGSAWSHPQFEK	StrepTag	petDuet	NdEI, XhoI	9626	9495	N/A	9,99	5500
6	USrc D10A (NMT)	MGSNKS KPK AASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGSAWSHPQFEK	StrepTag	petDuet	NdEI, XhoI	9568	9437	9647	10,27	5500
7	USrc P8G (NMT)	MGSNKS KPK GKASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGSAWSHPQFEK	StrepTag	petDuet	NdEI, XhoI	9572	9441	9651	9,99	5500
8	USrc D10A P8G (NMT)	MGSNKS KPK GK AA SQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGSAWSHPQFEK	StrepTag	petDuet	NdEI, XhoI	9528	9397	9607	10,27	5500
9	USrc E19D (NMT)	MGSNKS KPK DASQRRRS L DPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGSAWSHPQFEK	StrepTag	petDuet	NdEI, XhoI	9598	9467	9677	9,99	5500
10	USrc 6His (NMT)	MGSNKS KPK DASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGSAWSHPQFEK GH	StrepTag and HisTag	petDuet	NdEI, XhoI	10492	10361	10571	9,99	5500

11	USH3 (NMT)	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDTVTSPQRA GPLAGGVTTFVALYDYESRTETDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16550	16418	16628	6,52	16960
12	USH3 A3 (NMT)	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKAAAAGFNSSDTVTSPQRA GPLAGGVTTFVALYDYESRTETDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16445	16314	16524	6,49	16960
13	USH3 A2G A3 (NMT)	MASNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKAAAAGFNSSDTVTSPQRA GPLAGGVTTFVALYDYESRTETDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, NcOI	16460	16327	N/A	6,53	16960
14	USH3 A2G (NMT)	MASNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDTVTSPQRA GPLAGGVTTFVALYDYESRTETDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16564	16432	N/A	6,53	16960
15	USH3 D10A (NMT)	MGSNKS KPKAASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDTVTSPQRA GPLAGGVTTFVALYDYESRTETDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16506	16375	16585	6,82	16960
16	USH3 N68A (NMT)	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFASDTVTSPQRA GPLAGGVTTFVALYDYESRTETDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16507	16376	16586	6,49	16960
17	USH3 D71A (NMT)	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSATVTSPQRA GPLAGGVTTFVALYDYESRTETDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16506	16375	16585	6,82	16960

18	USH3 N68A D71A (NMT)	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFASSATVTSPQRA GPLAGGVTTTFVALYDYESRTETDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16463	16332	16542	6,82	16960
19	USH3 SS69AA (NMT)	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNAAADTVTSPQR AGPLAGGVTTTFVALYDYESRTETDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQ TGYPISNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16518	16387	16597	6,52	16960
20	USH3 IHH (NMT)	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGVTTTFVALYDYESIHHHTDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16551	16420	16630	6,62	16960
21	USH3 A2G IHH (NMT)	MASNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGVTTTFVALYDYESIHHHTDLSFK KGERLQIVNNTTEGDWWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	petDuet	NdEI, XhoI	16565	16434	N/A	6,63	16960
22	USH3 T37E (NMT)	MGSNKS KPKDASQRRRSLEPAENVH GAGGGAFPASQEPSKPASADGHRGP SAAFAPAAAEPKLFGGFNSSDVTSP QRAGPLAGGVTTTFVALYDYESRTETD LSFKKGERLQIVNNTTEGDWWLAHSL TGQTYIPSNYVAPSDSIQAEHHHH HH	HisTag	petDuet	NdEI, XhoI	16578	16447	16657	6,31	16960
23	UGFP (NMT)	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDVTSPQRA GPLAGGSAWSHPQFEKLEGGMVSKG EELFTGVVPILVELDGDVNGHKFSVSG EGEGDATYGKLTLLKFICTTGKLPVPWP TLVTTLTLYGVQCFSRYPDHMKQHDFF KSAMPEGYVQERTIFFKDDGNYKTRA EVKFEGDTLVNRIELKGIDFKEDGNILG HKLEYNYNSHNVYIMADKQKNGIKVN FKIRHNIEDGSVQLADHYQQNTPIGDG PVLLPNNHYLSTQSALS KDPNEKRDH MVLLEFVTAAGITLGMDELYKGHHHH HH	StrepTag and HisTag	petDuet	NdEI, PacI	37828	37697	37718	6,52	27515 at 280 55000 at 488

24	UGFP A3 (NMT)	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKAAAAGFNSSDTVTSPQRA GPLAGGSASWHPQFEKGLEGGMVSKG EELFTGVVPILVELDGDVNGHKFSVSG EGEGDATYGKLTCLKFICTTGKLPVPWP TLVTTLTYGVQCFSRYPDHMKQHDF KSAMPEGYVQERTIFFKDDGNYKTRA EVKFEGDTLVNRIELKGIDFKEDGNILG HKLEYNYNSHNVYIMADKQKNGIKVN FKIRHNIEDGSVQLADHYQQNTPIGDG PVLLPNNHYLSTQSALS KDPNEKRDH MVLLEFVTAAGITLGMDELYKGHHHH HH	StrepTag and HisTag	petDuet	NdEI, PacI	37724	37593	37803	6,54	27515 at 280 55000 at 488
25	UGFP A2G (NMT)	MASNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDTVTSPQRA GPLAGGSASWHPQFEKGLEGGMVSKG EELFTGVVPILVELDGDVNGHKFSVSG EGEGDATYGKLTCLKFICTTGKLPVPWP TLVTTLTYGVQCFSRYPDHMKQHDF KSAMPEGYVQERTIFFKDDGNYKTRA EVKFEGDTLVNRIELKGIDFKEDGNILG HKLEYNYNSHNVYIMADKQKNGIKVN FKIRHNIEDGSVQLADHYQQNTPIGDG PVLLPNNHYLSTQSALS K DAN EKRDH MVLLESYKSAGITLGMDELYKGHHHH HH	StrepTag and HisTag	petDuet	NdEI, PacI	37842	37711	N/A	6,54	27515 at 280 55000 at 488
26	USH3 6His	MGSNKS KPKDASQRRRSLEPAENVHG AGGGAFPASQTPSKPASADGHRGPSA AFAPAAAEPKLFGGFNSSDTVTSPQRA GPLAGGVTTFVALYDYESRTETDLSFK KGERLQIVNNTGWDWLAHSLSTGQT GYIPSNYVAPSDSIQAEHHHHHHH	HisTag	pET14b	NdEI, NcoI	16550	16418	N/A	6,52	16960
27	NMT (Nef)	MNSLPAERIQEIQKAIELFSVGQPAKT MEEASKRSYQFWDTPVPKLG EVVNT HGPVEPKDNIRQEPYTLPPQGFTWDAL DLGDRGV LKELYTLLNENYVEDDDN MFRFDYSPEFLWALRPPGWLPQWHC GVRVSSRKL VGFISAIPANIHIYDTEK KMVEINFLCVHKKLRSKRVAPVLIREI TRRVHLEGIFQAVYTAGVVL PKPVGTC RYWHRSLNPRKLIEVKFSHLSRNMTM QRTMKLYRLPETPKTAGLRPMETKDIP VVHQLLTRYLKQFHLTPVMSQEEVEH WFYPQENIIDTFVVENANGEVTDFLSF YTLPSTIMNHPTHKSLKAAYSFYNVHT QTPLLDLMSDALVLAKMKGFDFVFNAL DLMENKTFLEKLKFGIGDGNLQYYLY NWKCPMSMGAEKVGLVLQ	N/A	petDuet	N/A	48141	48009	N/A	8,2	69380 / 69330

*petDuet and pet14b both contain the ampicillin resistance

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