Role of phospholipid synthesis and phospholipase C in the regulation of diacylglycerol required for membrane trafficking at the Golgi complex

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Departament de Biologia Cel·lular, Immunologia i Neurociències

Facultat de Medicina

ROLE OF PHOSPHOLIPID SYNTHESIS AND PHOSPHOLIPASE C IN THE REGULATION OF DIACYLGLYCEROL REQUIRED FOR MEMBRANE TRAFFICKING AT THE GOLGI COMPLEX

Dissertation submitted by Adrià Sicart Casellas to Facultat de Medicina de la Universitat de Barcelona in partial fulfilment of the requirements for a Doctoral degree in Biomedicine.

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Programa de Doctorat de Biomedicina

I a vegades se’ns baixa la verge

i de sobte ens revela que ens en sortim.

Manel – Captatio Benevolentiae

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RESUM

Introducció

El complex de Golgi és l'orgànul encarregat d'organitzar el transport de la via secretora. Aquest es dóna mitjançant elements de transport, com vesícules o túbuls, per a la formació dels quals és necessària l'acció coordinada de moltes proteïnes i una composició lipídica determinada. El diacilglicerol (DAG) és un lípid senyalitzador necessari per al transport des del complex de Golgi a la membrana plasmàtica i per la formació de vesícules COPI, responsables del transport retrògrad del complex de Golgi al reticle endoplasmàtic. En principi, els nivells de DAG podrien ser regulats tan per les seves vies de producció com les de consum. El DAG necessari per al transport al complex de Golgi podria ser produït per tres vies diferents: mitjançant la fosfolipasa D (PLD), que converteix fosfatidilcolina (PC) en àcid fosfatídic (PA), acoblada a l'acció de les fosfatases del PA (PAPs) ; per acció de la fosfolipasa C (PLC), que produeix DAG a partir de fosfatidilinositol bifosfat (PIP2) i, com també s'ha proposat, de fosfatidilinositol fosfat (PIP); i per acció de l'esfingomielina sintasa (SMS) a partir de PC i ceramida. Els nivells de DAG també podrien ser regulats pel seu consum, mitjançant la síntesi de fosfolípids com la PC, a través de la via de la CDP-colina; o el fosfatidilinositol (PI), per la via que comença amb la fosforilació del DAG per la DAG quinasa.

Existeixen treballs que suggereixen que la producció de DAG mitjançant la SMS així com l'acció combinada de la PLD i les PAPs pot regular el transport a nivell del complex de Golgi. En canvi, no hi ha evidències que les vies que consumeixen DAG o la hidròlisi de polifosfoinosítids per part de la PLC participin en la regulació de la formació d'intermediaris de transport. A més a més, es desconeix quina de les vies metabolitzadores de DAG, ja sigui de producció o de consum, és regulada de forma específica dins de la cèl·lula i en condicions fisiològiques per estimular la formació d'intermediaris de transport.

Objectius

El primer objectiu d'aquesta tesi és determinar si les vies de consum de DAG, les vies de síntesi de fosfolípids, poden regular els nivells de DAG al complex de Golgi i si aquestes tenen algun paper en la regulació del transport intracel·lular. Per això necessitem confirmar la localització de les reaccions que consumeixen DAG de les vies de síntesi de PI i PC al complex de Golgi, determinar si canvis en la seva activitat poden variar els nivells de DAG en aquest orgànul i si aquestes variacions es tradueixen en una alteració del transport intracel·lular des del complex de Golgi.

El segon objectiu és estudiar la possible participació de la PLC en la regulació dels nivells de DAG del complex de Golgi, i la seva possible implicació en el transport intracel·lular a nivell d'aquest orgànul. Per a això, hem d'estudiar la presència d'activitat PLC al complex de Golgi, així com els efectes del seu silenciament en la dinàmica funcional i estructural d'aquest orgànul. Com que la PLC és una molècula senyalitzadora, estudiarem els possibles senyals activadors de la seva activitat al complex de Golgi.

<u>Resultats</u>

De cara al primer objectiu, primer vam voler confirmar la localització al complex de Golgi de les reaccions consumidores de DAG de les vies de síntesi de PI i PC. Per això, vam determinar l'activitat enzimàtica d'aquests enzims en fraccions de membrana enriquides en Golgi provinents de cèl·lules Vero i de fetge de rata. Aquestes van ser incubades durant 20 minuts en presència o no de CMP. En els dos casos, els nivells de DAG van augmentar en presència de CMP, com a conseqüència de la reversió de la CDP-colina:diacilglicerol transferasa (CPT), l'últim enzim de la síntesi de fosfatidilcolina. A aquesta pujada dels nivells de DAG en presència de CMP també hi pot contribuir la reacció reversa de la fosfatidilinositol sintasa, encara que el principal producte d'aquesta reacció seria el CDP-DAG. Aquests resultats suggereixen que els enzims responsables de les reaccions consumidores de DAG es troben localitzats al complex de Golgi.

Posteriorment, vam determinar que canvis en l'activitat de les vies de síntesi de PI i PC poden regular els nivells de DAG del complex de Golgi en un context cel·lular. Per això, vam posar a punt dues estratègies experimentals per a modificar els nivells de DAG regulant aquestes rutes metabòliques. La primera consisteix en l'ús de la línia cel·lular CHO-MT58, amb l'enzim regulador de la síntesi de fosfatidilcolina, la CTP:fosfocolina citidililtransferasa (CTT), termosensible. Vam observar que incubacions en temps curts a 40ºC, la temperatura restrictiva, de la línia CHO-MT58 es tradueixen en un augment dels nivells de DAG, respecte als de les CHO-K1 *wt*, que es mantenen constants. Aquest augment de DAG ja és visible als 30 minuts i dura almenys fins a les 3 hores. L'altra estratègia per a modular l'activitat de les vies de síntesi de PC i PI consisteix en controlar la presència o l'absència de colina i inositol, substrats necessaris per el consum de DAG mitjançant aquestes rutes. En absència de substrats, vam afegir liti per reduir la presència d'inositol endogen. Els nivells de DAG observats són més alts tant en cèl·lules NRK com en CHO-K1 incubades durant 1 hora en absència d'aquests substrats que no en presència d'ells. Per assegurar que la

inhibició de la síntesi de fosfolípids produïda en les nostres condicions experimentals només varia els nivells de DAG, vam mesurar els nivells dels principals fosfolípids així com de triacilglicerols. En les dues condicions experimentals descrites no veiem canvis en els nivells de PC, PI, fosfatidiletanolamina, esfingomileina, fosfatidilserina, àcid fosfatídic (PA), ni en els nivells de triacilglicèrids.

Per a determinar si aquest augment en els nivells de DAG causats per la inhibició de la síntesi de fosfolípids podien tenir lloc al complex de Golgi, vam estudiar la ultraestructura d'aquest orgànul en cèl·lules CHO-K1 i CHO-MT58 a 33ºC i 40ºC en presència o no de propanolol. Estudis previs del grup havien indicat que el propanolol, un inhibidor de les PAPs, reduïa el DAG del complex de Golgi, augmentant els perfils de *budding*, fet que reflectia un bloqueig en la fissió de vesícules. L'augment de perfils de *budding* associats al complex de Golgi causat pel propanolol va ser revertit en cèl·lules CHO-MT58 incubades a 40ºC, suggerint que la augment de DAG causat per la inhibició de la síntesi de PC pot contrarestar la inhibició de la formació de DAG al complex de Golgi per acció del propanolol.

Per determinar si l'augment de DAG produït per la inhibició de les vies de síntesi de fosfolípids tenia un efecte en el transport a través de la via secretora, vam analitzar primer la distribució del receptor KDEL en cèl·lules NRK i CHO-K1. Vam observar que la inhibició de la síntesi de fosfolípids per absència de colina i inositol es tradueix en un augment en el número de partícules amb receptor KDEL. Aquest també es dóna en incubar la línia MT-58 a 40ºC durant 1 hora a la temperatura no permissiva. Vam analitzar el transport del VSVG des del complex de Golgi a la membrana plasmàtica, i aquest es veia frenat quan es consumia DAG en presència de colina i inositol, ja que el VSVG està més temps al complex de Golgi i arriba més lentament a la membrana plasmàtica que en absència d'aquests substrats. Vam observar un efecte similar en la secreció de la proteïna soluble ssHRP, que és menor quan es consumeix DAG en presència dels substrats colina i inositol que en la seva absència, quan el DAG no és consumit.

De cara al segon objectiu, estudiar la possible implicació de la PLC en el transport al complex de Golgi, primer vam voler determinar si hi podia haver activitat PLC en fraccions enriquides en membranes de Golgi. Per a això, vam incubar membranes de Golgi amb [^{32}P]ATP i vam mesurar la producció de[^{32}P] PA, com a reflex de la producció de DAG. L'addició de citosol (les PLCs són proteïnes citoplasmàtiques) va augmentar la producció de [^{32}P]PA i els nivells d'aquest eren molt més alts en afegir PLC recombinant. A més, la depleció de Ca^{2+} i GTPγS, activadors de l'activitat PLC,

produeix una inhibició de la producció de [^{32}P]PA. A continuació vam voler determinar si els nivells de PIP, el fosfoinosítid majoritari al complex de Golgi, es veien disminuïts en afegir citosol, per confirmar la presència d'activitat PLC. En presència de citosol, el nivells de [^{32}P]PIP es veien disminuïts, i aquest consum era depenent de Ca^{2+}, però no de GTPγS. Aquests resultats suggereixen que si la PLC actua al complex de Golgi, aquesta podria ser la PLCγ, ja que l'activitat d'aquesta, és sensible al Ca^{2+}, però no és regulada per proteïnes G.

Per això, vam estudiar la dinàmica funcional i estructural del complex de Golgi en cèl·lules silenciades per la isoforma expressada de forma ubiqua PLCγ1. Vam observar que l'arribada del VSVG a la membrana plasmàtica és més lenta en cèl·lules HeLa silenciades per la PLCγ1, comparades amb les cèl·lules control. En la mateixa línia, la secreció de proteïnes marcades amb [^{35}S] també es troba significativament disminuïda en silenciar la PLCγ1. Aquests resultats mostren que la PLCγ1 participa en el transport anterògrad a nivell del complex de Golgi. Per contra, la internalització de la toxina B de *Shigella* amb la seqüència de retenció al reticle KDEL (STxB-KDEL) no es veu alterada en silenciar la PLCγ1, suggerint que aquesta no intervé en el transport retrògrad.

També vam analitzar per immunofluorescència la morfologia del complex de Golgi de les cèl·lules silenciades per la PLCγ1. Aquestes mostraven una morfologia lleugerament més compacta que les cèl·lules control. Vam quantificar aquesta morfologia mitjançant l'índex de compactació del complex de Golgi, on vam trobar que les cèl·lules silenciades per la PLCγ1 presentaven un valor de compactació més elevat que les cèl·lules control. En concordança amb aquest resultat, cèl·lules que sobreexpressen la PLCγ1, però no de la seva forma catalíticament inactiva PLCγ1(H335Q), presenten una morfologia del complex de Golgi més fragmentada.

Posteriorment, vam analitzar si la PLCγ1 podia estar regulant els nivells de DAG al complex de Golgi. Per això, vam mesurar la localització del sensor de DAG C1-PKCθ-GFP al complex de Golgi en cèl·lules silenciades per la PLCγ1. La localització del C1-PKCθ-GFP a aquest orgànul era lleugerament menor en les cèl·lules silenciades per la PLCγ1 que en les control, i en afegir PMA per desplaçar el C1-PKCθ-GFP a la membrana, vam veure que la presència d'aquest sensor de DAG al complex de Golgi era significativament menor en cèl·lules silenciades per la PLCγ1 que en les control, indicant així la implicació de la PLCγ1 en la regulació dels nivells de DAG al complex de Golgi.

Com que la PLCγ1 és un enzim que participa en la senyalització intracel·lular, vam buscar quin podria ser un dels seus activadors al complex de Golgi. Recentment, s'ha descrit que l'arribada de *cargo* al complex de Golgi inicia una senyal necessària pel correcte funcionament de la via secretora Vam analitzar la hipòtesi de que aquesta mateixa senyal, l'arribada de *cargo*, podia controlar la producció de DAG al complex de Golgi i que aquest procés era depenent de la PLCγ1. Per a això, vam analitzar la localització del sensor de DAG C1-PKCθ-GFP al complex de Golgi abans i després de l'arribada de VSVG, com a model d'arribada de *cargo*. Vam observar que els nivells de C1-PKCθ-GFP al complex de Golgi es trobaven augmentats després de l'arribada de *cargo*, i que aquest increment no tenia lloc en cèl·lules que no expressaven el VSVG. Aquests resultat ens indica que l'arribada de *cargo* augmenta els nivells de DAG al complex de Golgi. Per estudiar si aquesta producció de DAG és deguda a la PLCγ1, vam fer el mateix experiment en cèl·lules silenciades per la PLCγ1, on vam observar que l'arribada de *cargo* provocava un menor augment en la localització del C1-PKCθ-GFP al complex de Golgi en les cèl·lules silenciades per la PLCγ1 que en les cèl·lules control. Aquests resultats ens indiquen que la PLCγ1 pot participar en la producció de DAG induïda per l'arribada de cargo.

Com que el mecanisme d'activació descrit més comú de la PLCγ1 és la fosforil·lació de la seva tirosina 783, vam analitzar els nivells d'aquesta fosforil·lació després d'una arribada de cargo. Contràriament al que esperàvem, els nivells de la fosforil·lació de la tirosina 783 de la PLCγ1 no es veien modificats per una arribada massiva de VSVG. Aquest resultat ens indica que si la PLCγ1 actua al complex de Golgi després d'una arribada de cargo, tal com suggereixen els nostres resultats, el seu mecanisme d'activació ha de ser diferent a la fosforil·lació.

Discussió

En conjunt, els resultats obtinguts a la primera part de la tesi indiquen que la síntesi de fosfolípids, a part de tenir lloc al reticle endoplasmàtic, també pot tenir lloc al complex de Golgi. Aquestes vies de síntesi de PC i PI, que són consumidores de DAG, poden regular els nivells d'aquest metabòlit al complex de Golgi. A més a més, una inhibició de l'activitat d'aquestes vies consumidores de DAG pot accelerar el transport tant retrògrad com anterògrad al complex de Golgi. D'aquesta manera, podem concloure que les vies de síntesi de fosfolípids participen en la regulació del DAG necessari per al transport, tan retrògrad com anterògrad, al complex de Golgi.

Per altra banda, els resultats obtinguts en la segona part de la tesi ens indiquen que la PLCγ1 pot participar al complex de Golgi en el control del *pool* de DAG d'aquest

orgànul, regular el transport post-Golgi i la morfologia d'aquest orgànul. A més, el fet que la PLCγ1 participi en la producció de DAG induïda per l'arribada de *cargo* situa la PLCγ1 en un punt clau de la cascada de senyalització per al correcte funcionament del complex de Golgi, ja que la converteix en un dels nexes entre l'arribada de *cargo* a aquest orgànul i la producció de DAG per la sortida d'aquest *cargo* al seu destí cel·lular.

Conclusions

1.- Les vies metabòliques per a la síntesi de fosfolípids que són consumidores de DAG regulen els nivells d'aquest al complex de Golgi.

2.- La síntesi de fosfolípids controla els nivells de DAG necessaris per al transport tan retrògrad com anterògrad al complex de Golgi.

3.- L'arribada de *cargo* al complex de Golgi estimula la producció de DAG.

4.- La PLCγ1 està involucrada en la producció de DAG estimulada per l'arribada de *cargo* al complex de Golgi.

5.- La PLCγ1 és necessària per al transport post-Golgi i pel manteniment de l'estructura del complex de Golgi.

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ABBREVIATIONS

Arf	ADP-ribosylation factor
BARS	Brefeldin-A ADP-ribosylated substrate
CaR	Ca^{2+}-receptor
CCT	CTP:phosphocholine cytidylyltransferase
CDS	cytidine diphosphate-diacylglycerol synthase
CERT	ceramide transfer protein
CK	choline kinase
COPI	coat protein complex I
COPII	coat protein complex II
CPT	CDP-choline: diacylglycerol phosphotransferase
DAG	diacylglycerol
DAGK	diacylglycerol kinase
DOG	2-dioctanoyl-sn-glycerol
Endo-H	endo-β-N-acetylglucosaminidase H enzyme
ER	endoplasmic reticulum
ERES	endoplasmic reticulum exit sites
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
GAP	GTP-ase activating protein
GEF	guanine exchange factor
GFP	green fluorescent protein
GlcCer	glucosylceramide
GPCR	G-protein coupled receptor
GSL	glycosphingolipids
IP_3	inositol(1,4,5)triphosphate
IQ	ilimaquinone
KDELR	KDEL receptor
LPA	lysophpsphatidic acid
LPP	lipid phosphate phosphatase
MAPK	mitogen activated protein kinase

OSBP	oxysterol binding protein
PA	phosphatidic acid
PAP	phosphatidic acid phosphatases
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PFA	paraformaldehyde
PH	pleckstrin homology
PI	phosphatidylinostiol
PI(4)P	phosphatidylinositol 4-phosphate
PI(4)PK	phosphatidylinositol 4-phosphate kinase
PI(4,5)P_2	phosphatidyl 4,5-bisphosphate
PIP	phosphatidylinositol phosphate
PIS	phosphatidylinositol synthase
PITP	phosphatidylinositol transfer protein
PKC	protein kinase C
PKD	protein kinase D
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
RasGRP	Ras guanine-realising protein
SERCA	sarcoplasmic and ER Ca^{2+} ATPase
siRNA	small interference RNA
SM	sphingomyelin
SMS	sphingomyelin synthase
SPCA	secretory pathway Ca^{2+} ATPase
ssHRP	secretory form of horseradish peroxidase
STxB	Shiga toxin B

STxB-KDEL	Shiga toxin B tagged with the KDEL sequence
TAG	triacylglycerol
TBS	tris-buffered saline
TBS-T	tris-buffered saline containing 1% Tween-20
TGN	*trans*-Golgi network
TIM	triose phosphate isomerase
U73122	(1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione)
U73343	(1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione)
VSVG	vesicular stomatitis virus G protein
YFP	yellow fluorescent protein

I. INTRODUCTION

1.- INTRACELLULAR MEMBRANE TRAFFICKING

Along evolution, the eukaryotic cell increased its complexity by acquiring intracellular membranes, which allowed the segregation of the cell in different subunits, known as cell organelles. This compartmentalization into different organelles vastly improves the efficiency of many cellular functions and prevents potentially dangerous molecules from roaming freely within the cell. But when distinct cellular processes are compartmentalized, a new problem arises: these different compartments need to communicate with each other and exchange specific molecules or need to be exported them to the cell exterior (Zierath and Lendahl, 2013). All intracellular membranes are interconnected by bidirectional traffic, yet each maintains a unique lipid and protein composition (Van Meer, 2010). This intracellular traffic is achieved by vesicular or tubulovesicular structures known as transport carriers, which detach from one intracellular membrane to fuse to another one, thus allowing the communication and exchange of material between different organelles.

1.1.- The composition of cellular membranes

Cellular membranes are composed by a bilayer of amphipathic lipid molecules. The propensity of the hydrophobic moieties to self-associate in an aqueous environment and the tendency of the hydrophilic moieties to interact with it and with each other is the basis of the spontaneous formation of a membrane. The major structural lipids in eukaryotic membranes are the glycerophospholipids (Fig. 1A); phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA). Their backbone is diacylglycerol (DAG), which contains a glycerol with two saturated or *cis*-unsaturated acyl chains of varying lengths esterified to its first and second alcohol groups. PC is the most abundant phospholipid of eukaryotic cells, accounting for more than 50% of the total phospholipid mass. PC, in an aqueous environment, is self-organized as a planar bilayer, where it has a nearly cylindrical molecular geometry. Most PC molecules have one *cis*-unsaturated fatty acyl chain, which renders them fluid at room temperature and increases the diamater of the hydrophobic part of the molecule. The shape of each phospholipid class in the membrane bilayer depends not only on the nature of its acyl chains, but also on the size of their polar group. For example, PE has a more conical shape than PC, since its polar headgroup it is relatively small compared to PC (Van Meer, 2008).

Although PI and its derivates, known as phosphoinositides, account for less than 15% of the total phospholipids, they play a key role in organizing cellular membranes. Inositol, the PI polar headgroup, can be phosphorylated in different positions. PI

kinases specifically modify the D-3, D-4 and D-5 positions of the inositol ring in all of the possible combinations, generating up to seven different classes of phosphoinositides. These different classes of phosphoinositides are specifically segregated in different organelles and in different amounts. For example, phosphatidylinositol 4-phosphate (PI(4)P) is the major phosphoinositide of the Golgi complex, while phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) is mainly localized at the plasma membrane. Moreover, phosphoinositides also are important in cell signalling and in the recruitment of specific proteins for each phosphoinositide class to cellular membranes (Di Paolo and De Camilli, 2006).

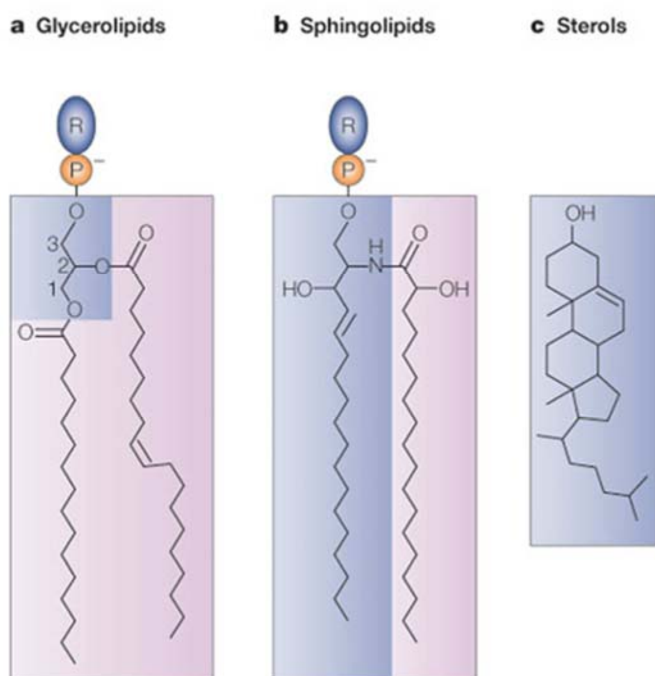

Figure 1. The three main classes of eukaryotic membrane lipids. *Structure of glycerophospholipids, sphingolipids and sterols, which are based in glycerol, sphingosine and cholesterol, respectively (blue shading). P stands for a phosphate group and R for a head group, which can be either neutral, as for PS and PI; or basic, as for PE and PC. (Adapted from Holthuis and Levine, 2005)*

In addition to the glycerol-based phospholipids, the eukaryotic cell membranes also possesses sphingolipids and sterols (Fig. 1B,C). Having ceramide as their backbone, sphingolipids constitute another class of structural lipids. The major sphingolipids in mammal cells are sphingomyelin (SM) and the glycosphingolipids (GSLs), which contain mono-, di- or oligosaccharides based on glucosylceramide (GlcCer) and sometimes galacotosylceramide. Sphingolipids have saturated or *trans*-unsaturated acyl chains so they are able to form taller, narrower cylinder than PC lipids of the same chain length. Sphingolipids pack more tightly, and are self-assembled in a more ordered membrane and can be fluidized by sterols, the major non-polar lipids of cell membranes. Cholesterol is the major sterol in mammals (Van Meer, 2008, Pruett, 2008; Hannich, 2011).

The main lipid biosynthetic organelle is the endoplasmic reticulum (ER), which produces the bulk of the structural lipids and cholesterol (Bell, 1981). However, significant levels of lipid synthesis also occur in the Golgi complex. Synthesis of SM and complex sphingolipid occurs at the Golgi complex, although the synthesis of their precursor ceramide also takes place at the ER (Bartke and Hannun, 2009). Moreover, the Golgi complex also possesses the capacity of synthesis of glycerophospholipids as PC and PI (Jelsema and Morré, 1978).

Cellular membranes are also loaded with proteins, which can account for roughly half the mass of most cell membranes. Some of these proteins are transmembrane proteins, which are inserted into the membrane bilayer and stick out on both sides. Other proteins are found associated with the membrane, usually bound to other proteins (Van Meer, 2008).

1.2.- Intracellular trafficking pathways

Since the eukaryotic cell is compartmentalized in different organelles, exchange of material as proteins or lipids between compartments is necessary to maintain the integrity and function of the cell. This exchange of material is achieved by trafficking pathways that communicate the different organelles. These trafficking pathways are responsible to deliver specific molecules, known as cargo, which could be lipid, protein, carbohydrate or inorganic substances; from one compartment to another. Intracellular trafficking is mediated by transport carriers that could be vesicles or tubulovesicular structures, which are detached from the donor organelle, moved by motor proteins using the cytoskeleton as a structural platform for their movement and finally fused to its target organelle. The different intracellular trafficking pathways can be classified by a functional criterion (Fig. 2).

1.2.1.- Endocytosis and the recycling pathway

Endocytosis is the pathway that is used to incorporate exogenous soluble molecules and membrane components inside the cell. Cargo is internalized to the early endosomes, from where it is delivered to late endosomes and then to the lysosomes, the *trans*-Golgi network (TGN) or to recycling endosomes (Jones, 2007). Endocytosis can be classified into phagocytosis or pinocytosis, depending on the size and the nature of the internalized cargo (Conner and Schmid, 2004). Phagocytosis is used by specialized cells, as macrophages, to internalize large objects as viruses, bacteria or other intracellular parasites, which are then digested in the lysosomes. Pinocytosis is used to internalize macromolecules and fluids, and also is responsible of the constant

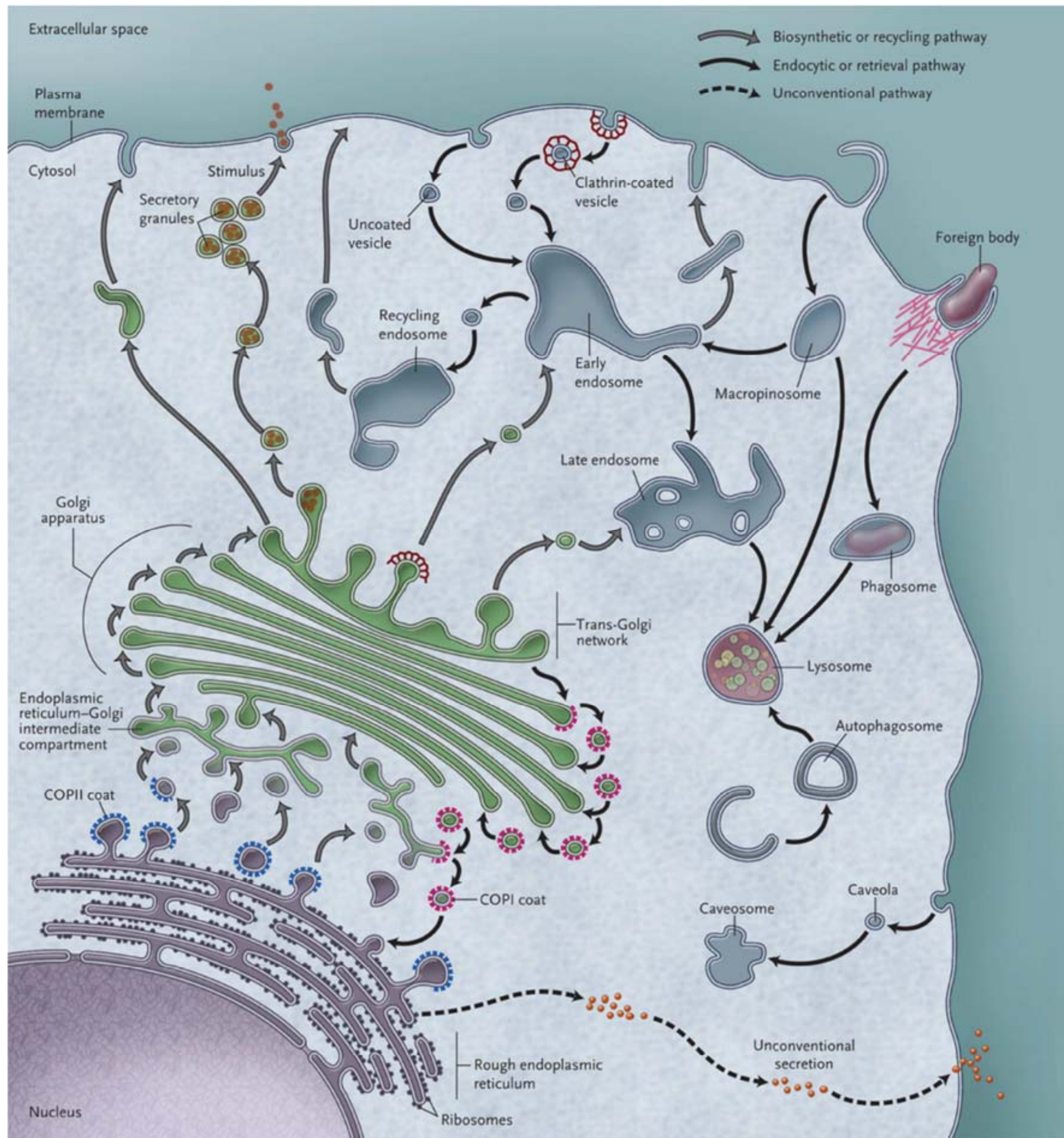

Figure 2. Membrane trafficking pathways. *In the secretory pathway, cargo synthesised at the ER is first sent to the ERGIC by COPII transport carriers and then to the Golgi complex. Once at the TGN, cargo sorted and sent to the plasma membrane, to the endo/lysosomal system, to secretory granules in specialized cells or sent back to the ER in COPI transport carriers. In the endocytic pathway, cargo is internalised at the plasma membrane and sent to the endosomal compartments and to the Golgi complex or the lysosomes. The recycling pathway delivers part of the endocyted cargo to the cell surface by the action of the recycling endosomes. The unconventional secretion pathway transport proteins to the cell surface without entering the Golgi complex (De Matteis and Luini, 2011).*

exchange of material on the plasma membrane. On the other hand, pinocytosis can be classified into different subclasses: macropinocytosis, clathrin-mediated endocytosis, and clathrin- independent endocytosis. In micropinocytosis the plasma membrane is deformed in order to internalize the exogenous element. In clathrin-mediated endocytosis the transport carriers of this kind of endocytosis are coated with clathrin, a

molecule that after ligand recognition by the receptor is assembled into polygonal structures that deform the plasma membrane in its inner side (Robinson and Bonifacino, 2001). Clathrin-independent endocytosis could be divided in processes dependent on RhoA and Cdc42, flotillin-mediated endocytosis and Arf6-associated uptake of cargo (Sandvig, 2011). Caveolin-mediated endocytosis was proposed to be another mechanism of clathrin-independent endocytosis (Van Deurs, 2003), but nowadays the role for caveolae in endocytosis is questioned (Doherty and McMahon, 2009; Sandvig, 2011).

After endocytosis, some molecules are selected and sent back to the plasma membrane, where they will exert their function again. The responsible of this process is the recycling pathway, which is followed in part by most membrane receptors, which after interacting with their ligand are endocyted. Once internalized, they split from their ligand and are sent back to the plasma membrane through the recycling endosomes (Maxfield and McGraw, 2004).

1.2.2.- The secretory pathway

Newly synthesized components are transported from their site of synthesis to their final destination by the secretory pathway. This trafficking pathway begins at the ER, where cargo is synthesized and folded if necessary. After that, cargo enters the exit sites of the ER (ERES), where it is sorted into either small or large vesicles that are generated through the membrane bending properties of coat protein complex II (COPII). Once detached from the ER, these vesicles move cargo to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and after this it reaches the Golgi complex. Once at the *cis* side of the Golgi, cargo can be sent back to the ER, in a process known as retrograde trafficking, or can continue forward to the *trans* pole until reaching the TGN. At the TGN, cargo is sorted and packaged in different vesicles, which then carry them to their final destinations, such as lysosomes, plasma membrane or secretory granules in specialized cells (De Matteis and Luini, 2011). Recently, another secretory pathway has been described, known as unconventional secretion, in which cargo is not packed in transport carriers and does not enter the Golgi complex. Proteins that follow this pathway exit the ER directly to the cytoplasm to finally reach the extracellular media, (Malhotra, 2013).

1.3- The Golgi complex: structure and function

The Golgi complex is an organelle present in all eukaryotes that acts as a central station of the secretory pathway and its main function is to organize most of the

intracellular trafficking of this pathway (Egea, 2001). It is localized at the perinuclear region, associated to the centrosome and the microtubule cytoskeleton (Fig. 3A; Ríos and Bornens, 2003). The Golgi complex has a characteristic structure that is conserved amongst nearly all eukaryotes, comprising flattened membrane discs called cisternae that are layered on top of each other to generate the Golgi stack (Lowe, 2011). Each stack is morphologically and functionally polarized, where it can be distinguished an entrance face, the *cis*-Golgi, where cargo synthesised at the ER is received; and an exit zone, the *trans*-Golgi, where processed cargo is sorted and released (Fig. 3B). The group of cisternae between the *cis* and the *trans* pole form the *mid*-Golgi region. Both *cis*- and *trans*-Golgi are associated to a highly dynamic network of tubulovesicular structures that act as an interface between the Golgi complex and other compartments of the secretory pathway. Thus, the *cis*-Golgi network is found between the ER and the *cis* side of the Golgi complex, while the TGN is localized between the *trans* pole and the plasma membrane or other compartments of the secretory pathway (Rambourg and Clermont, 1990).

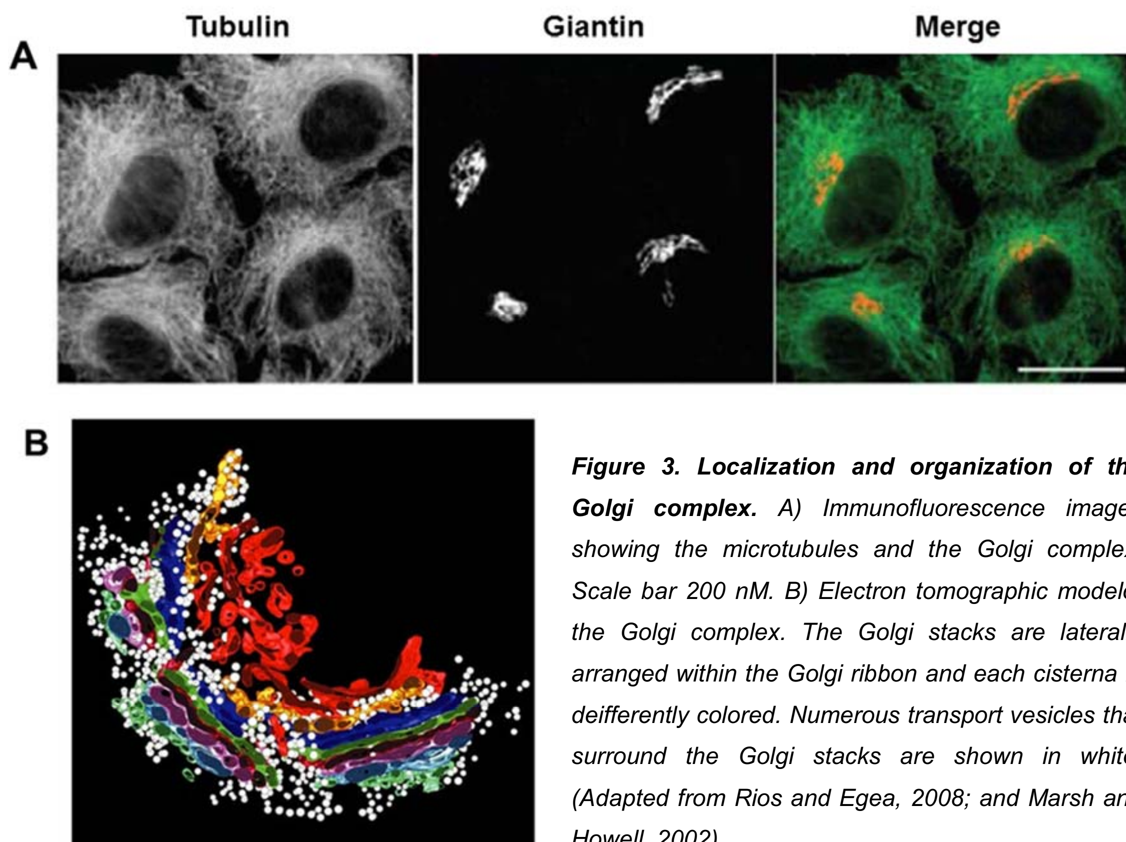

Figure 3. Localization and organization of the Golgi complex. *A) Immunofluorescence images showing the microtubules and the Golgi complex. Scale bar 200 nM. B) Electron tomographic modelof the Golgi complex. The Golgi stacks are laterally arranged within the Golgi ribbon and each cisterna is deifferently colored. Numerous transport vesicles that surround the Golgi stacks are shown in white. (Adapted from Rios and Egea, 2008; and Marsh and Howell, 2002)*

The Golgi complex provides a spatial compartmentalization for post-translational modifications of cargo synthesised at the ER. Glycosylation of proteins is the most

common of these modifications and is a fundamental process for the final function and the correct sorting of cargo, but other processes as phosphorylation also take place in this organelle. One example of protein post-translational modifications is the phosphorylation of mannose residues to produce mannose-6-phosphate residues, a signal that is recognized by the sorting machinery to send lysosomal enzymes to the lysosome (Hille-Rehfeld, 1995). As mentioned before, the Golgi complex is also fundamental for lipid metabolism, since is the place for sphingolipid synthesis (Van Meer, 2008) and phospholipid synthesis also occurs at this organelle (Jelsema and Morré, 1978).

Recently, the Golgi complex has also been proposed to be a central hub for intracellular signalling (Cancino and Luini, 2013), since numerous components of signalling pathways, such as kinases and G proteins, are found associated with this organelle. The signalling events at the Golgi can either control the secretory pathway or other cellular functions (see below, chapter III).

1.4.- Golgi-associated membrane trafficking

The molecular components of the Golgi complex are constantly renewed. Cargo synthesised at the ER, together with membrane and other components of the transport carriers, is constantly arriving and fusing with the Golgi complex (Cancino, 2013). Moreover, the Golgi complex also receives cargo and membrane from the endosomes. On the other hand, a large of proportion membrane also moves out from the Golgi complex by fission of transport carriers that are sent to other cell organelles. This high and constant flux of membrane makes the Golgi complex a very dynamic organelle. IN order to maintain its structure and function, the net gain of the membrane flux should be null. These highly regulated membrane fluxes to and from the Golgi complex are described below.

1.4.1.- Membrane trafficking at the ER-Golgi interface

Membrane traffic between the ER and the Golgi complex is bidirectional. The membrane flux that moves from the ER to the Golgi complex is called anterograde transport, while retrograde transport drives the movement of membrane in the opposite direction, from the Golgi complex to the ER. Distinct machineries facilitate the formation of carriers for anterograde and retrograde transport, which are thought to ensure fidelity and directionality of trafficking (Fig. 4). COPII operates in the anterograde pathway from the ER to the Golgi complex, and COPI functions in the retrograde route from the Golgi complex to the ER (Brandizzi and Barlowe, 2013), although COPI has also been

proposed to participate in the anterograde transport from the ERGIC to the Golgi complex (Orci, 1997).

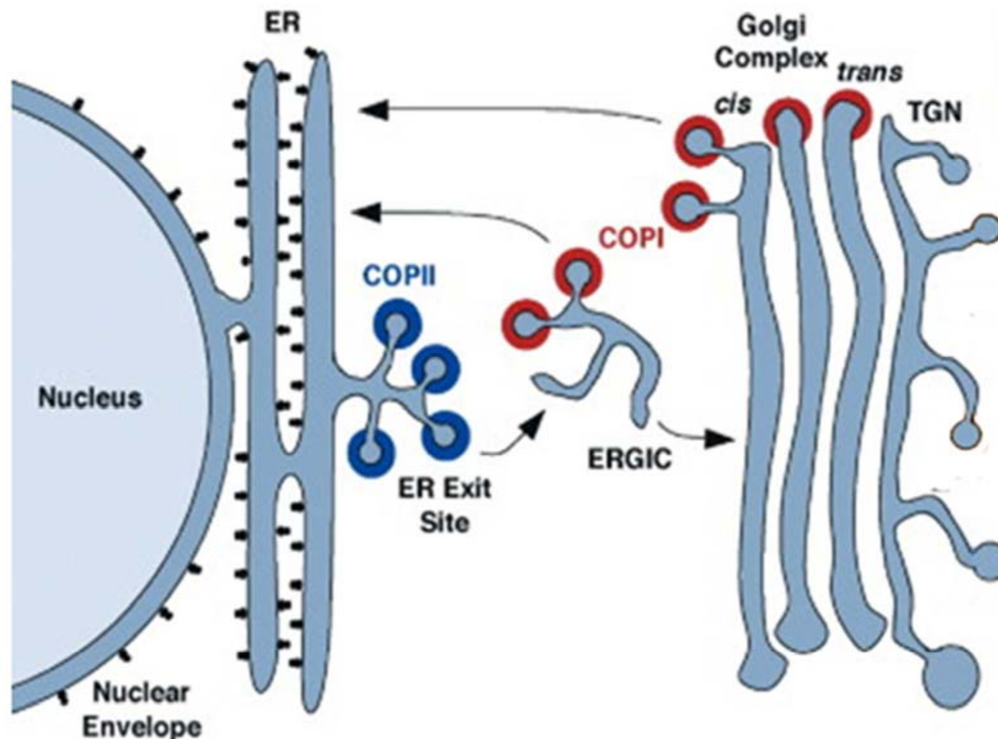

Figure 4. Membrane trafficking at the ER-Golgi interface. *Transport between the ER and the Golgi complex occurs in different steps. Cargo is loaded to COPII vesicles, which bud at the ERES and are sent to the ERGIC and next to the Golgi complex. ER resident proteins that have reached the ERGIC or the Golgi complex are sent back to the ER in COPI vesicles (adapted from Bonifacino and Glick, 2004).*

1.4.1.1.- Anterograde transport

Most proteins and lipids synthesised at the ER are transported to the Golgi complex. To exit the ER, fully folded soluble and membrane cargos are packaged into COPII-coated transport carriers. The generation of these COPII transport carriers is localized at specialized, long-lived subdomains of the ER, termed ERES, which are enriched in COPII coat proteins. In addition to the core COPII components, large multidomain Sec16 localizes at the ERES and is required for export site assembly and function. The core components of COPII complex are the proteins Sec23/24, Sec 13/31 and the Sar1 GTPase (Kirchhausen, 2007; Barlowe 1994). Sar1 and Sec23/24 form the inner layer of the COPII complex, which binds and selects specific cargo for packaging into ER-derived transport vesicles, while Sec13/31 is found in the outer layer and their polymerization leads to deformation of ER membranes to drive transport vesicle formation (Brandizzi and Barlowe, 2013).

COPII coated vesicles do not travel directly to the Golgi complex. These vesicles fuse with themselves and generate a tubulovesicular structure between the ER and Golgi complex, known as the ER/Golgi intermediate compartment (ERGIC), which concentrates and sorts cargo (Bannykh, 1998; Hauri and Schweizer, 1992). Transport from the ERGIC to the *cis*-Golgi can be explained by two models. The first one suggests that the ERGIC is a transient organelle that fuses to generate the first Golgi cisternae. The second one suggests that the ERGIC is a stable compartment and that COPI vesicles deliver cargo from the ERGIC the first Golgi cisternae (Rabouille and Klumperman, 2005).

1.4.1.2.- Retrograde transport

ER resident proteins possess signals for its retention in this organelle; however some of them reach the Golgi complex, either for post-translational modification or for miss-sorting at the ERES. Retrograde transport is used to send back to the ER these soluble or transmembrane ER resident proteins. Proteins that cycle between these organelles, as the KDEL receptor (KDELR), also use this trafficking pathway to travel from the Golgi complex to the ER. Retrograde membrane trafficking also compensates the large amount of membrane that arrives at the Golgi complex from the ER, which could lead to disruption of Golgi structure and function if not compensated.

Retrograde transport is mainly driven by coat protein complex I (COPI) vesicles, which are formed by the protein complex coatomer (Kreis and Pepperkok, 1994). The most accepted model postulates that COPI vesicle function is to accumulate, sort and transport cargo in a retrograde manner (Bethune, 2006; Lippincott-Schwartz and Liu, 2006). However, the prominent presence of COPI vesicles around the cisternae of the Golgi also suggests a role of these carriers in intra-Golgi trafficking (Duden 1991; Serafini 1991a). Soluble ER resident proteins possess a four aminoacid sequence (KDEL, where K stands for lysine, D for aspartic acid, E for glutamic acid and L for leucine) at its carboxyl end, which is recognized by a seven transmembrane protein that cycles between the ER and the Golgi complex, the KDEL receptor (KDELR; Lewis and Pelham, 1990; Capitani and Sallese, 2009). When KDELR binds a protein with the KDEL tag at the Golgi complex, it sends it back to the ER through COPI vesicles. Transmembrane ER resident proteins also possess a four aminoacidic tag at its carboxyl end, KKXX (where K stands for lysine and X for any other aminoacid). This aminoacidic sequence interacts with proteins of the COPI coat and acts as a recovery signal that stimulates retrograde transport of these proteins from the Golgi complex or the ERGIC back to the ER (Nilsson, 1989).

Retrograde transport is not limited by COPI vesicles. First evidences of COPI independent retrograde transport rose with the identification the Golgi GTPase Rab6. Overexpression of a constitutively active form of Rab6-GTP leads to a retrograde flux of membrane (Martínez, 1997). Studies with bacterial toxins as the *Shigella* toxin B (StxB), showed that inhibition of COPI transport did not alter the transport of the STxB from the Golgi complex to the ER and that this transport was dependent of Rab6 (Girod, 1999).

1.4.2.- Intra-Golgi transport

Once the cargo has arrived at the *cis*-Golgi, it must go through all Golgi compartments, where it will be modified, and after reaching the *trans* face of the organelle will be finally sent to its final destination. Cargo, either small as the vesicular stomatitis virus G protein (VSVG) or large as procollagen, traverse the Golgi within COPI vesicles and/or through tubular connections that communicate different compartments (Mironov, 2001). However, there is lots of controversy about how the cargo goes through the Golgi, and debate between two classical models for intra-Golgi transport is not just still open, but some other models are arising (Glick and Luini, 2011). The vesicular transport model views the Golgi complex as a set of stable compartments where cargo is delivered to its *cis* side and would then move from one compartment to the next in COPI vesicles (Rothman and Wieland, 1996). On the other hand, in the cisternal progression/maturation model the Golgi cisternae are viewed as transient carriers, formed by homotypic fusion of COPII vesicles or other ER-derived carriers, which nucleate to form a new *cis*-Golgi cisterna (Bannykh and Bach, 1997, Mironov 2003). As the cisterna carry the secretory cargo forward, COPI vesicles would recycle Golgi proteins from older to younger cisternae (Glick and Malhotra, 1998; Rabouille and Klumperman, 2005). This model can be extended to incorporate tubular connections between cisterna. These tubular connections have been shown (Marsh, 2004; Trucco 2004) and enzymes of phospholipid metabolism as phospholipase A_2 may be implicated in its formation (San Pietro, 2009). These connections would allow cargo to move faster through cisternae and also allow the retrograde trafficking of resident Golgi proteins (San Pietro, 2009). Another model, known as rapid partitioning model, suggests that the Golgi operates as a single compartment that contains processing domains and export domains, and that cargo would partition between these domains and exit the Golgi from every level to its final destination (Patterson, 2008; Lippincott-Schwartz and Phair, 2010). Finally, the cisternal progenitor model proposes that the Golgi complex is a set of stable compartments that are segregated into domains

defined by Rab GTPases, where cargo transport would involve continual fusion of fission of these stable compartments (Pfeffer, 2010).

1.4.3.- Post-Golgi transport

After crossing the Golgi complex, cargo finally reaches the TGN, a tubulovesicular network where last post-translational modifications are done and cargo is sorted and sent to its final cellular destination as endosomes, lysosomes, the plasma membrane or the extracellular media. In polarized cells different molecular machineries regulate the transport to the basolateral plasma membrane and to the apical membrane (De Matteis and Luini, 2008). Proteins that are destined for basolateral domains possess a cytoplasmic tag that consists of the aminoacidic sequence YXX0 (where Y stands for tyrosine, X for any aminoacid and 0 for a hydrophobic one), which is recognized by coat or adaptor proteins (Bonifacino and Traub, 2003). Sorting of apical cargoes occurs by lipid-lipid or lipid-protein interactins within transmembrane or luminal domains, being the latter a process mediated by N- and O-glycans (Potter, 2004), or by interactions with the tethering domain GPI of the lipid rafts (Schuck and Simons, 2004). Once cargo is correctly sorted, this is retained in specific membrane domains of the TGN, in which cargoes with other destination and resident proteins are excluded. Finally, this cargo is embedded into a transport carrier that will bud and fission from the TGN (De Matteis and Luini, 2008).

1.5.- Biogenesis of transport carriers at the Golgi complex

When a membrane export domain is ready for export, it interacts with a suitable motor, it is extruded from the Golgi area and is followed by the detachment of these tubular precursors into transport carriers (De Matteis and Luini, 2008). These steps are also common in the biogenesis of COPI transport carriers for retrograde transport. The first step for the formation of the transport carrier is its budding, where a region of the membrane loaded with cargo will protrude into the cytoplasmic face. After the budding of the transport carrier, this must be detached from the membrane in a process called fission. Membrane fission requires local distortion and remodelling of the lipid bilayer to create a separate membrane-bound compartment without compromise the integrity of the maternal bilayer and avoiding any exposure of the vesicle lumen with the external milieu (Campelo and Malhotra, 2012). Membrane fission has been proposed to proceed via hemifusion-like pathway (Kozlovsky and Kozlov, 2003). Briefly, membrane fission starts with the formation a constricted neck, where membranes of the fission neck come into close proximity. Then contacting monolayers merge in a fission stalk

intermediate and the decay of this fission stalk leads to the completion of the fission reaction (Campelo and Malhotra, 2012; Fig. 5).

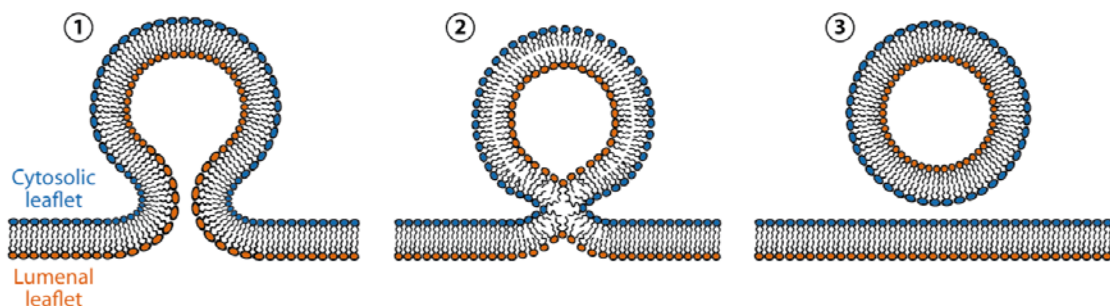

Figure 5. The steps of the fission of a vesicular transport carrier. *1) Membrane budding, 2) formation of a hemifission intermediate and 3) the eventual separation of the carrier . (Campelo and Malhotra, 2012)*

The process of membrane remodelling required for fission must be energetically favourable. The system free energy before remodeling has to be higher than after fission, which means that membrane remodeling must result in relaxation of the free energy. In other words, the fission process must go energetically “downhill” (Kozlov, 2010). Then, a large energy barrier has to be overcome to prevent the arrest of the system in a bud/tube configuration. The energy requirement for this late step can be reduced by changes in membrane curvature that promote fission, which can be driven by protein insertions into the bilayer or changes in the lipid composition of the membrane (Campelo and Malhotra, 2012).

1.5.1.- Membrane curvature for transport carrier formation

Both budding and fusion require changes in membrane curvature, which can be positive or negative. Conventionally, the curvature of a monolayer is defined as positive if the monolayer bulges in the direction of the polar heads, and negative for the opposite direction of bending (Semesh, 2003). Transport carrier budding needs the generation of both positive and negative curvature and their fission is highly dependent of negative curvature. In the formation of a membrane bud, positive curvature is required at the outer bilayer, while negative is needed at the inner. In the neck, there is a “saddle” geometry, which means that at any point both positive and negative curvatures are needed (Fig. 6). To proceed, the membrane bud needs to promote negative curvature to destabilize this “saddle” structure to finally achieve the fission of the transport carrier from the membrane of the donor organelle.

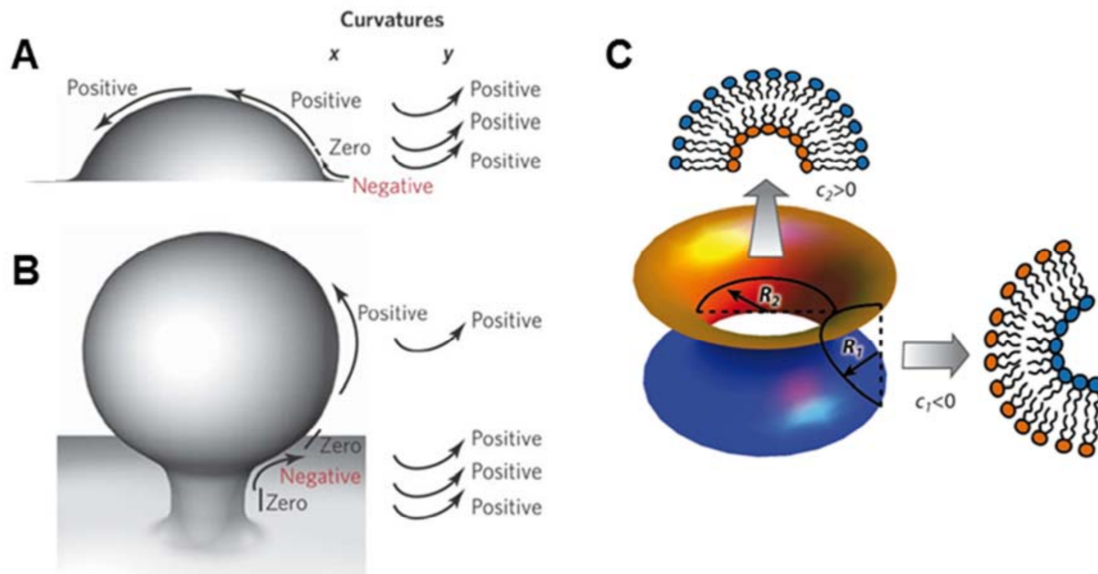

Figure 6. Membrane curvature in transport carrier formation. *A) Membrane curvatures found in the A) budding and B) scission of a transport carrier. C) Detailed view of the different curvatures at the neck of a membrane bud, where is shown that both negative and positive curvature are needed for membrane fission. The cytosolic and luminal leaflets of the membrane are depicted in blue and orange, respectively. (Adapted from McMahon and Gallop, 2005; and Campelo and Malhotra, 2012).*

The changes in membrane curvature that makes fission energetically favourable can be driven by different mechanisms (Fig. 7): changes in cytoskeletal polymerization and pulling of tubules by motor proteins, interaction of proteins with the membrane and/or changes in the lipid composition of the bilayer (McMahon and Gallop, 2005). First, the external force required to expand a piece of membrane for budding can be done by the combined action of the cytoskeleton and its motor proteins (De Matteis and Luini, 2008). Second, the interaction of proteins with the membrane bilayer can modify its curvature by three different ways. Transmembrane proteins can deform the membrane bilayer, since some of them have intrinsic curvature or present curvature on oligomerization. Peripheral membrane proteins can also modify the membrane by direct or indirect scaffolding. One example of this bending of the membrane by protein scaffolding is the oligomerization of the COPI coat proteins, which leads to the budding of COPI vesicles. Third, the insertion of the amphipathic helix into the membrane can also increase positive membrane curvature (McMahon and Gallop, 2005).

Finally, the lipid composition also plays a role in the generation of membrane curvature. The structure of their polar head and the nature of their acyl chains can influence the shape of the membranes. Depending on these parameters, three groups of lipids can be distinguished: cylindrical, inverted cones and conical lipids. Membrane lipids with a

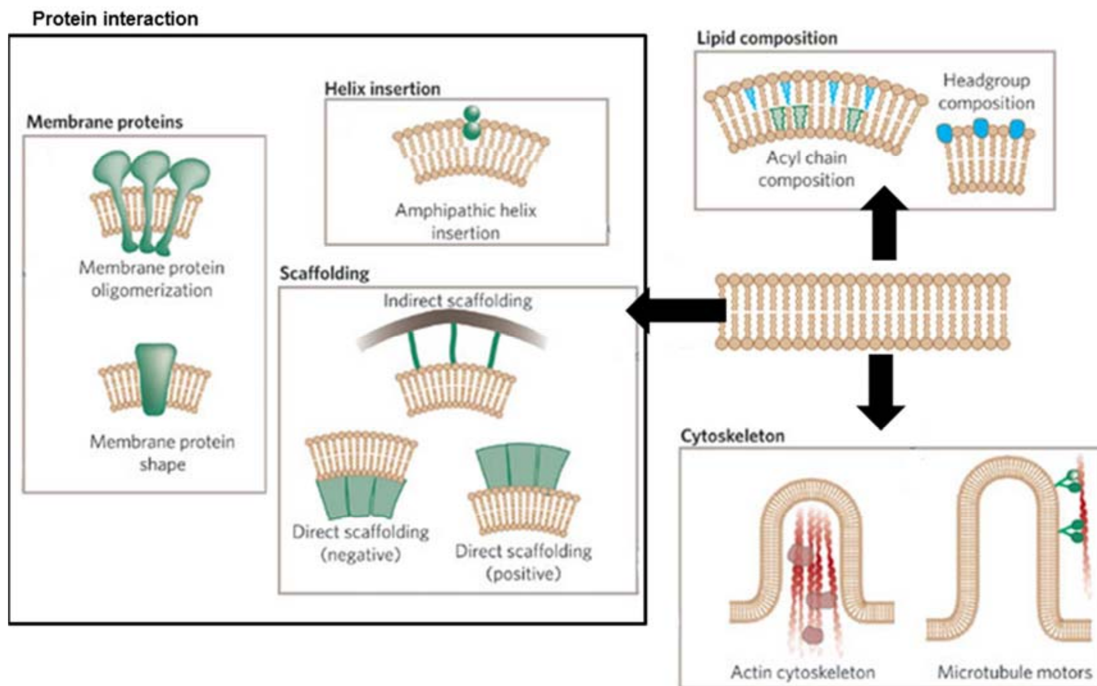

Figure 7. Mechanisms of membrane deformation. *The phospholipid bilayer can be deformed causing positive or negative membrane curvature. There are three main categories: 1, changes in lipid composition; 2, changes in cytoskeletal polymerization and pulling of tubules by motor proteins; and 3, by protein interaction , which can be by integral membrane proteins that have intrinsic curvature or have curvature on oligomerization; by direct and indirect scaffolding of the bilayer; or by active amphipathic helix insertion into one leaflet of the bilayer (adapted from McMahon and Gallop, 2005).*

polar head size similar to its hydrophobic moiety are known as cylindrical lipids, which spontaneously form a planar bilayer, without membrane curvature. PC, the most abundant lipid on cell membranes is a cylindrical lipid. The second group of lipids, the inverted cones, possess a polar head thicker than their hydrophobic part, which adopt a convex structure and promote positive curvature. Lysophospholids, as lysophospahtidic acid (LPA), are examples of inverted cones. The structure of conical lipids is opposite to the inverted cones, with a polar group smaller than its hydrophobic part. DAG and PA are examples of conical lipids, which adopt a concave structure and promote negative curvature of the membrane (Fig. 8; Voet and Voet, 1995). However, the negative charge of the polar headgroup of PA prevents that several PA molecules are localized together in order to promote negative curvature.

Therefore, changes in the lipid composition of the membrane can promote its curvature. It has been reported that an increase of DAG concentration increased the negative curvature of PC monolayers *in vitro* (Szule, 2002). The conversion of one kind of lipid to another can promote membrane curvature, as it does phospholipase A, which

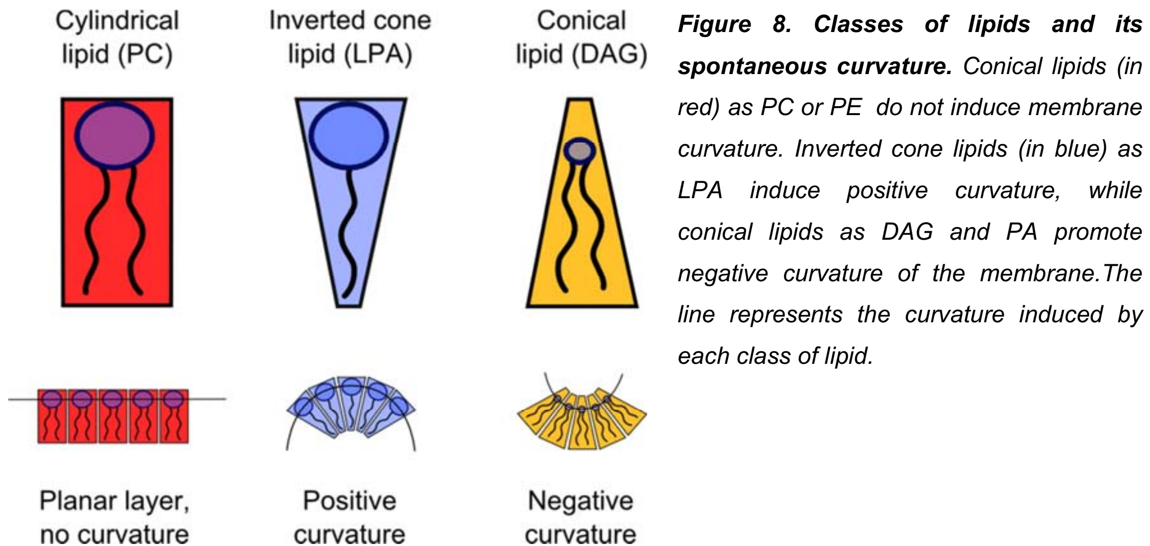

Figure 8. Classes of lipids and its spontaneous curvature. *Conical lipids (in red) as PC or PE do not induce membrane curvature. Inverted cone lipids (in blue) as LPA induce positive curvature, while conical lipids as DAG and PA promote negative curvature of the membrane.The line represents the curvature induced by each class of lipid.*

transforms PA (a conical lipid with two acyl chains) to LPA (an inverted cone with one acyl chain); which favour opposite curvature (Kooijman, 2005). Enzymes that change lipid headgroup size also can influence the area occupied by the lipids, affecting membrane curvature. DAG formation by phospholipase C (PLC) also promoted the increase of negative curvature of liposomes (Riske and Döbereiner, 2003). The action of flippases, which transfer lipids from one leaflet to the other, could generate membrane asymmetry, which could lead to membrane bending; or disrupt it. Thus, the metabolism of membrane lipids can play an important role in the generation or stabilization of membrane curvature and its particular distribution in the shape of a membrane, since most of the headgroups and acyl groups of different membrane lipids can be changed, going from the cylindrical PC to the highly conical DAG (McMahon and Gallop, 2005).

1.5.2.- Biogenesis of COPI-coated vesicles

The generation of COPI vesicles can be conceptually subdivided into different steps: coat recruitment, uptake of cargo, budding, membrane separation or scission and uncoating (Fig. 9; Popoff, 2011). The COPI, also called coatomer, consists of heptameric (α, β, β´, γ, δ, ε, ζ) complex, with two main subcomplexes: the γ-COP–δ-COP–ζ-COP–β-COP tetrameric complex, which constitutes the inner layer core; and the α-COP–β´-COP–ε-COP trimeric complex, which forms the outer layer of the COPI coat (Waters, 1991; Eugster, 2000). Recruitment of the coatomer to Golgi membranes is tightly correlated to ADP-ribosylation factor 1 (Arf1) activation (Donaldson, 1991; Serafini, 1991b; Palmer, 1993). Arf1 contains Golgi localization signals encoded in its sequence, and, once at this organelle can bind to dimeric complexes of the p24 family of Golgi-resident transmembrane proteins (Gommel, 2001). In contrast to clathrin1 and

COPII, which are composed of two successively recruited layers of protein, the coatomer is recruited *en bloc* (Hara-Kuge, 1994).

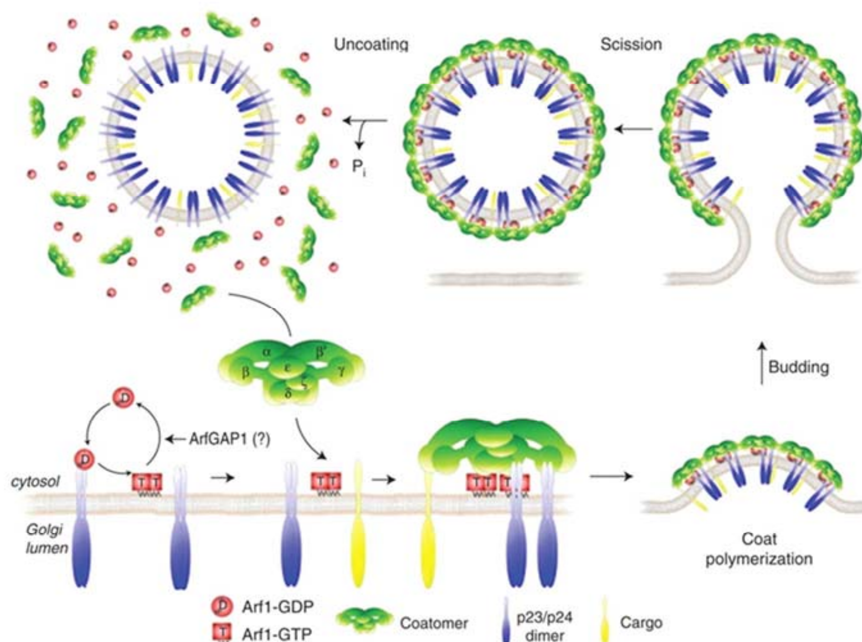

Figure 9.Scheme of the individual steps of the formation of a COPI vesicle. *See detailed information in the text (Popoff, 2011.)*

Cargo proteins are directed into COPI vesicles by different mechanisms based on direct or indirect binding to the coat. The KDELR interacts at the luminal side of soluble proteins with the C-terminal KDEL-sequence and its cytosolic tail interacts with coatomer and, as a result, KDEL-proteins are included into COPI vesicles and retrieved to the ER (Pelham 1991, Majoul 1998). Membrane proteins to be included into COPI vesicles can also be recognized directly by coatomer through sorting motifs present in their sequence (Popoff, 2011). It has also been proposed that ArfGAP1 can mediate cargo concentration at the center of a growing bud (Liu, 2005).

During the formation of a COPI bud, two different mechanisms have the potential to deform membrane into a curved bud. These are the polymerization of coatomer and the activity of Arf. Coatomer and Arf-GTP alone are sufficient to form COPI vesicles *in vitro* (Spang, 1998). Coatomer forms multiple protein-protein interfaces that result in conformational changes in γ-COP and α-COP subunits (Reinhard, 1999; Bethune, 2006; Langer, 2008). The spatial rearrangement of these subunits causes aggregation of the complex and is likely to initiate coatomer polymerization (Reinhard, 1999) providing the energy to bend the membrane to form a COPI-coated bud (Popoff, 2011). Other proteins as Brefeldin-A ADP-ribosylated Substrate (BARS), endophilin and

ArfGAP1 are thought to play a role in membrane scission of COPI-coated buds (Popoff, 2011). However, scission of COPI vesicles is a process still poorly understood.

Once a COPI vesicle is formed, it needs to be uncoated in order to allow fusion with its target membrane. This process is coupled to hydrolysis of GTP by Arf1 (Tanigawa 1993), triggered by ArfGAPs (Cukierman, 1995), which can be regulated by membrane curvature (Bigay, 2003).

In addition to the protein machinery involved in COPI vesicle formation, it has been shown that lipids as PA, DAG and PI also have active roles in the generation of these transport carriers. Our group showed that pharmacological inhibition of DAG production impaired the fission of COPI vesicles and the recruitment of ArfGAP1 at the Golgi complex (Fernández-Ulibarri, 2007). These results were confirmed by another study, where it was proposed that DAG was needed for bud formation (Asp, 2009). It has also been proposed that PA participates in COPI vesicle formation, since it has been shown that BARS requires PA to induce tubulation *in vitro* (Yang, 2008). Moreover, knockdown of PI transfer protein β (PITPβ) leads to an impairment of COPI-mediated retrograde transport (Carvou, 2010), suggesting that PI is required for COPI vesicle formation, since it is the substrate of PI(4)P.

1.5.3.- Biogenesis of TGN-to-plasma membrane transport carriers

In contrast to COPI-, COP-II and clathrin-coated vesicles, thus far there is no specific coat protein identified for the formation of transport carriers that drive the trafficking of cargo from the TGN to the plasma membrane (Campelo and Malhotra, 2012). However, it is well known that a serine/threonine kinase termed protein kinase D (PKD) plays a critical role in the formation of these transport carriers (Bard and Malhotra, 2006; Malhotra and Campelo, 2011).

Early studies with the sponge metabolite ilimaquinone (IQ) suggested the existence of regulated membrane fission machinery at the Golgi complex. IQ addition led to a complete vesiculation of the Golgi complex and after IQ removal the Golgi complex assembled into a normal, fully functional, organelle (Takizawa, 1993). Further studies showed that trimeric G protein subunits βγ and PKD were involved in the IQ-mediated fragmentation of the Golgi (Jamora, 1997; Jamora 1999; Díaz-Añel and Malhotra, 2005). Chemical inactivation of PKD or expression of an inactive form inhibited trafficking of proteins from the Golgi to the cell surface, retaining cargo into large tubules attached to the TGN (Liljedahl, 2001). Overexpression of a constitutive active PKD caused extensive vesiculation of the TGN, while PKD depletion by small

interference siRNA (siRNA) led to cargo accumulation in tubular membranes at the TGN (Bossard, 2007). These results led to the proposal that PKD was required for events leading to membrane fission specifically of carriers that transport cargo to the cell surface.

DAG has a critical role in the generation of TGN-to-plasma membrane transport carriers. The first cysteine rich domain of PKD, known as C1a, binds DAG at the TGN (Maeda, 2001) and depletion of cellular pools of DAG inhibits the binding of PKD to the TGN and blocks protein trafficking to the cell surface (Baron and Malhotra, 2002). In addition to recruiting PKD, DAG in the TGN is required for protein kinase C η (PKCη) activation, which in turn phosphorylates and activates the DAG-associated PKD (Díaz-Añel and Malhotra, 2005). Moreover, the local increase of DAG should promote the negative curvature needed for the fission of these transport carriers (Bard and Malhotra, 2006). In a proposed model, Golgi-associated DAG recruits PKD, which in turn indirectly increases DAG production by the action of shingomyelin synthase to promote fission of transport carriers at this organelle (Malhotra and Campelo, 2011, see below chapter III).

2.- DIACYLGLYCEROL AT THE GOLGI COMPLEX

2.1.- Structure and function of diacylglycerol

Diacylglycerol (DAG) is a lipid consisting of a glycerol molecule linked through ester bonds to two fatty acids. This composition confer exceptional properties on DAG as a lipid intermediate in metabolism, as a component of biological membranes and as a second messenger (Fig. 10; Carrasco and Mérida, 2007).

Based on its acyl chain nature, more than 50 DAG species have been identified in mammals, which differ in the length and the number of insaturations of acyl chains (Pettitt and Wakelam, 1993). Palmitic acid (16:0; 16 carbon length: 0 double bonds), estearic acid (18:0), and arachidic acid (20:0) are the most common saturated fatty acids in cell membranes. Oleic acid (18:1) is the most common monounsaturated acyl chain while linoleic acid (18:2) and arachidonic acid (20:4) are the most abundant poliinsaturated fatty acids.

DAG has an important role as a precursor for phospholipid synthesis. Although DAG is found in small amounts, its presence is a requisite for most phospholipid synthesis. DAG is a precursor for PC and PE, whose synthesis requires the incorporation of the activated alcohols CDP-choline and CDP-ethanolamine into DAG (Henneberry, 2002). In addition, DAG can be phosphorylated by DAG kinases (DAGK) to produce PA,

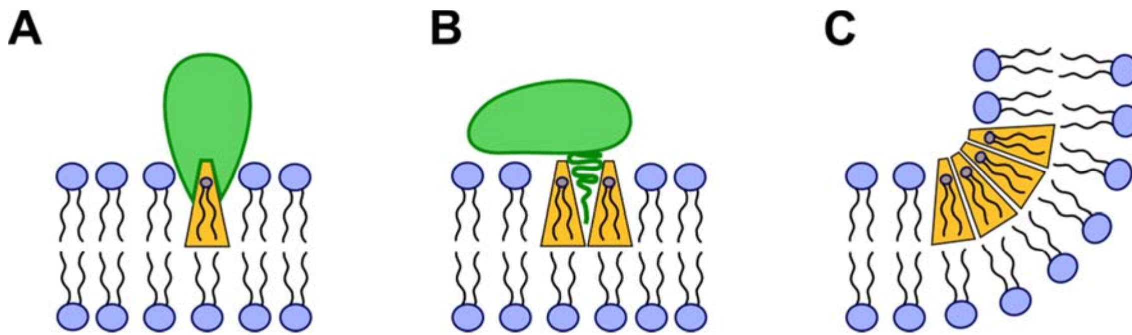

***Figure 10. Functions of DAG as a component of biological membranes**. DAG (highlighted in yellow) can participate in different events at the biological membrane. A) Some proteins are recruited to the membrane by the binding of its C1 domain to DAG. B) DAG can interfere to some protein activities by facilitates the insertion of amphipathic helixes of proteins into the bilayer. C) DAG, as a conical lipid, promotes negative membrane curvature.*

which is essential for the synthesis of PI and cardiolipin. Moreover, DAG can be metabolized into triacylglycerol (TAG) by esterification of a new fatty acid in the free alcohol group of the glycerol moiety (Carrasco and Mérida, 2007).

DAG also acts as a second messenger. One of the most known producers of DAG as a second messenger PLC, a family of phospholipases that classically, after extracellular stimuli, hydrolyses PI(4,5)P_2 producing DAG and IP_3, a soluble second messenger that is essential for Ca^{2+} exit from the intracellular Ca^{2+} stores (Gresset, 2012, Khadamur and Ross, 2012). DAG is able to bind and activate different signalling proteins, which have at least one sequence of 50 amino acids known as C1 domain (Yang and Kazanietz, 2003). C1 are conserved domains rich in cysteine that were first described in the PKC family, but that are also found in other five protein families as chimaerins, DGKs, PKD, Munc13, Ras guanine-realising protein (RasGRP) and myotonic dystrophy kinase-related Cdc42-binding kinase (Carrasco and Mérida, 2007). C1 domains were initially described as domains that bind DAG analogues as phorbol esters, and their capacity to bind DAG and other related compounds was confirmed later (Kazanietz, 2000).

Compared to other membrane phospholipids, DAG possesses a small polar headgroup, comprising only an alcohol group of the glycerol moiety. This makes DAG the lipid with the most spontaneous negative curvature, involving it in events that require high membrane curvature as fission of transport carriers (Shemesh, 2003). If membrane curvature is impaired, the presence of DAG in a bilayer induces the generation of small areas in which the apolar regions of neighbouring lipids are partially exposed (Goñi 1999). By promoting the hydrophobic interaction of proteins with these membrane areas, DAG can affect the activity of some proteins that are integrated or

interact with cell membranes (Lee, 2004; Bigay, 2005; Ahyayauch 2005). Moreover, DAG molecules with saturated fatty acids adopt a more conical structure than those with unsaturated ones, thus promoting membrane curvature or interaction of proteins with hydrophobic tails. One example of this is found in ArfGAP1, which is more active in membranes enriched DAG molecules with one monounsaturated acyl chain (Antonny, 1997), which are the most common fatty acids of the hydrophobic backbone of PC.

2.2.-DAG in the fission of transport carriers at the Golgi complex

DAG has a critical role at the Golgi complex, where is needed for the generation of transport carriers. First evidences were shown in yeast, where DAG depletion by overexpression of a DAGK impaired protein transport from the Golgi complex (Kearns, 1997). DAG requirement for transport to the plasma membrane was confirmed in mammals, where pharmacological reduction of DAG levels impaired the recruitment of PKD to the Golgi complex and blocked protein trafficking from the TGN to the plasma membrane (Baron and Malhotra, 2002). A theoretical model suggested that DAG was not only needed for PKD recruitment, but also for generating the membrane curvature needed for fission of transport carriers (Semesh, 2003). Reduction of Golgi DAG levels after Nir2 inhibition impaired Golgi secretory function, which can be recovered by PC synthesis inhibition (Litvak, 2005). By pharmacological inhibition of DAG production, our group demonstrated the need of DAG for fission of COPI vesicles at the Golgi complex (Fernández-Ulibarri, 2007), a result confirmed by others, where DAG was proposed to be needed for vesicle budding (Asp, 2009). In this context, inhibition of sphingomyelin synthase (SMS), which also produces DAG; by pharmacological and siRNA approaches also impaired protein secretion (Subathra, 2011)

These results showed the involvement of DAG in transport carrier formation and led to models of transport carrier formation where DAG has a pivotal role in both PKD recruitment and vesicle fission (Bard and Malhotra, 2006, Campelo and Malhotra, 2011). They also showed the enzymatic pathways that may be targeted by cells to control DAG at the Golgi compelx. However, how cells regulate DAG for the generation of transport carriers is unknown. It has been postulated that, cargo arrival could be the more straightforward activator of DAG production at the Golgi complex for transport carrier formation (Bard and Malhotra, 2007), but this issue has not been experimentally adressed.

2.3. DAG metabolism at the Golgi complex

DAG is an intermediate metabolite that is involved in different metabolic pathways (Fig. 11). At the Golgi complex DAG can be produced by at least three different mechanisms: 1) by SMS (Holthuis and Luberto, 2010), 2) the coupled reactions of phospholipase D (PLD) and lipid phosphate phosphatases (LPPs; Sciorra and Morris, 1999), and 3) by PLC (Bard and Malhotra, 2005). However, DAG concentration not only depends on these mechanisms that produce it, but also on metabolic pathways that consume DAG. These pathways are the ones involved in phospholipid synthesis, which are the synthesis of PC by the CDP-choline pathway or PI synthesis. The key enzymes of DAG-consuming reactions are also localized at the Golgi complex (Jelsema and Morré, 1978). This way, an increase of DAG for the generation of transport carriers at the Golgi complex could be caused, theoretically, by a stimulation of mechanisms that produce DAG, by an inhibition of the ones that consume it or by both.

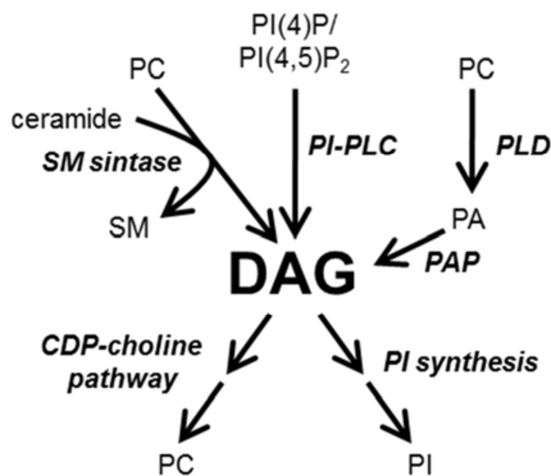

Figure 11. Metabolic pathways that can regulate DAG at the Golgi complex. *The name of the enzyme or pathway that produces or consumes DAG is written in bold and italic.*

2.3.1.- DAG production by the sphingomyelin synthase

Production of DAG by SMS is one of the mechanisms that can regulate DAG homeostasis at the Golgi complex, since it is well established that SMS is found at the *trans*-Golgi (Holthuis and Luberto, 2010). SMS, also named phosphatidylcholine: ceramide cholinephosphotransferase; transfers phosphocholine from PC to ceramide to yield SM and DAG. SM synthesis is initiated by the production of ceramide, the key intermediate for the biosynthesis of most sphingolipids. Briefly, L-serine and palmitoyl CoA are condensated to generate 3-ketodihydrosphingosine, which is rapidly reducted and N-acylated to generate dihydroceramide, which is desaturated to finally generate ceramide. These reactions that produce ceramide have been described to occur in the

cytosolic leaflet of the ER bilayer (Hanada, 2009). Then, ceramide is delivered to the luminal side of the Golgi complex by the ceramide transfer protein (CERT; Hanada, 2003), and converted to SM by SMS.

Mammals express two isoforms of SMS (SMS1 and SMS2), both localized at the Golgi complex, although SMS2 is also found at the plasma membrane (Villani, 2008; Huitema 2004). Availability of ceramide seems to be the limiting step for SM synthesis. LY-A, a cell line that expresses a mutated CERT, has an impaired SM synthesis that can be restored with addition of functional CERT (Hanada, 2003).

There are evidences that DAG production by SMS at the TGN has a role in secretory function. Treatment with fumonisin B1, which inhibits ceramide synthase and thus deprives SMS of its substrate, lowers DAG levels in the Golgi complex, and this inhibition also results in block of Golgi-to-cell-surface transport (Baron and Malhotra 2002). Addition of short chain ceramide leads to an increase of DAG at the Golgi complex, suggesting that SMS can regulate the Golgi-associated DAG pool (Villani, 2008). Pharmacological inhibition of SMS leads to tubulation of the TGN and silencing of both SMS1 and SMS2 reduces the trafficking of VSVG to the plasma membrane and insulin secretion (Subathra, 2011), suggesting a role for SMS in the TGN secretory function. Moreover, a recent study suggests that a regulated production and organization of SM is necessary for transport carrier formation (Duran, 2012), showing that both products of SMS are needed for Golgi secretory function. However, if SMS is regulated to control the amount of DAG in order to promote fission at the Golgi and/or SMS is activated after cargo arrival is not known.

2.3.2.- DAG production by phospholipase D and lipid phosphate phosphatases

DAG can also be produced by the combined action of PLD and LPPs. PLD converts PC into PA, which can be dephosphorylated to DAG by LPPs.

Two mammalian PLD enzymes that convert PC to PA have been identified: PLD1 and PLD2. Both enzymes have been reported on the plasma membrane and on internal vesicles, although PLD2 appears less abundant at internal sites than PLD1. However, their precise localization is controversial. Both enzymes have been reported to be localized at the Golgi complex (Freyberg 2001, Freyberg 2002), but this localization has not been confirmed by other studies (Sarri 2003, Du 2004).

LPPs are members of a larger family of proteins, the phoshpatidic acid phosphatases (PAP) that hydrolyze PA. LPPs were formerly known as Mg^{2+}-independent and N-ethylmaleimideinsensitive phosphatidate phosphatases (PAP2). There are three LPPs

(LPP1, LPP2, and LPP3 and a splice variant, LPP1a), which hydrolyze lipid phosphates, including PA, LPA, sphingosine1-phosphate, ceramide 1-phosphate and diacylglycerol pyrophosphate (Brindely 2009). All LPPs are present to some extent in the plasma membrane, while LPP2 and LPP3 have been variably localized in endomembranes such as ER, endosomes, vesicular structures and the Golgi complex (Alderton, 2001). In a recent study from our group, LPP3 was found in early compartments of the secretory pathway including the Golgi complex, and particularly enriched at the ERGIC (Gutiérrez-Martínez, 2013).

PLD has been suggested to be involved in membrane traffic since it was discovered that Arf1, an important regulator of membrane traffic localized at the Golgi complex, was an activator of PLD activity (Brown 1993, Cockcroft 1994). Studies that revealed a role for PLD in formation of Golgi coated vesicle (Ktistakis 1996), transport from the ER to the Golgi (Bi, 1997) or in regulation of Golgi structure and protein secretion in endocrine cells (Siddhanta, 2000) have not been confirmed by siRNA experiments that knockdown PLD (Roth, 2008). PA, the product of PLD, has also been suggested to be necessary for COPI vesicle formation (Yang, 2008) and if metabolized by LPPs can generate DAG. There are some evidences that inhibition of LPPs causes defects on the Golgi function. Treatment with propanolol, an inhibitor of general PAP activity, leads to a lesser recruitment of PKD in the Golgi complex and alters anterograde transport (Baron, 2002), lowers DAG levels in this organelle and impairs COPI vesicle fission (Fernández-Ulibarri, 2007) and budding (Asp, 2009), with the subsequent alteration of retrograde transport from the Golgi complex to the ER. Moreover, the depletion of LPP3 impairs the Rab6-dependent retrograde transport of STxB from the Golgi to the ER and induces a high accumulation of Golgi-associated membrane buds (Gutiérrez-Martínez, 2013). However, none of these studies show evidence that LPPs or PLD may be regulated by the presence of cargo or specifically stimulated during DAG production for transport carrier formation at the Golgi complex.

Nevertheless, the structure of PA and DAG produced by PLD activity also support the idea that PLD could be involved in transport carrier formation. As explained before, the most abundant forms of PC, the substrate for PLD in mammalian cells, have one saturated and one unsaturated acyl chain. Membrane lipids with an unsaturated acyl chain have a relative bigger hydrophobic part than those with two saturated acyl chains. PA and DAG derived from PLD activity would conserve this unsaturated acyl chain, being even more conical than PA and DAG with two saturated acyl chains, promoting negative membrane curvature and, on the other hand, facilitating the insertion of hydrophobic amino acids into the membrane (Roth, 2008).

2.3.3.- DAG consuming pathways

2.3.3.1.- DAG consumption by PC synthesis: the CDP-choline or Kennedy pathway

DAG can be consumed by the synthesis of PC in the CDP-choline or Kennedy pathway. PC is the most abundant phospholipid of eukaryotic membranes, generally comprising more than 50% of the total phospholipid mass. In mammalian cells, PC is mainly synthesised *de novo* by the CDP-choline or Kennedy pathway.

Choline is an essential nutrient provided by the diet. Choline is internalized by gradient-driven choline transporters. Once inside the cell, choline is phosphorylated by the choline kinase (CK) producing phosphocholine. Because CK has a high affinity for choline, choline taken up into cells is readily trapped as phosphocholine, which constitutes the largest pool of intermediates in the CDP-choline pathway (Fagone and Jackowski, 2013), and it also maintains the choline gradient that allows its internalization to the cell. Phosphocholine can be also supplied as a result of sphingomyelin degradation by a lysosomal sphyngomyelinase (Jansen, 2001).

Phosphocholine and CTP give CDP-choline and pyrophosphate, in a reaction catalyzed by the CTP:phosphocholine cytidylyltransfarase (CCT). CCT is the key regulatory enzyme in the CDP-choline pathway. Moreover, CDP-choline, the product of the reaction, is present in extremely small amounts in cells, indicating that newly synthesized CDP-choline is readily incorporated to PC. CCT presents a biochemical regulation that enables its response to local changes in membrane lipid. This mechanism relies on the insertion of the hydrophobic face of its α-helix into membranes with lipid packaging stress. This way, CCT has low affinity with membranes that are made up of a large fraction of PC; and introduction of DAG or free fatty acids into these membranes introduces negative elastic stress and enhances CCT association and activity (Yang, 1995; Johnson, 1998). Thus, CCT can sense changes on membrane composition in order to increase PC synthesis when this is depleted (Fagone and Jackowski, 2013).

DAG is consumed in the last step of the CDP-choline pathway, a reaction catalysed by the enzyme CDP-choline: diacylglycerol phosphotransferase (CPT). In this reaction, phosphocholine is transferred from CDP-choline to DAG, producing the end product PC and CMP. This last, DAG-consuming step of the CDP-choline pathway can also be physiologically regulatory, since it has been reported that NMDA overactivation inhibits PC synthesis at the level of CPT (Gasull, 2003).

Although PC synthesis is mainly localized at the ER, evidences of its presence at the Golgi complex have been shown. Fractionation of subcellular organelles reveals that CPT activity in the Golgi complex is 37 % of that in the ER, the organelle where CPT activity is higher (Jelsema and Morré, 1978). Overexpression studies showed that CPT, the enzyme that incorporates DAG in the CDP-choline pathway, is localized at the Golgi complex (Henneberry 2002).

Some studies point that Golgi-specific CPT may have a role in regulating secretory processes by controlling DAG levels in this organelle. The yeast PITP Sec14p is essential for protein trafficking at the Golgi complex (Bankaitis, 1989). Sec14p requirement for yeast Golgi function can be bypassed by mutations in the yeast CK gene that impair PC synthesis (Cleves, 1991), suggesting a role for the CDP-choline pathway in the regulation of Golgi secretory function. Sec14p was proposed to act as a dynamic sensor of PI and PC content in Golgi membranes, regulating the CDP-choline biosynthetic pathway in this organelle (McGee, 1994). Defects on Sac1p that led to an increase of the DAG pool at the Golgi complex also bypassed Sec14p requirement for secretory function (Kearns, 1997). The CDP-choline pathway is not the primary route for PC synthesis in yeast, where is not essential (Fagone 2012). This observation suggests that, in yeast, one of the functions of this metabolic pathway, instead of being the main producer of PC, is to control the secretory function of the Golgi complex. Similar results were obtained with Nir2, the mammalian homologue of Sec14 (Litvak, 2005).

2.3.3.2.- DAG consumption by PI synthesis

DAG can also be consumed for PI synthesis. PI is a structural lipid and also the precursor of all polyphosphoinositides, which have critical roles in organelle identity, vesicular trafficking and signalling. DAG is consumed in this pathway by DAGKs, which phosphorylate DAG to produce PA. Then, PA is activated by CTP to CDP-DAG, which condensates with inositol to produce PI by the action of phosphatidylinositol synthase (PIS; Antonsson, 1997).

PIS is a membrane-bound enzyme that requires Mg^{2+} and has a high specificity for its substrate *myo*-inositol. *Myo*-inositol is an essential substrate and its availability could control PIS activity. It has also been suggested that PIS activity might be inhibited by its product, PI (Antonsson, 1997).

The substrate for PI synthesis CDP-DAG is formed in very low amounts by cytidine diphosphate-diacylglycerol synthase (CDS). The low levels of CDP-DAG reported

suggest that CDS activity may be the rate-limiting step for PI synthesis (Heacock and Agranoff, 1997), as CTT for the CDP-choline pathway; indicating that newly synthesised CDP-DAG is rapidly converted to PI or to phosphatidylglycerophosphate.

DAG is consumed for PI synthesis by DAGKs. DAGKs are members of a family of cytosolic lipid kinases, currently with ten members classified into five different subtypes. Most of its members are cytosolic proteins that translocate to the membrane, are regulated by phosphorylation and have tissue specificity. The main function attributed to DAGKs is the depletion of DAG, but also controls the levels of PA. DAGKs have been found to be crucial components for PI resynthesis, since some studies have shown that PA generation controls the PI cycle (Mérida, 2008).

It is well known that PI synthesis takes place at the ER. However, some PIS and CDS activity have been found in Golgi-enriched membrane fractions (Jelsema and Morré, 1978). A recent study showed that PIS is found in a mobile compartment of ER origin that makes ample contact with other organelles, while CDS resides in the Golgi complex. Moreover, catalytically inactive PIS is not found in this mobile compartment, suggesting that PI synthesis is needed for the generation of this mobile compartment where active PIS is found (Kim, 2011).

DAG consumption for PI synthesis takes place at the beginning of this metabolic pathway. However, regulation of PIS can also affect, theoretically, the Golgi-associated DAG pool, because changes on the rate of this enzyme can affect the rate of other steps of the pathway and, therefore, the amount of all the metabolic intermediates of this pathway, as DAG. Thus, changes in PIS and CDS activity could modify the activity of the other enzymes of the pathway, as the DAG consumption by DAGK. This step can also be taking place at the Golgi complex, since DAGK are cytosolic enzymes that can act on different cellular membranes.

On the other hand, PI is necessary for membrane trafficking and Golgi function, since it is the precursor of all polyphosphoinositides, which have critical roles in these processes (Mayinger, 2011). However, if regulation of PI synthesis can modulate DAG levels at the Golgi complex and the secretory function of this organelle has not been addressed.

2.3.4.- Phospholipase C

Another possible mechanism to produce DAG in the Golgi complex is by the action of PLC. Mammal PLC enzymes comprise 13 isozymes distributed in 6 different families (Fig. 12; Bunney and Katan, 2011). PLCs are calcium-dependent phosphodiesterases

that preferentially hydrolyze $PI(4,5)P_2$ into DAG and inositol(1,4,5)triphosphate (IP_3), but they also hydrolyse PI(4)P (Claro, 1993).

All PLCs share a conserved core structure in addition to a variety of other domains specific for each family. The core enzyme is composed of a pleckstrin homology (PH) domain, four tandem EF hand domains, a split catalytic triose phosphate isomerase (TIM) barrel and a C2 domain. The active site, all catalytic residues and a Ca^{2+} binding site are contributed by the TIM barrel (Katan and Williams, 1997). This domain has an autoinhibitory insert that leads to the naming of the N- and C- terminal halves of the TIM barrel as X and Y domains. This insert, known as the X-Y linker, differs among families in sequence and size, and is central to the regulation of PLC activity (Hicks, 2008).

All PLC isoforms can be found in soluble form, while their substrate is an integral part of the membrane. Therefore, PLC has to be, at least, in close proximity to the membrane to hydrolyse its substrate. Another requirement for PLC activity is Ca^{2+}, which facilitates the deprotonation of the inositol-phosphate moiety for subsequent PIP_2 hydrolysis. This essential Ca^{2+} is ligated by four acidic residues, and mutation of them impairs PLC activity or increases the concentration of Ca^{2+} required for catalysis. PLCs initially form a weakly enzyme-bound cyclic intermediate, inositol 1,2-cyclic phosphodiester, which is then hydrolysed to form IP_3. All the residues in the active site participating in substrate specificity, Ca^{2+} coordination and the catalytic activity are highly conserved across the PLC family (Hicks, 2008; Jezyk, 2006; Waldo, 2010).

Regulation of PLC activity depends on each isozyme, but all families share a common mechanism of autoinhibition. PLCs are autoinhibited by the X-Y linker of the TIM barrel (Hicks, 2008), which it apparently occludes the active site. Removal of this X-Y linker is needed for activation of PLC isozymes, although regulation of this step may be different in each family. Both proteolysis and genetic deletion of sections of the X-Y linker increase PLC activity in most of the PLC families (Kadamur and Ross, 2013).

Two compounds have been proposed as general PLC inhibitors. 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U73122) is an aminosteroid first reported as an inhibitor of PLC-dependent processes (Bleasdale, 1989). Many studies have used U73122 as a direct and general PLC inhibitor, in

combination with (1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione) (U73343) a close structural analog of U73122 containing N-alkylsuccinimide moiety in place of N-alkylmaleimide, which is supposed to not inhibit

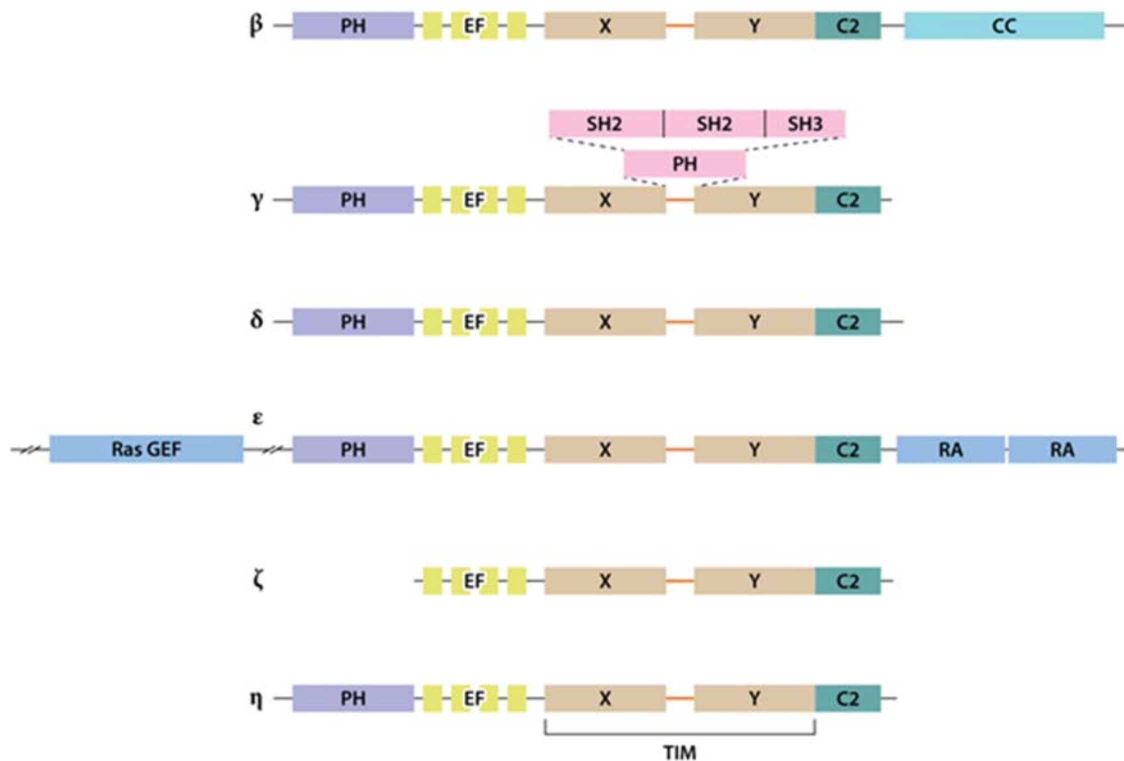

Figure 12. Domain structures of mammalian PLC isozymes. *Different domains are abbreviated as follows: PH, pleckstrin homology; EF, EF hands; CC, coiled coil; SH2/SH3, Src homology 2/3; RA, Ras association; Ras GEF, Ras GDP/GTP exchange factor; X and Y, N- and C-terminal portions of the TIM barrel (Kadamur and Ross, 2013).*

PLC. U73122 has been used to involve PLC in different cellular process, although its mechanism is not known. Recently it has been reported that U73122, but not U73343, can stimulate PLCβ and PLCγ *in vitro* (Klein, 2011). The other compound frequently used as a PLC inhibitor is the ether lipid analogue 1-octadecyl-2-methyl-rac-glycero-3-phosphocholine, also known as edelfosine (Powis, 1992). Although its exact mechanism of inhibition is also not known, it has been proposed that edelfosine may be a more selective inhibitor than U73122 (Horowitz, 2005).

2.3.4.1.- PLC families and their regulation

2.3.4.1.1.- PLCδ

The three PLCδ proteins (numbered 1, 3 and 4) appear to be the progenitor animal PLCs, since they are already found in early eukaryotes. PLCδs consist of only the core domain and their only well regulatory ligands are PI(4,5)P_2 and Ca^{2+}. The PH domain of PLCδ binds tightly PI(4,5)P_2 and this binding drives membrane association and promotes PLCδ activity. The residues of the PH domain that ligate PI(4,5)P_2 in PLCδ are not conserved in other PLC isozymes, and mutation of them impair PLCδ activity.

However, $PI(4,5)P_2$-mediated membrane binding is not sufficient to activate PLCδ and Ca^{2+} is required for maximal activation (Gresset, 2012). Ca^{2+} binding to the C2 domain, in addition to Ca^{2+} at the active site, is the only known regulator that enhances the activity of PLCδ isoforms and is sufficient to promote its activity (Allen, 1997; Kim, 1999).

All PLCδ isozymes are expressed widely. PLCδ1 is mainly cytosolic, although it shuttles between the nucleus and the cytosol depending on the phase of the cell cycle. PLCδ3 has been detected in membrane fractions and PLCδ4 localizes at the nucleus. It has been proposed that the role of PLCδ is to intensify and prolong Ca^{2+} signals by responding to elevated cytosolic Ca^{2+} that has been evoked by the action of other PLC isoforms (Gresset, 2012).

2.3.4.1.2.- PLCβ

The structure of all four PLCβ isozymes is unique in having, in addition to the whole core of PLCs described in PLCδ isoforms, a C-terminal three-stranded coiled coil domain, which is required for stimulation by heterotrimeric G-proteins. PLCβ isozymes are effectors of heterotrimeric G-proteins downstream of G-protein coupled receptors (GPCR) that activate Gα subunits of the G_q family. The G_q family consists of four different Gα subunits ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{16}$) and all of them can strongly activate PLCβ in both intact cells or *in vitro*. Although individual PLCβ can be regulated by other factors as Rac, PA or phosphorylation, all isozymes are also stimulated by subunits Gα and Ca^{2+} (Kadamur and Ross, 2013). All PLCβ also are GTPase-activating proteins (GAPs) for $G\alpha_q$, promoting the hydrolysis of GTP and consequent $G\alpha_q$ inactivation (Biddlecome 1996; Ross 2008).

All PLCβ isozymes are cytosolic but they can be found associated with the plasma membrane. PLCβ1 can also be found in the nucleus, where it can hydrolyse PI(4)P. The four PLCβ differ in expression pattern and only PLCβ3 is broadly expressed. The main physiological role of the PLCβ family is to mediate the actions of many extracellular stimuli, through activation of GPCR and consequent activation of Gα subunits of the G_q family that regulate their activity (Kadamur and Ross, 2013).

2.3.4.1.3.- PLCε

PLCε has a single isoform that, in addition to the core lipase domains contains an N-terminal cysteine-rich domain, an N-terminal CDC25 domain and two C-terminal Ras-associating domains. PLCε is a direct target of Ras, Rap and Rho. The GTP-bound forms of Rap and Ras stimulate PLCε by binding to the second of its C-terminal Ras-

associating domain. Therefore, PLCε can respond to all imputs that regulate these GTP-binding proteins, as GPCR coupled to G proteins or tyrosin kinase receptors. Moreover, Ras and Rap also participate in recruiting PLCε to specific organelles, as the cell periphery or to the perinuclear area, respectively (Gresset, 2012; Kadamur and Ross, 2013). Recently, it has been described that PLCε can generate DAG from PI(4)P in the Golgi complex in response to hypertrophic stimuli in mouse cardiac myocytes (Zhang, 2013).

2.3.4.1.4.- PLCζ

PLCζ is found only in vertebrate sperm and is structurally characterized for lacking an N-terminal PH domain. PLCζ is the smallest PLC isozyme and has the highest sensitivity to Ca^{2+}, its only known regulatory ligand. PLCζ contains two nuclear localization signals, one localized at the X-Y that can promote its shuttling in and out of the nucleus coinciding with Ca^{2+} oscillations (Gresset, 2012; Kadamur and Ross, 2013).

2.3.4.1.5.- PLCη

A novel class of PLC isozymes that includes two isozymes, PLCη1 and PLCη2, was discovered in 2005. Its expression is enriched in the brain. PLCη structure is similar to PLCδ, with the addition of an extended C-terminus after the C2 domain. To date, only Ca^{2+} has been reported to stimulate PLCη, and some data suggest that PLCη2 can be activated also by Gβγ subunits (Gresset, 2012; Kadamur and Ross, 2013).

2.3.4.1.6.- PLCγ

The two PLCγ isoforms are structurally characterized by a large insertion in the X-Y linker that is central to its regulation. This insertion consists of a split PH domain, two SH2 domains and a SH3 domain. Phosphorylation is a key step of removal of autoinhibition of this enlarged X-Y linker. Phosphorylation of Tyr783 PLCγ1 promotes conformational changes that remove autoinhibition of the X-Y linker and increases lipase activity (Gresset, 2010). This mechanism is likely to be analogous for PLCγ2 in its tyrosine Tyr759. PLCγ isoforms can be phosphorylated by receptors with intrinsic tyrosine kinase or by other cytosolic tyrosine kinases, both forming large signalling complexes in the plasma membrane (Gresset, 2012; Kadamur and Ross, 2013).

Although phosphorylation is the most acceped activator of PLCγ, other mechanisms can stimulate its activity. $PI(3,4,5)P_3$ can act as a specific ligand for PLCγ isozymes and can mediate their translocation to the plasma membrane. PLCγ2, but not PLCγ1, is

directly activated by Rac GTPases (Gresset, 2012). Moreover, activity of both isoforms is stimulated by increasing concentrations of Ca^{2+} (Piechulek, 2005).

PLCγ1 is found ubiquitously, while PLCγ2 expression is restricted to cells of the haematopoietic system. PLCγ, as PLCβ, responds to different extracellular stimuli, as almost all growth factors, and its main function is the transduction of extracellular stimuli (Gresset, 2012).

2.3.4.2.- PLC at the Golgi complex

The hypothesis of DAG production at the Golgi complex by PLC has the advantage that this DAG production can be activated by a signal cascade triggered at the Golgi complex. Since DAG is needed for vesicle budding for cargo exiting the Golgi complex, it is plausible that PLC could be activated by cargo to promote DAG formation. However, this particular role of PLC isozymes at the Golgi complex has not been widely addressed.

Our group found that incubation of U73122 led to a decrease of DAG at the Golgi and to a reduced number of particles of KDELR at the cytosol, suggesting that incubation with this PLC inhibitor was impairing retrograde transport (Fernández-Ulibarri, 2007). PLC activation by Ca^{2+} ionophores deplete the PI(4)P pool of the Golgi complex, showing that PLC can be acting at this organelle (Balla, 2005), but without resolving the PLC isozyme involved in this PI(4)P consumption.

Few PLC isozymes have been reported to be acting at or influence the Golgi complex. The localization of G protein signalling at the Golgi complex, where they can regulate vesicular transport, structure and secretion (Denker, 1996; Pimplikar and Simons, 1993; Stow, 1991) suggests that PLCβ could be acting at this organelle. In this context, a study shows that Gβγ could activate PLCβ3 at the Golgi complex and this PLC isozyme is a key component of generation of transport carriers (Diaz-Añel, 2007). Recently, as mentioned before, it has been described that PLCε could act at the Golgi complex in response to hypertrophic stimuli in mouse cardiac myocytes (Zhang, 2013).

PLCγ1 has been reported to mediate Ras activation at the Golgi complex. Activation of protein kinase receptors at the plasma membrane activates PLCγ1 in a Src dependent manner, which in turn promotes RasGRP1 translocation to the Golgi complex, where it activates H-Ras (Bivona, 2003). In this context, although PLCγ1 is controlling a Golgi event, its action is localized at the plasma membrane but not at the Golgi complex. However, direct evidences of stimulation of any PLC isozyme at the Golgi in order to generate DAG for transport carrier formation have not been described yet.

3.- SIGNALING AT THE GOLGI COMPLEX

Historically, the Golgi complex has been seen as one of the key regulators of the secretory pathway, where last post-transcriptional modification take place and cargo is sorted to its final destination. However, Golgi function may be wider than that and may be involved in more cellular processes. The Golgi complex may represent a new hub for cell signalling (Cancino and Luini, 2013), not only for its central position, but because a variety of cell-signalling molecules have been shown to be physically associated with this organelle, including G-proteins, kinases, phosphatases and phospholipases (Farhan and Rabouille, 2011; Wilson, 2010; Cancino and Luini, 2013).

This recent focus in the signalling function of the Golgi complex can be seen in the literature, in the increase in the number of papers of this field (Tillmann, 2013). In order to identify and study the relationship between the Golgi complex and signalling events, recent studies have used high throughput RNAi strategies to identify regulatory proteins involved in secretion and signalling proteins controlling Golgi structure and/or function. In these studies, 122 kinases/phosphatases were found to affect ER export and/or morphology of the Golgi (Farhan, 2010); secretion was influenced by 554 proteins (Simpson, 2012) and 159 signaling genes induced perturbations in the Golgi morphology (Chia, 2012). Signalling proteins that affected secretion or Golgi morphology were related with growth factor signalling, the Raf-MEK-ERK cascade, phosphoinositides regulation, acto-myosin dynamics and mitogen activated protein kinase signalling (Farhan, 2010; Simpson, 2012; Chia, 2012). These studies showed a strong relationship between the Golgi complex and signalling, and opened future directions in order to understand how these signals control the Golgi or the Golgi controls signalling.

Signaling cascades can regulate Golgi function and structure, and this signal can be generated at the Golgi complex itself or in other cellular locations. Moreover, the Golgi complex can be part of other transduction signals. In other words, as proposed recently, the Golgi complex can participate in the modulation of cell signalling in at least three different ways (Cancino and Luini, 2013). First, signalling might be generated at the Golgi complex itself, which will thus participate in the self-coordination of intracellular transport. Second, the Golgi complex might modulate an input generated at the plasma membrane that has an impact upon Golgi function. Finally, the Golgi complex can act as a modulator of cell signalling generated at the plasma membrane for the regulation of other cellular processes that do not specifically Golgi function or structure (Fig. 13; Cancino and Luini, 2013).

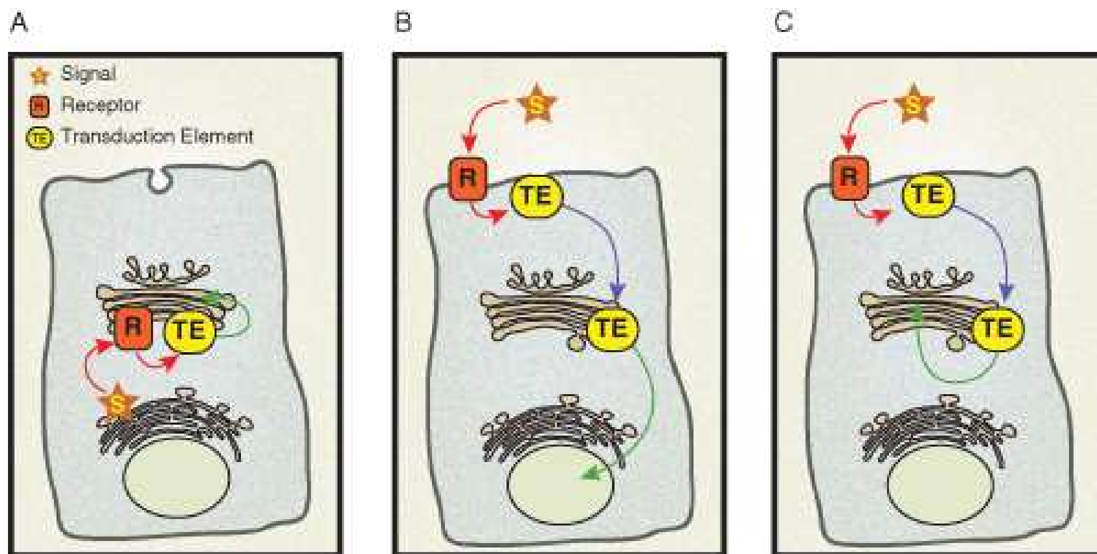

Figure 13. The Golgi complex as a signaling platform. *Golgi modulation of cell signaling: A) signaling might be generated by the Golgi complex itself, which will thus participate in the self-coordination of intracellular transport; B) cell signaling generated at the PM might be modulated at the Golgi complex, for the regulation of other cellular processes that do not specifically involve Golgi function or structure and C) the Golgi complex might modulate input initiated at the PM that has an impact upon Golgi function. (Cancino and Luini, 2013).*

One example of how the Golgi complex can act as a regulator for signalling initiated at the plasma membrane is the compartment-specific regulation of Ras (Bivona and Phillips 2003). Briefly, dimerization of protein tyrosine kinase receptors at the cell surface activates Ras on the PM through recruitment of Grb2/Sos complexes. In addition to Grb2/Sos, PLCγ and Src are also recruited and activated at the plasma membrane. PLCγ activation by Src generates the second messengers DAG and IP_3, which can then promote intracellular Ca^{2+} signaling. The Ras guanine nucleotide exchange factor (GEF) RasGRP1 responds to DAG and Ca^{2+} by translocation to the Golgi complex, where it activates H-Ras. Elevated Ca^{2+} also activates the RasGAP CAPRI, directing it to the plasma membrane, where it deactivates Ras. In this model, the Golgi complex provides selective activation of H-Ras, while plasma membrane Ras is deactivated (Bivona, 2003; Bivona and Phillips 2003).

3.1.- Control of Golgi function and structure by signalling events

As described before, multiple signalling molecules and signalling pathways are associated with Golgi function and structure. Some of them are well known to be localized at the Golgi complex and/or control the morphology and function of this organelle. Next, some of these signalling events that control the Golgi complex function and structure are described. As other cellular responses to signalling events, Golgi function and structure will be a result of the balance of all the signalling that controls it.

3.1.1.- Regulation of Golgi function by extracellular signals

The secretory function of the Golgi function is dependent on extracellular stimuli that influence the overall state of the cell. First evidences that extracellular stimuli can modulate membrane trafficking was based on the effects of the activation of IgE receptors and PKC, which was shown to regulate GTP-dependent binding of two COPI coat components, Arf and β-COP; and at the same time enhance secretory trafficking (De Matteis, 1993).

A more recent example of how secretory function can be influenced by extracellular stimuli is the regulation of PI(4)P by Sac1 in response to growth factors. Sac1 is a transmembrane phosphatase that is responsible for PI(4)P turnover at the ER and the Golgi (Foti, 2001; Schorr 2001; Rohde, 2003). In quiescent cells, Sac1 accumulates at the Golgi complex and down-regulates anterograde trafficking by depleting Golgi PI(4)P. When quiescent cells are stimulated by serum addition, the p38 mitogen-activated protein kinase (MAPK) pathway induces the COPI-mediated retrieval of Sc1 to the ER, thus releasing the break on Golgi secretory function by enabling the restoration of PI(4)P (Blagoveschenskaya, 2008).

3.1.2.- Regulation of Golgi function by a signal triggered by cargo

Golgi function and morphology can be controlled by other stimuli than extracellular ones. To prevent structural and functional disruption, the Golgi complex must regulate its high membrane fluxes. Thus, it must not only react to external stimuli as part of the integrated cellular response, but should also sense and generate a response to the passage of variable amounts of membranes and cargo. In other words, should respond to the passage of the traffic itself (Cancino and Luini, 2013). The ability of the Golgi complex to generate autonomous signalling in response to the arrival of cargo from the ER has been experimentally addressed only in the last few years (Pulvirenti, 2008; Giannotta, 2012). These studies show that cargo arrival from the ER can be sensed by a receptor in the Golgi complex, which initiates signalling pathways that can compensate the arrival of new membrane at the Golgi (Cancino and Luini, 2013).

It has been proposed that cargo arrival at the Golgi complex can be sensed by the KDELR, a seven transmembrane domain protein that cycles between the ER and Golgi complex and mediates the return of ER-resident proteins to the ER. The KDELR has structural similarities with GPCRs (Giannotta, 2012). The KDELR, upon binding to a KDEL-containing ligand, can activate G_q, and presumibily G_s, that in turn initiates a cascade that leads to the activation of a Golgi pool of the tyrosine kinase Src (Fig. 14;

Pulvirenti, 2008; Giannotta, 2012). Src activation is fundamental for Golgi function, since Src inhibition blocks intra-Golgi and post-Golgi traffic (Pulvirenti, 2008; Giannotta, 2012). One of the mechanisms by which Src can control secretion is by phosphorylation of dynamin 2, a protein required for the fission of transport carriers at the Golgi complex; which was found to be phosphorylated by Src family kinases (Weller, 2010). Moreover, Src has been also proposed to regulate Golgi structure and KDELR-dependent retrograde transport (Bard, 2003). It has been shown that after cargo arrival, Src promotes an increase of tyrosine phosphorylation at the Golgi complex, which is necessary for cargo exit from this organelle (Pulvirenti, 2008). These results are in accordance to a previous study that showed that reversible protein tyrosine phosphorylation at the *trans*-Golgi is required for albumin secretion. Moreover, the inhibition of tyrosine phosphatases led to a redistribution of *trans*-Golgi proteins (Webb, 2005), suggesting that the cycle of tyrosine phosphorylation and dephosphorylation is also required for maintenance of the *trans*-Golgi structure.

In addition to Src activation, cargo arrival at the Golgi complex also induces other signalling effetcts. Cargo arrival promotes the release of Ca^{2+} from the Golgi complex (Micaroni, 2010) and the recruitment of phospholipase A2 (PLA2) to this organelle, where is necessary for the formation inter-cisternal continuities for intra-Golgi trafficking (San Pietro, 2009). It is not known if these processes are independent or dependent of the KDELR activation, and/or if act upstream or downstream of Src or other signaling events at the Golgi complex.

3.1.3.- PKD signalling for TGN-to-plasma membrane transport carrier formation

As previously described, the generation of TGN-to-plasma membrane transport carriers depends on PKD (Bard and Malhotra, 2006). PKD activation requires production of DAG and the action of Arf1 for proper recruitment, but its activity can also be positively regulated by heterotrimeric G proteins (Saini, 2010) and inhibited by the MAPK p38δ (Sumara, 2009). Moreover, an increase of Ca^{2+} promotes the translocation of PKD at the TGN (Kunkel and Newton, 2010). Recruitment of PKD to the TGN is proposed to initiate the events that create and regulate the growth of export domains at the TGN for generation of transport carriers (Malhotra and Campelo, 2011). In the proposed model (Fig. 15), PKD phosphorylates and activates the enzyme PIKIIIβ (Hausser, 2005), thus

promoting the production of PI(4)P at the outer leaflet of the TGN, where it binds, among others, oxysterol binding protein (OSBP) and CERT (Nishikawa, 1998; Levine and Munro, 2002). CERT dependent transfer of ceramide leads to the generation of SM and DAG from PC and ceramide at the TGN (Hanada, 2009). SM and cholesterol

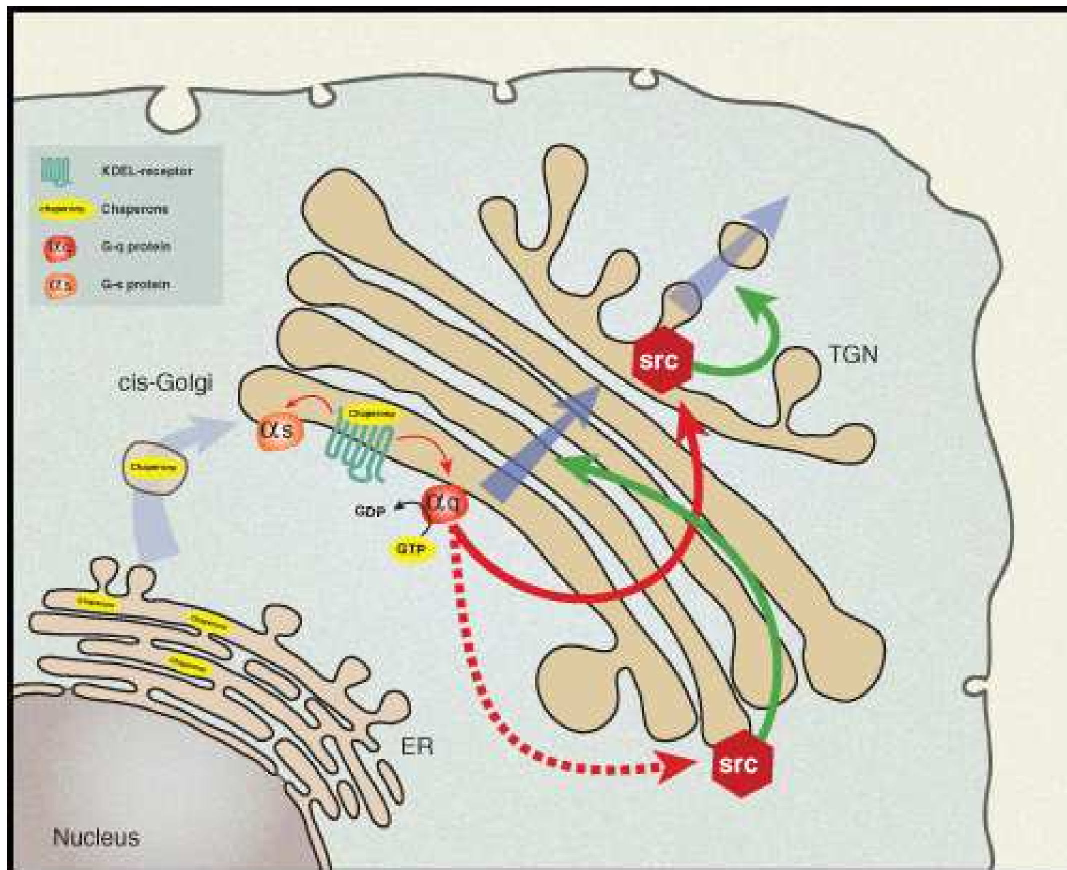

Figure 14. KDELR interacts with two major G proteins, G_q and G_s, and activates G_q at the Golgi. *When chaperones reach the Golgi complex via transport carriers, they can bind to the KDELR and return with it to the ER. Structural modeling predicts that KDELR has a three-dimensional fold that is similar to that of the GPCRs. Remarkably, the KDELR interacts with two major G proteins, G_q and G_s at the Golgi. Thus, upon binding to a KDEL-containing ligand (a chaperone), this can activate G_q (and presumably also G_s). G_q, in turn, initiates a phosphorylation cascade that leads to the activation of a Golgi pool of the tyrosine kinase Src. Through this Src activation, there is acceleration of anterograde intra-Golgi trafficking, which helps to maintain Golgi homeostasis (Cancino and Luini, 2013)*

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transferred by OSBP segregate from DAG creating non-overlapping domains at the TGN: one rich in SM and cholesterol and the other in DAG, where more PKD is recruited. This high recruitment of PKD by binding to DAG is proposed to prevent DAG from flipping across the membrane, thus concentrating DAG on the outer leaflet (Malhotra and Campelo, 2011). PKD then phosphorylates OSBP and CERT to release them from the TGN. At the same time, PKD binds Arf1, which can activate PLD to produce PA from PC, which is converted to LPA by PLA2 (San Pietro, 2009). This accumulation of different modified lipids leads to the fission of TGN-to-plasma membrane transport carriers. After fission, DAG will be consumed, thus dissociating PKD form the TGN membrane and resetting the system for another round of transport carriers in a cargo-dependent manner (Malhotra and Campelo, 2011). However, although it has been often proposed that cargo arrival could be the activator of this

PKD signalling pathway at the TGN (Bard and Malhotra, 2006; Malhotra and Campelo, 2011), there are no experimental evidences that cargo arrival is triggering PKD activation and/or DAG production at the TGN.

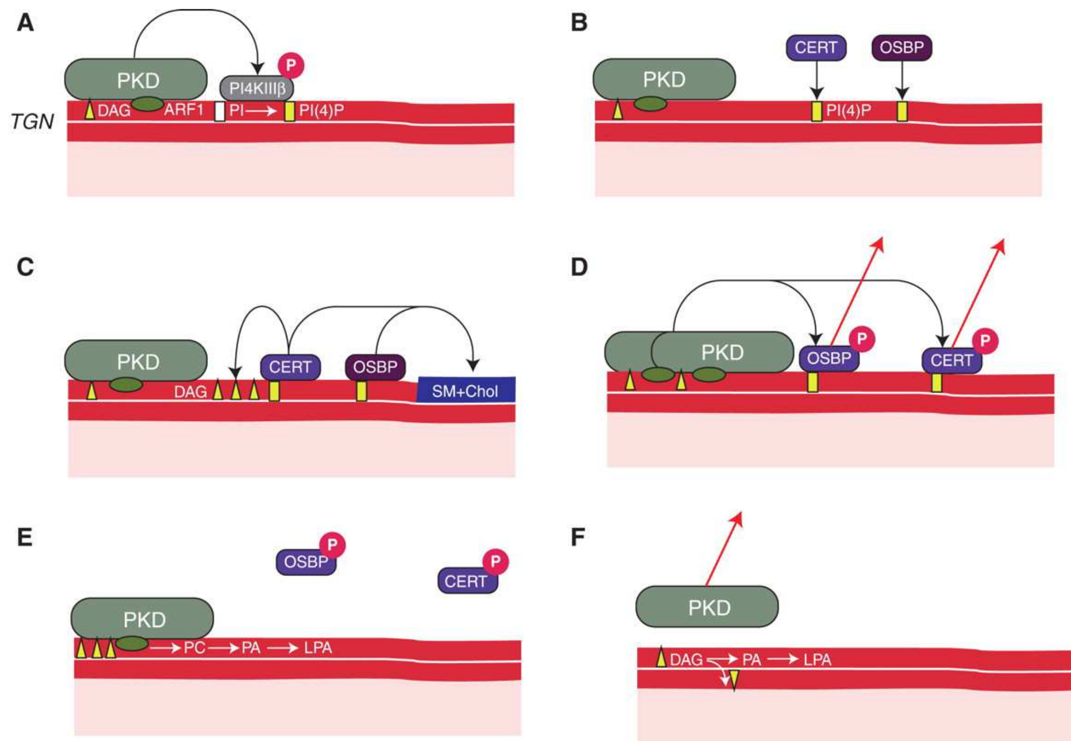

Figure 15. The effectors of PKD at the TGN. *A) PKD is recruited to the TGN by DAG and ARF1. Activated PKD phosphorylates to activate PI4KIIIß thus promoting the production of PI(4)P in the outer leaflet of the TGN. B) PI(4)P OSBP and CERT to the TGN. C) OSBP and CERT regulate the SM and sterol levels in the TGN and this is suggested to be required for separating the PI4P containing domain from the SM and sterol rich domain. CERT dependent ceramide transport to the TGN is required to generate and concentrate DAG at the TGN. D) This DAG in turn recruits more PKD to generate more DAG. PKD then phosphorylates OSBP and CERT to release them from the TGN. E) Arf1 mediated activation of PLD1 generates PA from the PC pool, which eventually is converted to LPA by the action of PLA2. The accumulation of these different modified lipids leads to fission of TGN to cell surface transport carriers. F) DAG is consumed thus dissociating PKD from the membrane thus resetting the TGN to generate another round of transport carriers in a cargo-dependent manner (Malhotra and Campelo, 2011).*

3.1.4.- Heterotrimeric G proteins signalling at the Golgi complex

The heterotrimeric G proteins are activated by the GPCRs and are made up of α and βγ subunits (Oldham and Hamm, 2008). Heterotrimeric G protein signals occur on the Golgi complex, where it has been shown to regulate its structure (Jamora, 1997; Saini, 2010) and secretion (Irannejad and Wedegaertner 2010; Saini, 2010). Heterotrimeric G proteins can translocate from the plasma membrane to the Golgi complex (Saini, 2010) or can be located in this organelle. In particular, $G\alpha_s$, $G\alpha_{i3}$, $G\alpha_{q/11}$ have been shown to

be located at the Golgi complex (Denker, 1996; Stow, 1991; Stow and Heimann, 1998), where they can control vesicle formation and secretion (Stow, 1991; Stow and Heimann, 1998). On the other hand, Golgi vesiculation occurs through the activity of the free βγ subunits (Jamora, 1997), which interact with PKD (Jamora, 1999, Díaz-Añel and Malhotra, 2005) after its translocation from the plasma membrane (Saini, 2010). $G\alpha_{q/11}$ has been proposed to participate in the signalling cascade initiated by cargo arrival at the Golgi complex and to be necessary for Src activation and regulation of anterograde transport (Giannotta, 2012).

3.1.5.- Protein kinase A and the Golgi complex

Protein kinase A (PKA) is a Ser/Thr kinase activated by the second messenger cAMP (Edelman, 1987). First evidences for the requirement of PKA for Golgi function were shown by pharmacological inhibition of PKA, which altered intra-Golgi trafficking and blocked the transport of VSVG from the TGN to the plasma membrane (Muñiz, 1996, Muñiz 1997). This inhibition of PKA altered secretion at the TGN level, where it blocked vesicle budding (Muñiz, 1997). PKA was also found to be required for retrograde transport, since PKA-mediated phosphorylation of the KDELR is needed for its retrieval from the Golgi complex to the ER (Cabrera, 2003). The mechanisms through which PKA exerts these effects on the secretory pathway have been identified in part. All components of the cAMP-PKA pathway have been located at the secretory pathway, and particularly on the Golgi complex (Cancino, 2013). However, Golgi-resident PKA can respond to extracellular signals to regulate Golgi structure and function (Mavillard, 2010). Moreover, it has been shown that the interaction between PKA and Golgi membranes is sensitive to modulators of heterotrimeric G proteins (Martin, 1999).

3.1.6.- Ca^{2+} and the Golgi complex

Ca^{2+} is the most ubiquitous second messenger in vertebrates, and even slight variations in its levels can greatly affect cell behaviour. The extracellular Ca^{2+} is usually in the low millimolar range, while the concentration of resting free cytosolic Ca^{2+} is in the order of 100 nM. According to the classical model, activation of some signalling pathways at the plasma membrane that produce IP_3 induce the release of Ca^{2+} from the ER, the main Ca^{2+} store of the cell (Micaroni, 2012). More recently, the Golgi complex has also been considered as a part of the intracellular Ca^{2+} response that can be triggered by extracellular stimuli (Pinton, 1998). Ca^{2+} release from the Golgi complex, as well from the ER, is mediated by activation of the IP_3 receptors (IP3Rs); while Golgi Ca^{2+} uptake involves two classes of Ca^{2+}-ATPase pumps, the sarcoplasmic and ER Ca^{2+} ATPase (SERCA), present in the ER and the *cis*-Golgi, and the Golgi

specific secretory pathway Ca^{2+}-ATPase (SPCA) (Missiaen, 2007; Micaroni, 2012). Ca^{2+} can be monitored by the Ca^{2+}-receptor (CaR), a GPCR that is coupled to various heterotrimeric G proteins (Ward, 2004). CaR is also present at the TGN, where it can sense the luminal concentration of Ca^{2+} of the Golgi complex and regulate Ca^{2+} uptake into this organelle by acting in concert with PLCγ1 and SPCA (Tu, 2007).

Ca^{2+} also participates in the control of membrane trafficking. After cargo arrival at the Golgi complex, the cytosolic concentration of Ca^{2+} is increased, and this temporary fluctuation of Ca^{2+} was shown to be necessary to allow the intra-Golgi trafficking of cargo and this Ca^{2+} was found to come within the Golgi complex (Micaroni, 2010). It has been proposed that the increase of cytosolic Ca^{2+} around the Golgi can affect the activities of Golgi-localized Ca^{2+}-dependant proteins and/or result in the recruitment of cytosolic signalling proteins that are involved in the initiation of signalling cascades similar to those seen at the plasma membrane (Micaroni, 2012). In accordance to this hypothesis, Ca^{2+} may allow intra-Golgi transport by activating cPLA2, a cytoplasmic phospholipase that requires Ca^{2+}, which is recruited to the Golgi complex after cargo arrival and is involved in the formation of intra-cisternal tubules for intra-Golgi transport (San Pietro, 2009).

Ca^{2+} requirement for membrane trafficking was demonstrated by the use of the Ca^{2+} chelator BAPTA, which impaired VSVG transport from the ERGIC to the Golgi complex and from the Golgi complex to the plasma membrane; and also promoted the detachment of the coatomer from membranes (Chen, 2002). Moreover, Ca^{2+} signalling from the plasma membrane promotes the increase of DAG at the Golgi complex and the recruitment of PKD to this organelle (Kunkel and Newton, 2010), steps needed for the transport of TGN-to-plasma membrane transport carriers.

II. AIMS

Previous studies have demonstrated that DAG is essential for protein transport at the Golgi complex in both yeast (Kearns, 1997) and mammals (Baron and Malhotra, 2002). It has been proposed that DAG may be playing a dual role in the generation of TGN-to-plasma membrane transport carriers. First, DAG acts as a signalling molecule that recruits PKD at the TGN (Baron and Malhotra, 2002), a kinase needed for TGN-to-plasma membrane transport carrier formation (Liljedahl, 2001). Second, the structure of DAG promotes the negative curvature needed for membrane fission (Semesh, 2003).

Our group demonstrated that DAG is also required for the generation of COPI vesicles at the fission step (Fernández-Ulibarri, 2007). Another study confirmed our results, but suggested that DAG may be participating in the budding of these transport carriers (Asp, 2009). These two studies were based on pharmacological inhibition of the formation of DAG by PAPs using propanolol (Fernández-Ulibarri, 2007; Asp, 2009). Other studies confirmed the requirement of DAG for proper Golgi function also by pharmacological inhibition of other metabolic pathways that produce DAG, as the SMS (Baron and Malhotra, 2002; Subathra, 2011) or PLC (Díaz-Añel, 2007). Silencing studies showed that the DAG-producing enzymes SMS1 and SMS2 are involved in anterograde trafficking at the Golgi complex (Subathra, 2011) and that LPP3 participates in the retrograde transport (Gutiérrez-Martínez, 2013).

However, the role of DAG-consuming pathways in the regulation of transport carrier formation at the Golgi complex remains unknown. PC and PI synthesis, which consume DAG, could be taking place at the Golgi complex, since their key enzymes are also localised at this organelle or associated with it (Jelsema and Morré, 1978; Hennerberry, 2002; Kim, 2011). In yeast, PC synthesis by the CDP-choline pathway is highly associated with Golgi secretory function (Cleves, 1991; McGee, 1994). In mammals, Nir2, a PITP protein that regulates the rate of the CDP-choline pathway at the Golgi complex, is also required for maintenance of the Golgi-associated DAG pool required for protein transport (Litvak, 2005).

On the other hand, neither of all the studies that show the requirement of DAG for Golgi function demonstrates which specific metabolic pathway is activated to produce DAG for the generation of transport carriers. All these studies show that the reduction of the Golgi-associated DAG pool, either by pharmacological agents or siRNA, leads to an impairment of transport carrier formation at the Golgi complex (Baron and Malhotra, 2002; Litvak, 2005; Díaz-Añel, 2007; Fernández-Ulibarri, 2007; Asp, 2009; Subathra, 2011). However, these works do not demonstrate that the inhibited metabolic pathways may be regulated in response to any stimulus and the effects described may derive

from an overall imbalance in DAG metabolism on Golgi membranes (Bard and Malhotra, 2005).

The potential stimulus that could trigger DAG production at the Golgi complex for the formation of transport carriers is the arrival of cargo. The main function of transport carriers is to transport cargo from one organelle to the other, thus it is plausible that cargo stimulates DAG production for transport carrier formation. Moreover, it has been recently described that the arrival of cargo at the Golgi complex triggers a signalling circuit that coordinates the secretory pathway (Pulvirenti, 2008). Cargo arrival at the Golgi complex activates G proteins (Giannotta, 2012), stimulates Src activity and increases the phospho-tyrosine levels at this organelle (Pulvirenti, 2008). Moreover, it also induces the release of Ca^{2+} from the Golgi complex, increasing the cytosolic Ca^{2+} concentration around this organelle (Micaroni, 2010). These events triggered by cargo arrival present the Golgi complex as a perfect site for PLC activation, a signalling protein that produces DAG. PLCβ and PLCγ isoforms have been related to the Golgi complex previously (Díaz-Añel, 2007; Bivona, 2003; Tu, 2007) All PLCs require Ca^{2+}, PLCβ isoforms respond to G protein signalling (Gresset, 2012; Khadamur and Ross, 2013) and PLCγ isoforms are activated by phosphorylation in one of their tyrosines (Gresset, 2010).

With all this background, the aims of this work are:

AIM 1.- To determine the implication of PC and PI synthesis in the regulation of diacylglycerol required for membrane trafficking at the Golgi complex.

1.1.- To design experimental approaches that, by tuning PC and PI synthesis, regulate the amount of DAG at the Golgi complex.

1.2.- To analyse membrane trafficking at the Golgi complex in conditions where DAG is altered by the regulation of PC and PI synthesis.

AIM 2.- To study the involvement of PLC in DAG production after cargo arrival at the Golgi complex.

2.1.- To determine which PLC isoform acts at the Golgi complex.

2.2.- To analyse DAG production at the Golgi complex after cargo arrival at this organelle and the involvement of PLC in this process.

III. MATERIALS AND METHODS

1.- MATERIALS

1.1- Reagents

In this table are found the different reagents used, a brief description of them and their use, their concentration of use and source.

Reagent	Description	Concentration of use	Source
Cicloheximide	Antibiotic that inhibits protein synthesis	100 μg/mL	Sigma-Aldrich (St. Louis, USA)
EGF (Epidermal Growth Factor)	Small mitogenic polypeptide which is present in many mammalian species. Used as an activator of PLCγ1	100 ng/mL	Invitrogen (Carlsbad, USA)
Phalloidin-TRITC	Fluorescent phallotoxin which may be used to identify filamentous actin .	0,5 μg/mL	Sigma-Aldrich
Phospholipase C, Phosphatidylinositol-specific, from *B.cereus.*	Recombinant phospholipase C	0,3 U/ml	Sigma-Aldrich
PMA (Phorbol 12-myristate 13-acetate)	Phorbol ester analogue of DAG.	100 nM	Sigma-Aldrich
Propanolol	Inhibitor of the lipid phosphate phosphatase (PAP/LPP)	60 μM	Calbiochem
STxBKDEL	Bacterial toxin that is internalized and travels from the plasma membrane to the ER. It is used as a model of retrograde transport	0,5 μg/mL	Dr. Ludger Johannes (Institute Marie Curie, Paris, France)
U73122	Inhibitor of agonist-induced phospholipase C activation in human platelets and neutrophils.	6 μM	Calbiochem (San Diego, USA)
U73343	A cell-permeable analog of U-73122 that acts as a very weak inhibitor of phospholipase C, used as a negative control.	6 μM	Calbiochem

1.2.- Antibodies

In the following tables are found the primary and secondary antibodies used for immunofluorescence (IF) and Western Blot (WB), showing their host specie, working dilution, concentration of use, if available (N.A. stands for not available) and source.

Primary antibody	Host specie	Working Dilution		Concentration of use (µg/mL)		Source
		IF	WB	IF	WB	
GFP	Rabbit	-	1:25000	-	0,008	Molecular Probes, Invitrogen
Actin	Rabbit	-	1:20000	-	0,02	
Calnexin	Mouse	-	1:1000	-		BD
Flag	Rabbit	-	1:1000	-	2	Sigma
GM130	Mouse	1:1000	1:10000	0,25	0,025	Transduction Laboratories (Lexington, KY, USA)
Golgin97	Mouse	1:500	-	0,002	-	Molecular Probes (Invitrogen)
KDELR	Rabbit	1:500	-	N.A.	-	Dr. H.D. Söling (University of Göttingen)
PentaHis	Mouse	1:200	1.2000	0,005	0,0005	Qiagen
PLCγ1	Rabbit	1:100	1:2000	N.A.	N.A.	Cell Signaling
PLCγ2	Rabbit	-	1:2000	-	N.A.	Cell Signaling
pPLCγ1	Rabbit	1:100	1:2000	N.A.	N.A.	Cell Signaling
RhoGDI	Rabbit	-	1:1000	-	0,2	Santa Cruz (Delaware, CA, USA)
TGN46	Sheep	1:500	-	0,5	-	Serotech (Oxford, UK)
VSVG Ectodomain	Mouse	1:500	-	N.A	-	Sigma-Aldrich

Secondary antibody	Working Dilution	Concentration of use (µg/mL)	Source
Alexa Fluor 488 Anti-Mouse	1:400	0,005	Invitrogen
Alexa Fluor 488 Anti-Rabbit	1:400	0,005	Invitrogen
Alexa Fluor 488 Anti-Sheep	1:400	0,005	Invitrogen
Alexa Fluor 546 Anti-Sheep	1:400	0,005	Invitrogen
Alexa Fluor 647 Anti-Mouse	1:250	0,008	Invitrogen
Cy3 Anti-Mouse	1:250	0,008	Jackson Laboratoties
Cy3 Anti-Rabbit	1:250	0,008	Jackson Laboratories
HRP Anti-Mouse	1:3000	0,0003	Promega (Eugene, USA)
HRP Anti-Rabbit	1:3000	0,0003	Promega

1.3.- Plasmids

In this table are described the plasmids used, together with their vectors of expression and its source.

Plasmid	Vector	Source
C1-PKCθ-GFP	pEGFP	Dr. I. Mérida (Consejo Superior de Investigaciones Científicas, Madrid)
FLAG-ssHRP	pEGFP	Dr. V. Malhotra (Centre de Regulació Genòmica, Barcelona)
PH-OSBP-GFP	pEGFP	Dr. T. Levine (University College London, London, UK)
PLCγ1 wt full length	pTriEx4	Dr. M. Katan
PLCγ1(H335Q) full length	pTriEx4	Dr. M. Katan
PLCγ2 (H327Q)(2-1198)	pTriEx4	Dr. M. Katan
PLCγ2 wt (2-1198)	pTriEx4	Dr. M. Katan
ts045VSVG-cherry	pEGFP	Dr. E.L. Snapp (Albert Einstein College of Medicine, NY, USA)
ts045VSVG-YFP	pEGFP	Dr. K. Simons (Max Planck Instiutute, Dresden, Germany)

1.4.- Radiolabelled molecules

In this table we find the radiolabeled molecules used and its specific activity. All of them were purchased from Perkinelmer Life Sciences.

Molecule	Isotope	Specific activity (mCi/mmol)
Choline chloride	^{3}H	50-62
Myo-inositol	^{3}H	10-25
Acetate	^{14}C	45-62
ATP	^{32}P	10
Ortho-phosphoric acid	^{32}P	850-912
EasyTag™ EXPRESS Protein Labeling Mix	^{35}S	1000

2.- METHODS

2.1.- Cell lines and cell culture

Chinese hamster ovary cells wild type (CHO-K1) and with a termosensitive CT (CHO-MT58) were grown at 33 ºC in F-12 media, while NRK, Vero, HeLa and HeLa

constitutively expressing VSVG-GFP were grown at 37 ºC in Dulbecco's modified medium nutrient mixture (DMEM) at 37 ºC. All of them were supplemented with 10 % inactivated foetal bovine serum, penicillin (100 U/ml), streptavidin (100 mg/ml), sodic pyruvate (100 mM) and L-glutamine (10 mM). Both F-12 and DMEM were from Gibco. All cells were grown in a humidified incubator at 5 % CO_2.

2.2.-Protein analysis, silencing and overexpression

2.2.1.- Cellular extracts and Western Blot

Cellular extracts were obtained from confluent cell culture dishes washed once with phosphate buffered saline (PBS; 137 mM NaCl, 2,7 mM KCl, 10 mM Na2HPO4 • 2H2O, 10 mM KH2PO4, pH 7.4) before addition of cold lysis buffer (50 mM Tris-HCl, 150 mM $NaCl_2$, 2mM EDTA, 50 mM NaF, 1% NP-40; pH 7,4) in the presence of protease inhibitors (1 µg/mL aprotinin, 1 µg/ mLleupeptin, 5µM pepstatin A, 2 mM sodium orthovanadate). Cells were scrapped, frozen, thawed and centrifuged at 1000 *g* for 10 minutes, and supernatant was collected and quantified for protein concentration by the Lowry method (Lowry, 1951) using the DC protein assay kit from Bio-Rad.

Western blot analysis was performed following a standard protocol. Protein were denatured in loading buffer 5x (62,5 mM Tris-HCl (pH 6,8), 2% (w/v) SDS, 10% glycerol, 140 mM β-mercaptoethanol and 0,1% (w/v) bromophenol blue) and heated at 100 ºC for 5 minutes. Electrophoresis was performed in denaturing polyacrylamide gels (SDS-PAGE) at different polyacrylamide concentrations at 35 mA. Proteins were then transferred to a nitrocellulose membrane (Whatman Maidstone, Kent, United Kingdom) during 1,5 hours at 100 V at 4 ºC. Nitrocellulose membranes were blocked in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7,4) containing 1 % Tween-20 (TBS-T) solution with the addition of 5% skimmed milk or 5% bovine albumin serum (BSA) for 1 hour at room temperature. After 3 washes of 10 minutes in TBS-T, membranes were blotted overnight at 4 ºC in the presence of the primary antibody diluted in TBS 1% BSA 0,002% sodium azide. After primary antibody incubation, membranes were washed 3 times for 10 minutes with TBS-T and incubated for 1 hour at room temperature with the appropriated secondary antibody in TBS-T with 2,5% skimmed milk or 2,5% BSA. Membranes were washed again three times for 10 minutes with TBS-Tto remove secondary antibody remains and the reaction, and they were incubated for 1 minute with the Western Blotting Luminal Reagent (Santa Cruz Biotechnology, Santa Cruz, USA) to finally be visualized by exposition on Hyperfilm (Amersham Pharmacia Biotec, Uppsala, Sweden). Actin, tubulin and RhoGDI

antibodies were used as loading controls. The film was scanned and the intensity of the bands was quantified using the GelPro analyser software version 4.0.

2.2.2.- Immunoprecipitation

Cellular extracts were obtained as described previously and 500 µg of protein in volume of 300 µL of lysis buffer were added to 50 µl of protein A sepharose beads (Santa Cruz) and 6 µL of PLCγ1 antibody and incubated overnight at 4 ºC on a rotary mixer. The next day, beads were centrifuged and washed with $RIPLA_2$ buffer (20 mM Tris, 137 mM NaCl, 10 % glycerol, 0,05% SDS, 0,5% Triton X-100, 0,1% sodium deoxycholate, 2 mM EDTA; pH 7,5) three times. 20 µL of loading buffer were added to the final immune complexes and were denatured boiling them for 10 minutes. Samples were then processed for western blot as previously described.

2.2.3.- DNA transfection

Plasmids were transfected using FuGene HD (Promega) for morphological analysis, as immunofluorescence and protein localization; or Lipofectamine (Invitrogene) for biochemical studies; as ssHRP secretion. For FuGene HD mediated transfection, the plasmid mixtures contained 1 µg of DNA per 1µL of FuGene HD, mixed in 100 µL of OptiMEM (Gibco). Lipofectamine transfection was prepared by mixing 2 µg of DNA and 3µL of Lipofictamine in 100 µL of OptiMEM and incubated for 20 min at room temperature. Plasmid mixtures were applied in cells in suspension, and then cells were plated onto glass coverslips or cell culture plates. If not specified, overexpression experiments lasted 12-16 hours of expression.

2.2.4.- siRNA transfection

20nM small interference RNA (siRNA) were transfected in cells in suspension using Lipofectamine, following manufacturer's instructions. In brief, siRNA mixtures were prepared by adding 100 pmols siRNA and 5µL of Lipofectamine in 100 µL of OptiMEM and incubated for 20 min at room temperature. Then, the siRNA mixture was added to a p6 well containg cells in suspension in 2 mL of DMEM. After 5-6 hours, media containing the siRNA mixture was washed with DMEM and cells were grown overnight. After 24 hour of transfection, cells were subcultured and grown in coverslips or cell culture plates. Experiments were performed 72 hours after siRNA transfection. siRNA pools for PLCγ1, PLCγ2 and *non-targeting* siRNA (siRNA control) were purchased from Dharmacon. The sequences of siRNA pools for PLCγ1 and PLCγ2 are found in the following table:

	PLCγ1	PLCγ2
Sequence from 5' to 3'	CCAACCAGCUUAAGAGGAA	GCAGAUGAAUCACGCAUUG
	GAAGUGAACAUGUGGAUCA	CCAAGUUCCUUAAAGAUAA
	GAGCAGUGCCUUUGAAGAA	GGAAAUAGGAGCACACAAA
	CCAAGAAGGACUCGGGUCA	GGGAAGAACUCCAAAGAUU

2.3.- Isolation of Golgi-enriched Fractions

2.3.1.- Preparation of Golgi-enriched fractions from Vero cells

Golgi fractions from Vero cells were prepared at 4 °C following a modification of a method reported previously (Balch, 1984). Cells were harvested, washed twice in PBS and twice in homogenization buffer (250 mM sucrose in 10 mm Tris-HCl, pH 7.4), and pelleted at 1500 g for 10 min. Cells were then resuspended in 4 volumes of homogenization buffer and homogenized using the Ball-Balch device. The homogenate was brought to a sucrose concentration of 37% by the addition of 62% sucrose in 10 mM Tris-HCl, pH 7.4, and EDTA (1 mM final concentration). Twelve millilitres of this solution were placed at the bottom of a centrifuge tube and carefully overlaid with 15 ml of sucrose at 35% and 9 ml of 29% sucrose in 10 mm Tris-HCl (pH 7.4). Gradients were centrifuged at 100,000 × g (25000 rpm) for 2.5 h with the swing rotor SW28 on a L7–55 Beckmann ultracentrifuge. The Golgi-enriched fraction was recovered at the 35%/29% sucrose interface and subsequently frozen in liquid nitrogen and stored in aliquots at −80 °C until use.

2.3.2.- Preparation of Golgi-enriched fractions from rat liver

For preparation of Golgi-enriched fractions from rat liver, adult female Wistar rats were starved for 24 hours. After being killed, livers were removed and placed into 200 mL of cold 0,5 M phosphate buffer pH 6,7 with 0,5 M sucrose. Livers were swirled, squeezed and cut to several pieces in order to remove as much as blood as possible. Excess buffer was removed and liver pieces were minced into small pieces and homogenized and sieved by gently pressing through a 150-μm-mesh stainless sieve with the aid bottom of a conical flask in grinding action. 13 mL of this homogenate were added to a discontinuous gradient and centrifuged in an SW-28 rotor at 28.000 rpm for 1 hour at 4ºC. 2-3 mL of the Golgi fractions were collected from between the 0,5 and 0,86 M sucrose fractions using a Pasteur pipette. Golgi fractions were then diluted to 0,25 M sucrose and centrifuged at 7000 rpm for 30 min at 4ºC in the SW-28 rotor. Supernatants were discarded and pellets were resuspended in 2mL of phosphate

buffer with 0,25 M sucrose and centrifuged again at 7000 rpm for 30 min at 4ºC. Supernatants were again discarded and the final pellet was resuspended in 4,5 mL of phosphate buffer with 0,25 M sucrose and subsequently aliquoted and frozen in liquid nitrogen and stored at −80 °C until use. Golgi-enriched fractions purity was checked by Western Blot, measuring the enrichment of the Golgi marker GM130

2.4.- Morphological analysis

2.4.1.- Immunocytochemistry

For morphological analysis, cells were grown on glass coverslips and fixed with 4 % paraformaldehyde (PFA) for 15 min at room temperature. After three washes with PBS, coverslips were treated for 20 min with 50 mM NH_4Cl 50, to block PFA's free reactive aldehyde groups. Coverslips were washed again three times with PBS and permeated with 0,1% saponin 1% BSA PBS for 10 minutes at room temperature. Primary antibodies were diluted in blocking solution (PBS 1% BSA) and coverslips incubated for 1 hour at room temperature or overnight at 4 ºC. After three PBS washes, coverslips were incubated with secondary antibodies in the same blocking solution for 45 minutes at room temperature, washed again with PBS and mounted on slides with mowiol-mounting media. Images were taken with an Olympus BX60 microscope coupled to an Orca-ER CCD camera (Hammamatsu Photonics, Japan). Confocal images were taken by a Leica TCS SL confocal microscope (Leica Microsystems, Heidelberg, Germany). Images were processed and quantified with Image J 1.43 (NIH, Bethesda, MD, USA).

2.4.2.- Quantification of Golgi compactness

To quantify Golgi compactness we measured the circularity of the Golgi as described before (Bard, 2003). 8 stacks of the Golgi area were taken by confocal microscopy and the mean of the Z projection of these stacks was analysed under a threshold between 80 and 90. The perimeter and the area of Golgi particles bigger than 3 pixels were measured. The circularity of the Golgi apparatus was then computed according to the formula 4π(sum (areas)/(sum(perimeters))^2). This dimensionless measurement varies from 0 to 1. The maximum value of 1 represents compacted, circular morphologies of the Golgi complex, while numbers below 0,1 represent fragmented structures.

2.4.3.- Electron microscopy

For transmission electron microscopy, CHO-K1 and CHO-MT58 cells were rapidly fixed with 1.25% glutaraldehyde in PIPES buffer (0.1 M, pH 7.4) containing sucrose (2%) and Mg_2SO_4 (2 mM) for 60 min at 37 °C. Cells were then gently scraped, pelleted at 100 × *g* for 10 min, rinsed three times in PIPES buffer, and postfixed with 1% (w/v) OsO_4, 1% $K_3Fe(CN)_6$ in PIPES buffer for 1 h at room temperature in the dark. Cells were then treated for 5 min with tannic acid (0.1%) in PIPES buffer, rinsed in distilled water, block-stained with 1% uranyl acetate in 70% ethanol for 1 h, dehydrated with graded ethanol solutions, and finally embedded in Epon plastic resin (EMS, Hatfield, PA). Ultrathin sections (50–70 nm thick) were stained with lead citrate and observed on a JEOL 1010 electron microscope (Peabody, MA). Micrographs of randomly selected areas were obtained with a Gatan Bioscan digital camera (Pleasanton, CA) at the same final magnification (×50,000) and analysed using point-counting procedures. The minimum sample size of each stereological parameter was determined by the progressive mean technique with a confidence limit of 5%.

2.5.- Lipid analysis

2.5.1.- Determination of DAG in Lipid Extracts

For DAG determination in lipid extracts cells were grown on 60-cm^2 plates to 80% confluence, corresponding to 1,2 mg of protein, and were used as individual samples for the different conditions. After incubation, cells were placed on ice, incubation medium was removed, and 1 volume of methanol was added (2 ml for 60-cm^2 culture dishes). Cells were then scraped and collected into glass test tubes containing 0.5 volumes of chloroform. After 5 min on ice, phases were separated by adding 0.65 volumes each of chloroform and distilled water. Tubes were vigorously vortexed and centrifuged at 3000 × g. The lower organic phase was washed in water/methanol (1:1) saturated with chloroform and evaporated under a stream of N_2. To determine the total amount of DAG in the lipid extract, samples were incubated with DAGK and radiolabeled [^{32}P]ATP, to phosphorylate DAG into [^{32}P]PA, which will be measured as a product of the total DAG of the lipid extract. For this, the lipid extract of each sample or 1,2-dioleoyl-sn-glycerol standards (in a range of 250–8000 pmol) were resuspended by sonication in 20 µl of a mixture of cardiolipin 5 mM, 7.5% N-octyl glucoside and 1 mM DETAPAC. The lipid suspension was then incubated at 37 °C for 1 h in a final volume of 100 µl with the reaction buffer (100 mM imidazole-HCl, 50 mM NaCl, 12.5 mM MgCl2, 1 mM EGTA, 0.5 mM DETAPAC, and 2 mM DTT), DAG kinase from *Escherichia coli* (0.022 unit) and 1 µCi of [^{32}P]ATP (2 mM), to phosphorylate the DAG

of the sample to [^{32}P]PA. The reaction was stopped by the addition of 0.6 ml of the mixture chloroform/methanol/HCl (100:200:1). After 15 min at room temperature, 0.25 ml of chloroform and 0.25 ml of water were added to separate two phases. The tubes were then shaken vigorously and centrifuged at 3000 × *g* for 5 min. The upper aqueous phase was removed, and the lower organic phase containing ^{32}P-lipids was washed in 0.75 ml of methanol/H2O (1:1) saturated with chloroform. The washed organic phase was evaporated under a stream of N_2. To separate [^{32}P]PA from the rest of ^{32}P-lipids, the lipid pellets were resuspended in 20 µl of chloroform/methanol (4:1) and spotted onto silica gel plates (HPTLC silica gel-60 plates, 10 × 10 cm), which were developed with chloroform/acetone/methanol/acetic acid/H2O (10:4:3:2:1). The area corresponding to [^{32}P]PA was identified by comigration with standards, counted for radioactivity with a PhosphorImager (Typhon TRIO, Amersham Biosciences), and analyzed with ImageQuant software (Amersham Biosciences). Standard values were plotted as counts of [^{32}P]PA versus pmol of 1,2-dioleoyl-sn-glycerol (250–8000 pmol), and the amount of DAG in each sample was interpolated after linear regression of the data.

2.5.2.- Determination of Major Phospholipid Classes and Triacylglycerol

For determination of major phospholipid classes and triacylglycerol, cells were radiolabeled with [^{3}H]choline (0,5 µCi/ml), [^{3}H]myo-inositol (0,5µCi/ml), [^{14}C]acetate (0,25 µCi/ml), or [^{3}H]orthophosphoric acid (5 µCi/ml) for 24 h in F-12 Ham culture medium at the permissive growing temperature. Then cells were extensively washed to remove the radiolabeled precursors and incubated at different conditions. After 90 min, lipid extracts were obtained as described above. Individual lipid classes were separated by TLC, identified with authentic standards, and counted for radioactivity. [^{3}H]PC in [^{3}H]choline-labeled lipid extracts and [^{3}H]PI in myo-[^{3}H]inositol-labeled lipid extracts were separated in one-dimensional TLC with the mobile phase, chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1 by volume). Major phospholipid classes (i.e. PE, PC, SM, PI, PS and PA in ^{32}P-labeled lipid extracts were separated in two-dimensional TLC with the following mobile phases: first, chloroform/methanol/ammonia (65:35:10 by volume); second, chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1 by volume). [^{14}C]triacylglycerol in [^{14}C]acetate-labeled lipid extracts was separated by one-dimensional TLC with the mobile phase, hexane/diethyl ether/acetic acid (70:30:1 by volume). For the ^{3}H- and ^{14}C-labeled lipids, the area corresponding to authentic standards on the TLC was scraped and counted by scintillation. The ^{32}P-labeled phospholipids were counted and analysed for radioactivity as described above.

2.5.3.- Lipid metabolism assays in Golgi-enriched membrane fractions

2.5.3.1.- Measurement of CDP-choline:diacylglycerol phosphotransferase activity

CDP-choline:diacylglycerol phosphotransferase (CPT) activity was measured in the presence of CMP to force the reaction to produce DAG and CDP-choline at the expense of endogenous Golgi membrane PC, which is the reverse of the reaction that takes place under physiological conditions. Golgi membranes (50 µg of protein) isolated from Vero cells and rat liver were incubated in 100 mM Tris/HCl buffer containing 5 mM EDTA, 5 mM DTT, 10 mM MgCl2, pH 8.2, and, when indicated, 10 mM CMP for 30 min at 37ºC. Incubations were stopped by adding 1 volume of chloroform/methanol (1:2) saturated with chloroform. Then, lipid samples were processed for determination of DAG as described previously.

2.5.3.2.- [^{32}P]PA production in Golgi-enriched membrane fractions

Cytosol and PLC-dependent [^{32}P]PA production was determined in Golgi-enriched membrane fraction. 25 µg of Golgi-enriched membrane fractions from rat liver were resuspended in 10 mM Tris/Maleate, 3 mM EGTA, 4 mM $MgCl_2$ 0,62 mM Ca^{2+} (100nM of free Ca^{2+}) and 20 µM GTPγS in the presence of 1µCi of [^{32}P]ATP in a final volume of 200 µL, and incubated at 37 ºC for 30 minutes. 60 µg/ml of cytosol, 6µM U73122, 6 µM U73343 or 0,3 U/ml of recombinant PLC were added to the incubation when indicated, or Ca^{2+} and GTPγS were not supplied as indicated. Reaction was stopped with chloroform/methanol (1:2) saturated with chloroform. The lipid phase was obtained as previously described and [^{32}P]PA was separated from other lipids by one dimensional TLC with the mobile phase chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1).The [^{32}P]PA was identified by comigration with authentic standards and counted and analysed for radioactivity as previously described.

2.5.3.3.- [^{32}P]PIP metabolism in Golgi-enriched membrane fractions

For measurements of [^{32}P]PIP, 25 µg of Golgi-enriched membrane fractions from rat liver were resuspended in 10 mM Tris/Maleate, 3 mM EGTA, 4 mM $MgCl_2$ 0,62 mM Ca^{2+} and 20 µM GTPγS in the presence of 1 µCi of 0,5 µM ATP in a final volume of 200 µL and incubated at 37. After 30 minutes, membranes were washed three times to remove the remains of [^{32}P]ATP and incubated for other 30 minutes at 37 ºC in the presence of cytosol, Ca^{2+} and GTPγS, as indicated. The reactions were stopped by the addition of 0.6 ml of the mixture chloroform/methanol/HCl (100:200:1, v/v/v), and lipid extraxts were taken and processed as previously described .[^{32}P]PIP was separated from other lipids by one dimensional TLC treated with oxalate with the mobile phase

chloroform/methanol/ammonia/water (18:18:2:3,8 by volume), identified with comigration of authentic standards and counted and analysed for radioactivity as previously described.

2.5.4.- Determination of DAG and PI(4)P by its binding to lipid-sensor constructs

For the analysis of DAG and PI(4)P levels at the Golgi complex, we used the constructs C1-PKCθ-GFP and PH-OSBP-GFP, respectively (Carrasco and Mérida, 2004; Balla, 2005. The localization of these constructs at the Golgi complex is dependent on the amount of DAG and PI(4)P present at this organelle. HeLa cells grown on coverslips were transfected with cDNA of C1-PKCθ-GFP and PH-OSBP-GFP as previously described. After 12-16 hours of transfection, cells were fixed and processed for immunofluorescence and images were taken as previously described. To measure the localization of both constructs at the Golgi complex, cells were also stained for Golgin97 to define Golgi area. Localization of these constructs at the Golgi complex was expressed as the percentage of fluorescence of the construct in the Golgi area referred to the total fluorescence of the cell. To increase the resolution of DAG determination at the Golgi complex by the use of C1-PKCθ-GFP, 100 nM PMA was added to cells, in order to recruit C1-PKCθ-GFP to the plasma membrane, since it has been described that this construct also recognizes phorbol esters.

2.6.- Trafficking assays

2.6.1.- Transport of vesicular stomatitis virus G protein

For the analysis of the trafficking assays of VSVG we transfected VSVG-YFP or VSVG-cherry to CHO-K1 or we used HeLa cells that constitutively express VSVG-GFP. Cells were kept overnight at the restrictive temperature of 40ºC, where VSVG cannot fold properly and accumulates at the ER. Cicloheximide was added to the media (100 µg/ml) 30 minutes before the temperature shift to 32 ºC, in order to inhibit protein synthesis. Once at 32 ºC, the VSVG protein can fold properly and exits the ER, going through the Golgi complex and reaching the plasma membrane after 60 min. Cells were fixed at different times with 4% PFA and were processed for immunofluorescence.

To quantify VSVG arrival at the plasma membrane we stained non-permeated cells with the anti VSVG-EctoDomain antibody, that only stained the VSVG protein that has reached the plasma membrane. Images of the ectodomain fluorescence (membrane VSVG) and YFP fluorescence (total VSVG) were taken by epifluorescence microscopy.

The arrival of VSVG at the plasma membrane was represented as the ratio between ectodomain fluorescence and total YFP fluorescence of each cell.

To measure VSVG arrival at the Golgi complex we also used the endo-β-N-acetylglucosaminidase H enzyme (Endo-H, New England Biolabs, Ipswich, MA, USA,). Endo-H is a recombinant glycosidase that cuts the mannose-rich residues that proteins have when exiting the ER, but cannot act on the more complex N-glycan residues that proteins acquire at the Golgi complex. The VSVG protein that has not reached the Golgi complex is sensitive to Endo-H, but it becomes resistant to it once its glycan residues are modified at this organelle. To analyse the acquisition of resistance to Endo-H of the VSVG protein, HeLa cells that constitutively express the termosensitive GFP-VSVG protein were incubated 24 hours at 40 ºC and shifted to 32 ºC to allow the exit of VSVG of the ER. Cells were lysed at different times after the temperature shift with 0,5 % SDS and denaturalyzed at 100 ºC for 10 minutes. 20 µg of protein were treated with denaturing buffer and boiled again for 10 minutes at 100 ºC. After this step, the sample was incubated with 2µL of Endo-H enzyme at 37 ºC for 1 hour and reaction was stopped with 5 µL 5x loading buffer with 10% β-mercaptoethanol. Samples were processed for WB and GFP-VSVG was identified blotting against GFP. The VSVG band with higher electrophoretic mobility is the one sensitive to Endo-H. Arrival at the Golgi complex is represented as the ratio between the resistant VSVG and total VSVG in the sample.

2.6.2.- Secretion of a secretory form of horseradish peroxidase (ssHRP)

To study the secretion of a soluble protein we used a secretory form of horseradish peroxidase (ssHRP) as a model. CHO-K1 cells were transfected with Flag-tagged ssHRP as prieviously described and after 24 hours media was washed to start the secretion assay. CHO-K1 cells were incubated in 1 ml of saline buffer with choline 200 µM and inositol 5 mM or without them with the presence of LiCl 5 mM. 100 µL of media were collected and centrifuged for 5 minutes at 1000 *g* at indicated times. The enzymatic activity of ssHRP of the supernatants was measured with ECL detection, producing a chemical signal which was quantified with a Luminometer (Synergy 2 from Biotek). Cellular ssHRP was measured by chemiluminiscence after lysing cells with water or by Western Blot.

2.6.3.- Secretion of [^{35}S] radiolabeled soluble proteins

To study soluble protein secretion, we adapted the protocol from Cheong *et al* 2003. HeLa cells were silenced as described and grown on poli-D-lysine for 48 hours on 6-well plates. Cells were starved for 30 minutes in DMEM without cysteine and methionine and radiolabeled with 20 µCi of EasyTag™ EXPRESS^{35}S Protein Labeling Mix in 750 µL of media for 10 minutes. After that, cells were washed twice with ice-cold DMEM and kept at 18 ºC for 3 hours, in order to accumulate newly synthetized [^{35}S]proteins at the Golgi complex. Cells were washed twice with PBS 5%BSA and 1 ml of DMEM per well was added to start secretion at 37 ºC. At indicated times, 100 µl of culture media was collected, centrifuged and protein in the supernatant was precipitated with 200 µl of 20% tichloroacetic acid. Pellets were washed twice with cold acetone, and left overnight with 500 µl NaOH 1 M to resuspend pellets. 500 µl of HCl 1 M were added to neutralize the solution before being quantified by scintillation counting. To determine total incorporation of [^{35}S]Met/Cys into cellular proteins, cells were lysed with 0.1 N NaOH in 0.1% sodium deoxycholate and quantified by scintillation counting.

2.6.4.- STxBKDELr internalization

To study retrograde transport we used the B fragment of the toxin of *Shigella* tagged with the KDEL sequence (STxB-KDEL). The KDEL sequence promotes the retention of this toxin in the ER, so we can monitor the transport of this toxin from the plasma membrane to the ER through the Golgi complex. For this purpose, HeLa cells grown on coverslips were washed with PBS and incubated without FBS for 30 minutes at 37 ºC. After this, 0,5 µg/mL STxB-KDEL was added to the media and cells were kept at 4ºC for 1 hour, allowing the binding of the toxin to globotriaosylceramide. The next step was to wash cells with DMEM in order to remove the remaining unbound toxin. Cells were shifted to 37ºC to induce its internalization to the Golgi complex and finally to the ER. Cells were fixed at indicated times with PFA 4% and processed for immunofluorescence.

IV. RESULTS

1.- Role of phospholipid synthesis in the regulation of DAG required for membrane trafficking at the Golgi complex

1.1.- Cellular content of DAG can be controlled by PC and PI synthesis

To study if phospholipid synthesis can control the formation of DAG at the Golgi complex and regulate trafficking at this organelle, we designed two strategies to inhibit phospholipid synthesis for a short time. The first one was using the mutant cell line CHO-MT58, which its CCT can not be folded properly at 40 ºC (Esko,1981). CCT is the rate limiting enzyme of the CDP-choline pathway, so when we shift CHO-MT58 cells to the non-permissive temperature, the cell's main pathway for PC synthesis is inhibited. The second strategy that we have set up to inhibit phospholipid synthesis was providing or not choline and inositol, essential precursors of PC and PI, respectively. Since regular growth media contain both choline and inositol, we used a saline buffer in these assays. As a control we added 200 μM choline chloride and 5 mM myo-inositol to the saline buffer. To inhibit phospholipid synthesis, we were not supplying choline and inositol and added 5 mM lithium to inhibit the recycling of inositol phosphates and deplete endogenous inositol. We expected that after a short time inhibition of phospholipid synthesis, DAG levels were increased in both experimental strategies.

DAG levels in CHO-MT58 were increased after shifting the cells to 40 ºC. This increase was already seen at 30 minutes, reached a maximum at 60 minutes and remained constant for at least 3 hours (Fig. 16B). As a control we used the wild-type cell line CHO-K1, where PC synthesis should not be compromised when shifted to 40 ºC. CHO-K1 DAG levels remained constant all the time that cells were at 40 ºC, being lower than those of CHO-MT58 from 30 minutes on (Fig. 16B). On the other hand, we observed that CHO-K1 and NRK cells incubated without choline and inositol for one hour had higher amount of DAG than those with choline and inositol (Fig. 16C). These results demonstrate that short time inhibition of phospholipid synthesis by our two different strategies increased cellular DAG.

1.2.- Inhibition of PC and PI synthesis for a short time does not affect PC and PI, other major phospholipids or TAG levels

To study the effects of DAG changes produced by inhibition of phospholipid synthesis, we first wanted to confirm that DAG was the only membrane lipid significantly altered in our experimental conditions. Since we are inhibiting PC and PI synthesis, a decrease of the amount of these phospholipids, and a possible increase of other lipids synthesised from DAG, such as TAG, could be expected. For this purpose, we

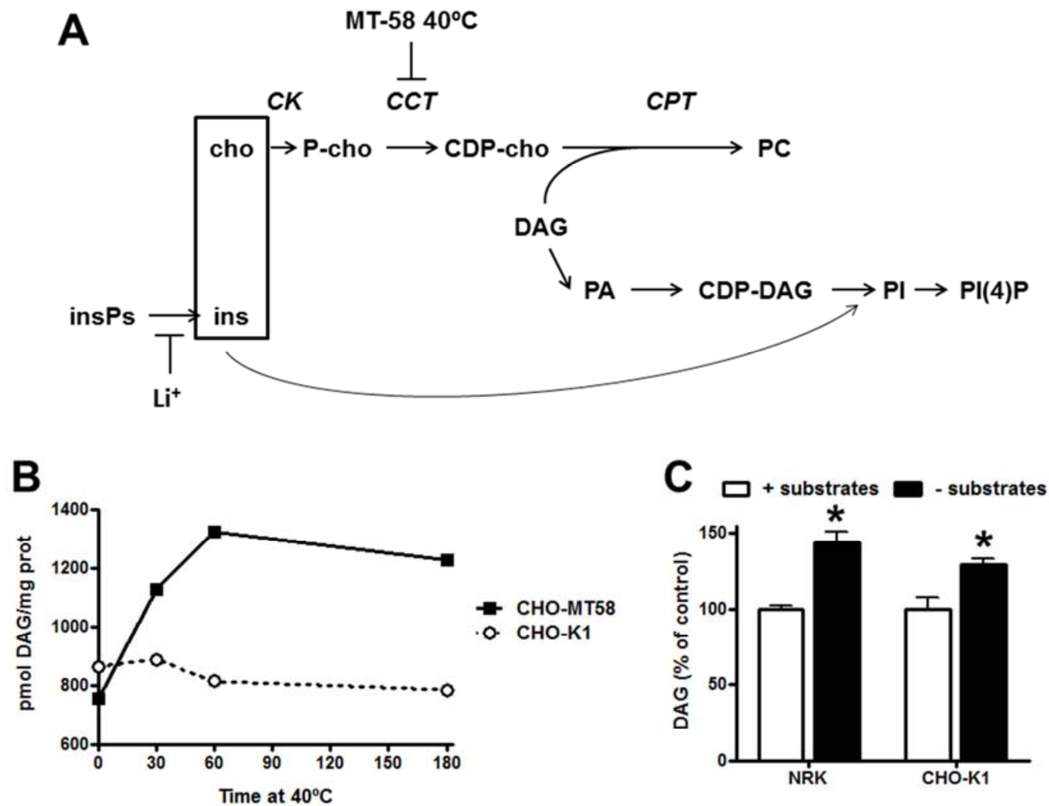

***Figure 16. The cellular content of DAG can be regulated by PC and PI synthetic pathways**. A, enzyme-catalyzed reactions for the synthesis of PC and PI from DAG and choline and inositol. B, CHO-K1 and CHO-MT58 cells were grown at 33 °C and then shifted to 40 °C for the indicated times, lipids extracted and DAG mass determined. Results are from one experiment representative of three experiments. C, CHO-K1 and NRK cells were incubated for 1 h in saline buffer with 200 µM choline and 5 mM inositol (white bars) or without these substrates in the presence of 5 mM LiCl (black bars). Lipids were extracted and DAG mass determined. Results are mean ± S.D. of three experiments performed in duplicate.*

incubated the cells with different radioactive tracers for 24 hours until the isotopic equilibrium, where the amount of radioactive labelling is proportional to the total mass of the molecule. We labelled CHO-K1 and CHO-MT58 cells with [^{32}P]orthophosphoric acid, [^{3}H]choline, [^{3}H]inositol or [^{14}C]acetate for 24 hours, then washed out the radioactive tracer and incubated them for one hour with our experimental conditions to inhibit PC and PI synthesis. We found that the relative amount of [^{32}P]PC was unaltered by any of the experimental conditions (Fig. 17A, 17B). [^{3}H]PC labelled with [^{3}H]choline, which is incorporated to PC through the CDP-choline pathway, was not altered after inhibiting phospholipid synthesis by the absence of choline and inositol (Fig. 17C). The level of [^{3}H]PC in CHO-MT58 cells at the permissive temperature was lower than in CHO-K1 (Fig. 17D), as previously described (Caviglia, 2004). However, during one hour at the non-permissive temperature, the level of [^{3}H]PC of CHO-MT58

remained stable, as it happened in CHO-K1 cells. These results confirmed that none of our strategies, which inhibit PC synthesis for a short time, changed significantly the total amount of PC.

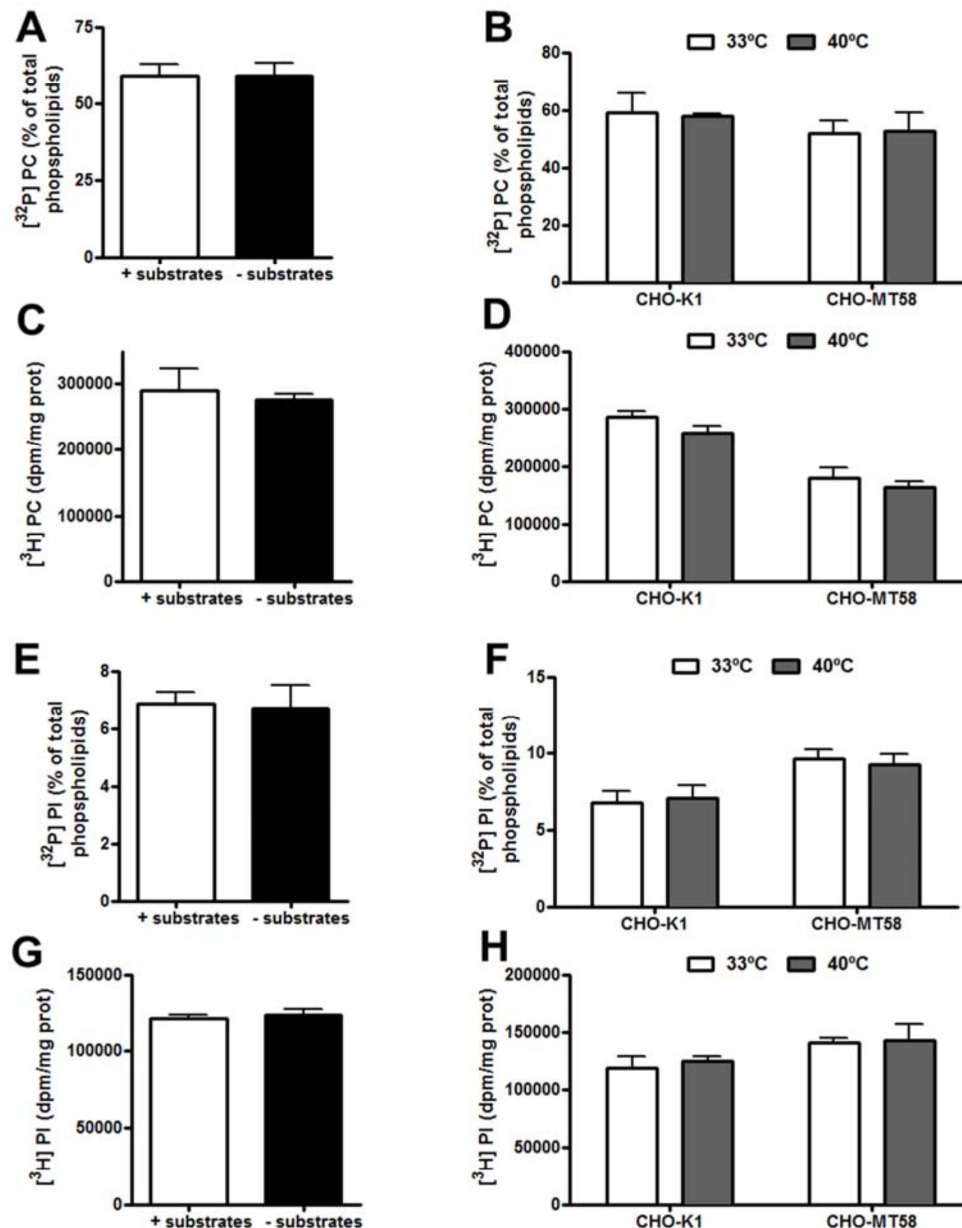

Figure 17. Modulation of PC and PI synthesis for a short time does not affect the cellular levels of PC or PI. *CHO-K1 and CHO-MT58 cells were labeled with [^{32}P]orthophosphoric acid (A, B, E, F), [^{3}H]choline (C, D) or [^{3}H]inositol (G, H) for 24 h. Cells were then washed and incubated for 1 h in saline buffer with 200 µM choline and 5 mM inositol (white bars), without these substrates in the presence of 5 mM LiCl (black bars) (A, C, E, F), or with growth medium (F12) at the temperature indicated (B, D, F, H). Lipids were extracted, separated by TLC, and counted for radioactivity. Results are mean ± S.D. of two experiments performed in duplicate (A, B, E, F) or triplicate (C, D, G, H)*

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[^{32}P]PI levels were not altered by any of our strategies used to inhibit phospholipid synthesis (Fig. 17E). The percentage of [^{32}P]PI was slightly higher in CHO-MT-58 than in CHO-K1 cells at the permissive temperature, but in both cell lines [^{32}P]PI levels remained unaltered after shifting to the non-permissive temperature (Fig. 17F). We also measured PI levels by labelling with [^{3}H]inositol and found that in both experimental conditions, [^{3}H]PI levels remained unaltered (Figs. 17G, 17H). These results showed that while DAG levels are increasing when we are inhibiting phospholipid synthesis for a short time, the total amount of PI is not significantly modified.

Once we checked that short inhibition of PI and PC synthesis was not lowering these phospholipids, we wanted to check if other major phospholipid classes were altered or not. Following the same protocol described before, we analysed the amount of SM, PS, PE and PA. None of them were altered in both strategies used to inhibit phospholipid synthesis (Figs. 18A, 18C).

Since DAG is a precursor of TAG synthesis, we also assessed if the amount of TAG was altered. For this purpose we labelled CHO-K1 and CHO-MT58 cells with [^{14}C]acetate and measured [^{14}C]TAG . The amount of [^{14}C]TAG was not altered in any of the conditions used to inhibit phospholipid synthesis (Figs 18B, 18D). CHO-MT58 had lower [^{14}C]TAG than CHO-K1 at 32 ºC, but their amount remained similar after PC synthesis inhibition at 40 ºC (Fig. 18D).

Altogether, these results showed that we have set up two different experimental conditions based in phospholipid synthesis inhibition for a short time, in which only cellular DAG levels increase, while other major membrane lipids remained unaltered. Then, the possible effects that we could see on the cell could be attributed to changes in DAG levels, rather than changes in other membrane lipids.

1.3.- Phospholipid synthesis controls DAG levels at the Golgi complex

Our next step was to check if the increase in DAG due to the inhibition of phospholipid synthesis for a short time could be taking place at the Golgi complex. For this purpose we wanted to confirm that DAG consuming reactions of phospholipid synthesis could take place in Golgi membranes. We isolated Golgi enriched fractions from Vero cells and rat liver and incubated them with or without 10mM CMP. CMP is one of the products of the last step of PC synthesis, a reaction catalysed by CPT that consumes DAG. CMP is also produced in the last reaction of PI synthesis, a reaction catalysed by the PIS. The addition of an excess of CMP to a Golgi membrane preparation will revert these PC and PI synthesis reactions if they are localized in the Golgi complex, and as a

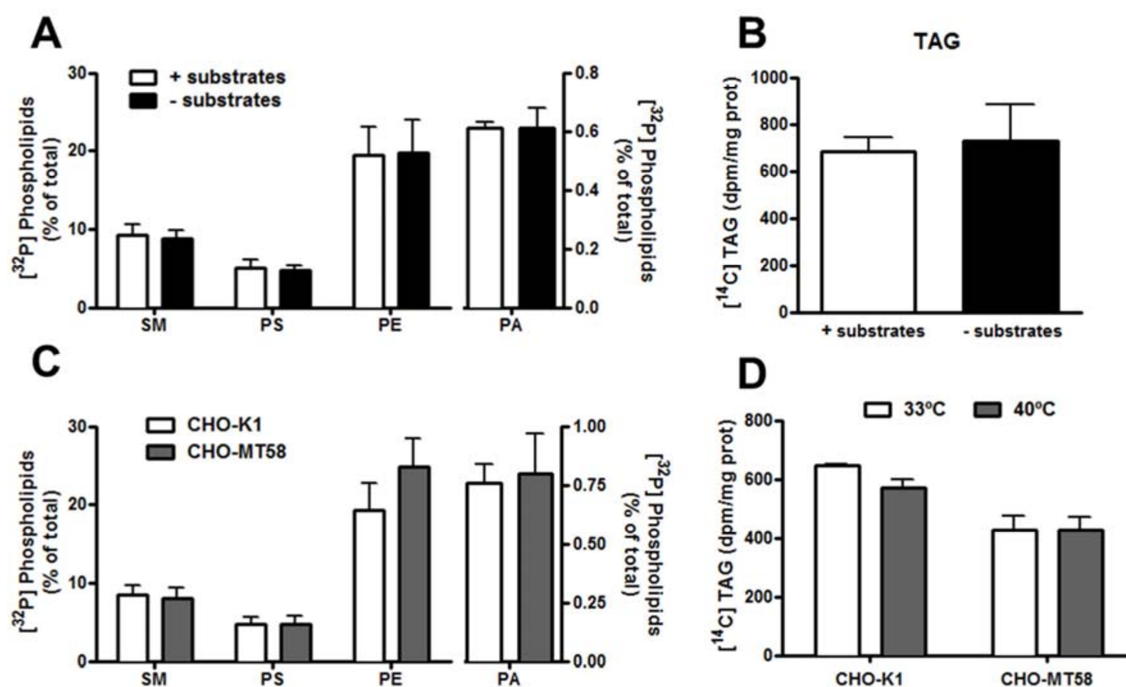

Figure 18. Modulation of PC and PI synthesis for a short time does not affect the levels of other than PI and PC major ^{32}P-labelled phospholipids or TAG levels. *CHO-K1 and CHO-MT58 cells were labelled with [^{32}P]orthophosphoric acid (A, C) or [^{14}C]acetate (B, D) for 24 h. Cells were then washed and incubated for 1 h in saline buffer with 200 µM choline and 5 mMm inositol (white bars), without these substrates in the presence of 5 mmM LiCl (−substrates) (A, B), or with F-12 growth medium (F12) for one hour at 40 ºC (C) or at the temperature indicated (D). Lipids were extracted, separated by TLC, and counted for radioactivity. Results are mean ± S.D. of two experiments performed in duplicate.*

result, DAG will increase. We observed that the addition of CMP led to a two-fold increase in the amount of DAG of Vero cells Golgi-enriched fractions (Fig. 19A), and a three-fold increase of DAG in Golgi membranes from rat liver (Fig. 19B). Since phospholipid synthesis, and so these DAG consumption reactions, is mainly localized at the ER, the CMP induced DAG rising in Golgi enriched fraction could be a consequence of ER membrane contamination in our samples. We analysed the purity of our Golgi-enriched fractions from rat liver by Western Blot, by comparing it to the initial homogenate. The Golgi marker GM130 was enriched in our membrane preparation, while the ER marker calnexin dropped to 10% in our membrane fractions (Fig. 19C). Besides, considering that the Golgi complex is an organelle rich in DAG, with 70 ± 17 nmols DAG/ mg protein, compared to 1,3 ± 0,4 nmols nmols DAG/ mg protein in cell lysates, the three-fold increase in DAG after CMP addition is plausible to be taking place in Golgi membranes, rather than as a consequence of ER contamination. These results indicated that DAG consumption reactions of phospholipid synthesis are localized at the Golgi, and suggest that the rise in DAG that

we observed after inhibiting phospholipid synthesis could be taking place, at least in part, at the Golgi complex.

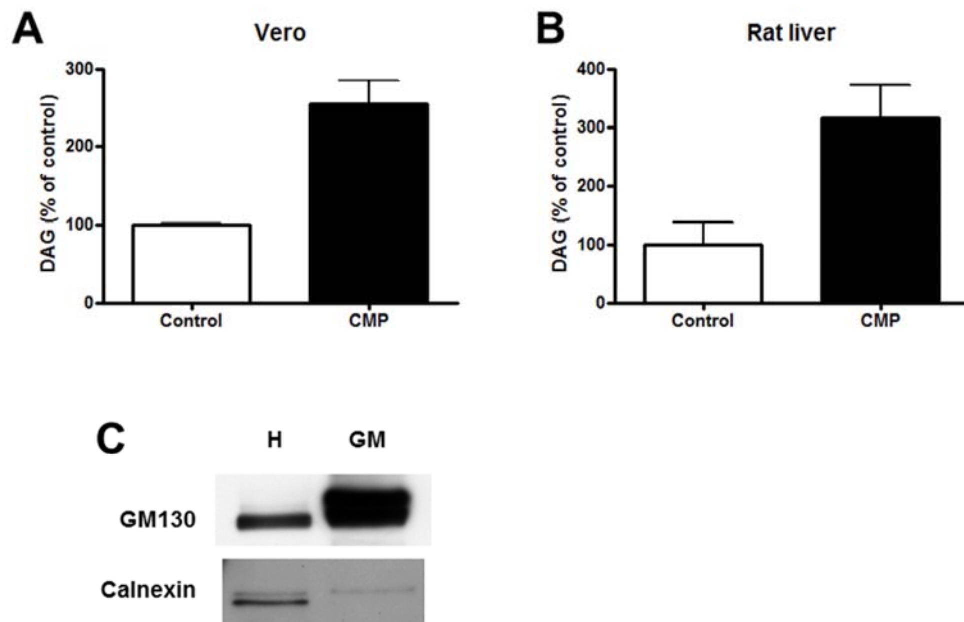

Figure 19. The content of DAG in Golgi membranes can be regulated by phospholipid synthetic pathways. *DAG production in the presence of CMP in isolated Golgi membranes from Vero cells (A) and rat liver (B). Golgi membranes (25 µg of protein) were incubated in Tris-HCl buffer for 20 min with or without of 10 mm CMP. Lipids were extracted, and DAG was determined. Results are mean ± S.D. of two experiments performed in duplicate. C, Western Blot analysis for GM130 and calnexin of homogenate (H) or Golgi-enriched fractions (GM) from rat liver.*

To determine if DAG at the Golgi is increased after inhibition of phospholipid synthesis, we used the overexpression of the C1b domain of PKCθ tagged with GFP (C1-PKCθ-GFP). This construct behaves as a sensor of DAG, that binds to the Golgi associated DAG pool (Carrasco and Mérida, 2004). We transfected CHO-K1 cells with C1-PKCθ-GFP and incubated them with saline buffer with or without substrates for phospholipid synthesis for one hour. Inhibition of phospholipid synthesis led to a small increase in C1-PKCθ-GFP recruitment to the Golgi, although it was not statistically significant (Fig. 20).

To determine if the inhibition of phospholipid synthesis that leads to an increase in the DAG pool of the Golgi produces a functional effect at the Golgi level, we incubated CHO-K1 and CHO-MT58 with propanolol during the inhibition of PC and PI synthesis. Propanolol is a drug that inhibits the formation of DAG from PA, and this inhibition leads to a decrease of DAG and an increase of the number of Golgi associated buds as a consequence of an impairment of vesicle fission at the Golgi (Fernández-Ulibarri, 2007). CHO-K1 and CHO-MT58 were incubated at 33 ºC or 40 ºC in the presence of

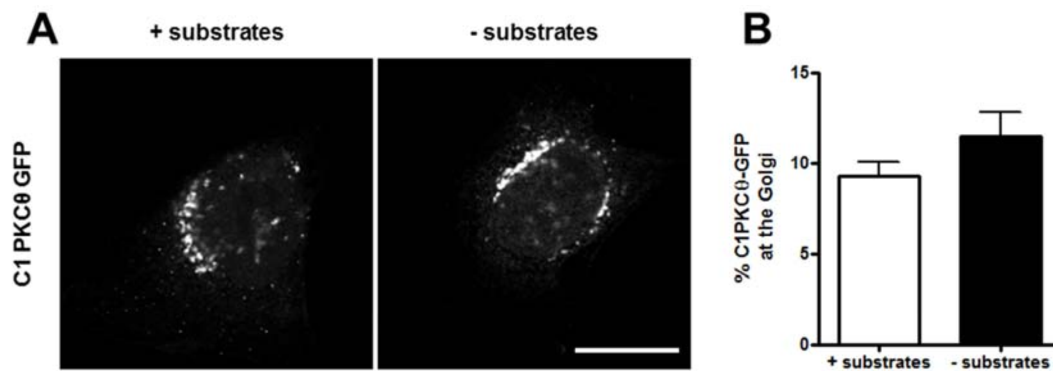

Figure 20. The recruitment of C1-PKCθ-GFP to the Golgi is slightly increased by inhibition of PC and PI synthesis. *A, CHO-K1 cells with transiently transfected C1-PKCθ-GFP cells were incubated for 1 h in saline buffer with 200 µM choline and 5 mM inositol or without these substrates in the presence of 5 mM LiC. Bar, 10 µm. B, Quantification of C1-PKCθ-at the Golgi complex was represented as a percentage of total fluorescence in the Golgi area. Golgi area was defined using staining with GM130. Results are mean ± S.D of more than 20 cells per condition from two different experiments.*

60 µM propanolol for the last 15 minutes and Golgi structure was analysed by electron microscopy. CHO-MT58 cells at 40 ºC prevented the increase in the number of Golgi-associated buds induced by propanolol (Fig. 21), which indicates that the DAG increase produced by PC synthesis inhibition may compensate the decrease of DAG produced by propanolol.

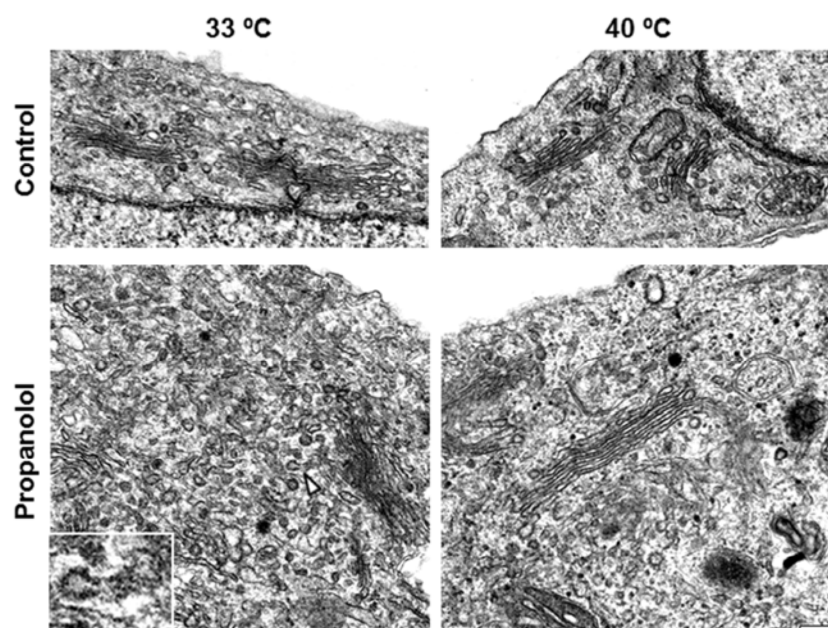

Figure 21. Inhibition of PC synthesis prevents the increase of Golgi-associated buds induced by propanolol. *Representative ultrastructural images for the Golgi area of CHO-MT58 cells incubated at 33 or 40 °C for 1 h with or without propanolol (60 µM) during the last 15 min. Bar, 200 nm.*

Quantitative analysis showed that propanolol induced an increase of the Golgi-associated budding vesicular profiles per μm^2 from 0,8 ± 0,3 to 2,9 ± 1,5 in CHO-K1 and from 0,7 ± 0,2 to 3,0 ± 1,4 in CHO-MT-58, at 32 ^oC. In CHO-MT58 cells at 40 ºC, where DAG is increased as a consequence of PC synthesis inhibition, the increase in the number of vesicular profiles after propanolol incubation was prevented(1,5 ± 0,7). In contrast, the rise to 40ºC in CHO-K1 did not prevent the propanolol effect in the number of vesicular profiles (0,9 ± 0,4 in control situation and 3,3 ± 1,6 with propanolol). This prevention of the propanolol effect on the Golgi ultrastructure suggests that PC synthesis leads to an increase of the Golgi-associated DAG pool, and that the experimental conditions that we had set up can control the level of DAG at the Golgi.

1.4.- Phospholipid synthesis controls both retrograde and anterograde transport at the Golgi complex

Once we confirmed that we are able to control the pool of DAG of the Golgi by tuning phospholipid synthesis, and this tuning had a structural effect on vesicle budding, we studied if this DAG plays a role in membrane trafficking at this organelle level. For this purpose we analysed three different models: the distribution of the endogenous KDELR, the transport of the VSVG protein and the secretion of ssHRP.

We first analysed the subcellular distribution of the KDELR, a transmembrane protein that cycles between the Golgi complex and the ER. Luminal ER resident proteins contain the KDEL sequence at their C terminus. Once these proteins bind to the KDELR at the Golgi complex or at the intermediate compartment, they are brought back to the ER in a COP-I dependent manner. Once at the ER, the protein-KDELR complex is split and KDELR returns to the Golgi complex. So, KDELR could be found in the Golgi complex, in the ER or cycling between these organelles, seen by immunofluorescence as cytoplasmic particles. Thus, the subcellular distribution of the KDELR reflects the membrane trafficking between the Golgi complex and the ER.

We analysed the number of KDELR particles by immunofluorescence in CHO-K1 and NRK cells after being incubated with saline buffer with or without substrates for 1 hour. The pattern of KDELR staining was different between these cell lines. CHO-K1 cells showed a strong Golgi staining and some particles in the cytoplasm, while NRK cells, although having a good Golgi pattern, presented more cytoplasm particles (Fig. 22A). In spite of this, both cell types had a higher number of KDELR positive structures per area when we deplete cells of choline and inositol to inhibit phospholipid synthesis (Fig. 22B). CHO-MT58 cells incubated for one hour at 40 ºC had a higher number of KDELR

positive particles per area than CHO-MT58 at 33 ºC and CHO-K1 at both 33 or 40 ºC (Fig. 22C). All together these results showed that the inhibition for a short time of PC synthesis led to an increase of KDELR particles cycling between the Golgi complex and the ER and that the amount of KDELR particles correlated with DAG levels, previously shown in figure 16B and 16C.

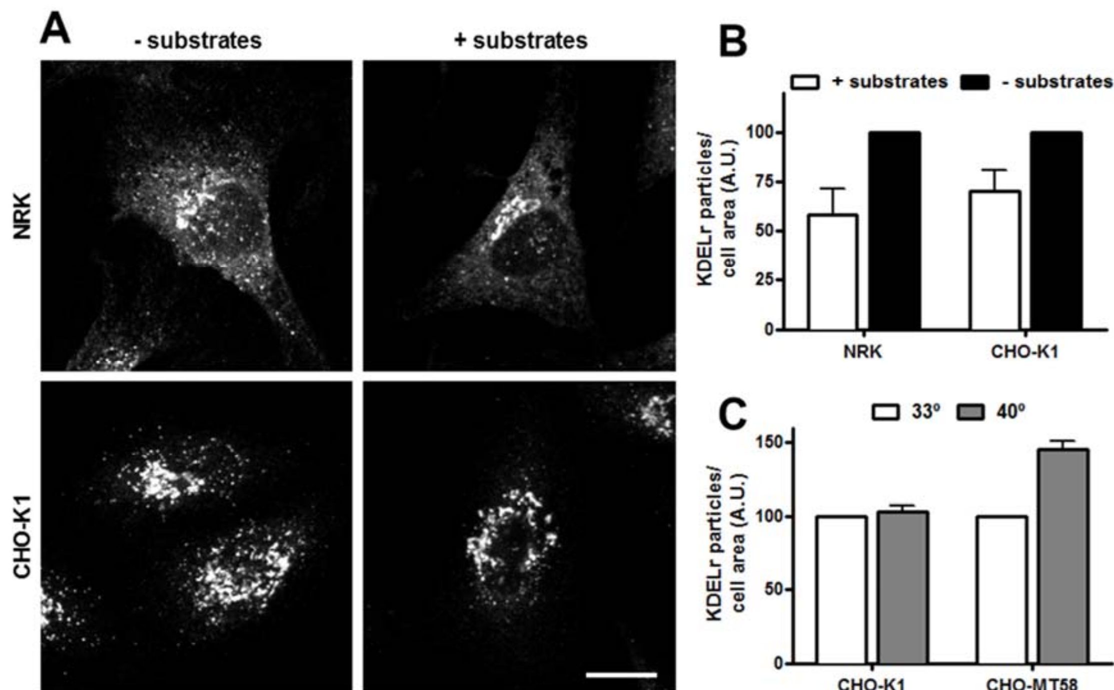

Figure 22. The number of KDELR particles per area is increased by the inhibition of PC and PI synthesis. *A, representative images for KDELR staining in NRK and CHO-K1 cells for the results presented in (B). Bar, 10 µm. B, NRK and CHO-K1 cells were incubated for 1 h in saline buffer with 200 µM choline and 5 mM inositol (white bars) or without these substrates in the presence of 5 mM LiCl (black bars).. C, CHO-K1 and CHO-MT58 cells were incubated for 1 h in growth medium at permissive (33 °C) or at non-permissive temperature (40 °C) for the PC-synthesis in CHO-MT58. Results are mean ± S.D. of three experiments performed in duplicate.*

To further analyse the dependence of KDELR distribution on DAG we tested a set of different experimental conditions and measured the number of KDELR positive particles and the amount of DAG. CHO-K1 and CHO-MT58 cells were incubated for one hour at 33 or 40 ºC, in saline buffer with or without substrates for phospholipid synthesis. The profile for the amount of DAG in the 8 conditions tested (Fig. 23A) was qualitatively similar to that for the KDELR particles per area (Fig. 23B). We next plotted the number of KDELR particles against the amount of DAG for each condition tested to characterize the correlation between these two parameters The data points were fitted to a rectangular hyperbola for one binding site (Fig. 23C), suggesting that the

distribution of KDELR particles can be controlled by the interaction of DAG to one effector protein.

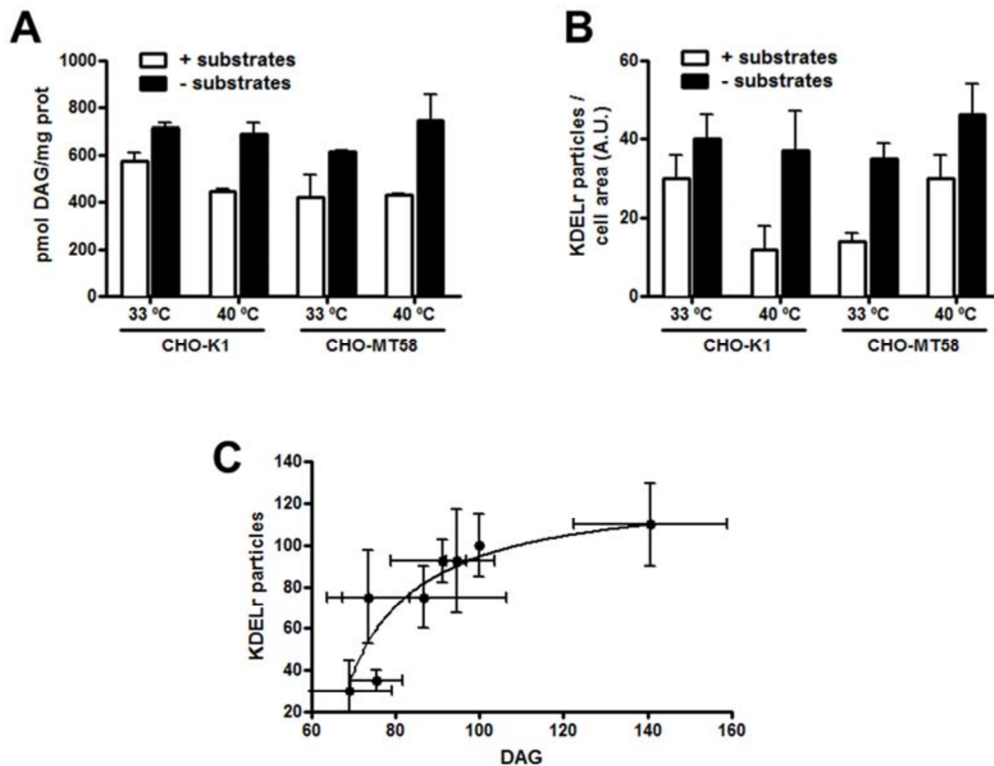

Figure 23. The density of KDELR particles correlates with the DAG content. *Shown are DAG levels (A) and density of KDELR particles (B) from a representative experiment. C, density of KDELR particles plotted against DAG for each different experimental condition tested in CHO-K1 and -MT58 cell lines incubated in saline buffer for 1 h. Data from individual experiments were normalized (the value for CHO-K1 cells at 33 °C in the absence of substrates was taken as 100%) and represented are means ± S.D. of three experiments performed in duplicate. Data points were fitted by non-linear regression to a one-binding site hyperbola (r = 0.896).*

We next studied the trafficking of VSVG to the plasma membrane after phospholipid synthesis inhibition. VSVG is a protein that is not folded properly at 40 ºC and is retained at the ER. After temperature shift to 32 ºC, it can fold and exit the ER, through the Golgi complex to the plasma membrane. Most protocols for VSVG synchronization keep VSVG expressing cells for at least 4 hours at 40 ºC, in order to accumulate VSVG at the ER. These protocols are incompatible with our strategy to inhibit PC synthesis in CHO-MT58 cells, because the restrictive temperature for PC synthesis is also 40 ºC. For this reason, we only analysed the trafficking in VSVG cells in CHO-K1 cells with or without choline and inositol. CHO-K1 cells transfected with VSVG were kept overnight at 40 ºC and its media was changed to saline buffer with or without substrates for phospholipid synthesis at the moment of the temperature shift to 32 ºC. Inhibition of phospholipid synthesis did not alter VSVG arrival at the Golgi complex (Figs. 24A,

24B). However, VSVG exited the Golgi complex faster in CHO-K1 cells incubated without choline and inositol than with them (Figs. 24A, 24B). In accordance with this result, inhibition of phospholipid synthesis by depletion of choline and inositol led to a faster VSVG arrival at the plasma membrane than in the presence of the substrates (Fig. 24C). Taken together, these results suggest that the increase of DAG in the Golgi complex due to phospholipid synthesis inhibition could be promoting the vesicle formation in the Golgi complex and its exit to the plasma membrane.

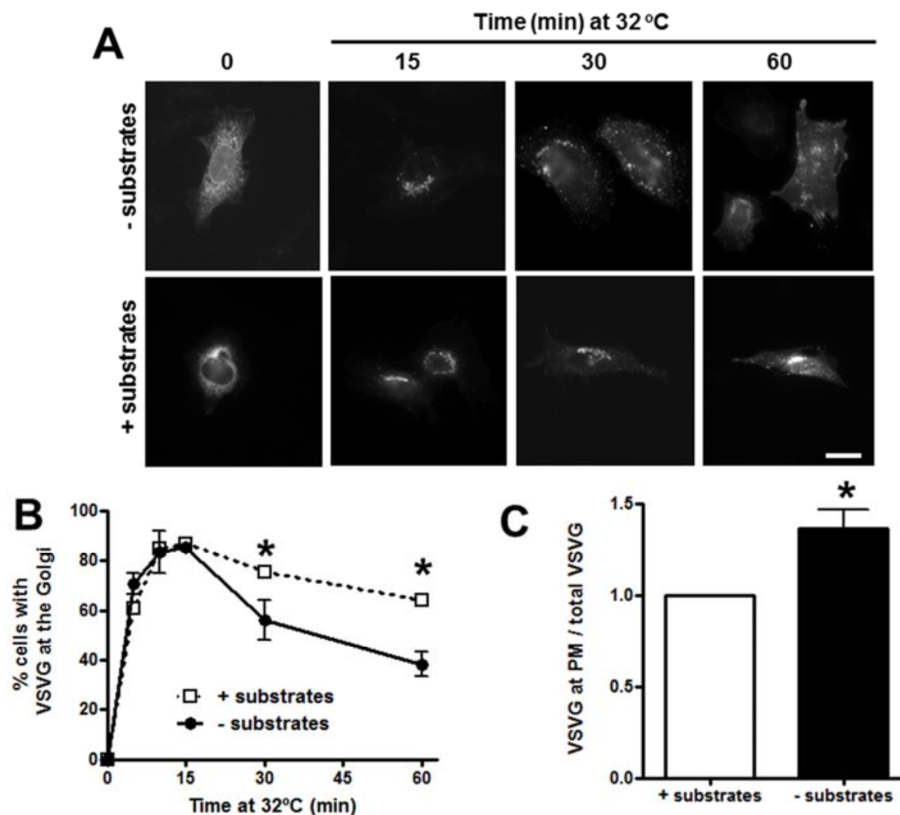

Figure 24. Post-Golgi transport of VSVG is increased by the inhibition of PC and PI synthesis. *CHO-K1 cells transiently transfected with VSVG were incubated overnight at 40 °C to accumulate the unfolded VSVG at the ER. Cells were then shifted to 32 °C and incubated for 1 h in saline buffer with 200 μM choline and 5 mM inositol or without these substrates in the presence of 5 mM .A, representative images for the two conditions. Bar, 10 μm. B, percentage of cells with VSVG at the Golgi for the times indicated. Results are mean ± S.D. of three experiments performed in duplicate. C, cells incubated for 60 min as in B were then labeled with anti-VSVG ectodomain antibody without permeabilization. Results represent the ratio between VSVG at the plasma membrane and the total VSVG. Results are mean ± S.E. of five experiments performed in triplicate.*

After analysing the traffic of KDELR and VSVG, both transmembrane proteins, we next wanted to check if the trafficking of soluble proteins could also be controlled by phospholipid synthetic pathways. For this purpose, we studied the secretion of a soluble form of the horseradish peroxidase (ssHRP). CHO-K1 cells were transfected

with ssHRP and incubated with saline buffer with or without choline and inositol. The amount of ssHRP was measured as the HRP activity accumulated in the media during every 30 minutes along 2 hours. CHO-K1 cells incubated without substrates for phospholipid synthesis had a higher HRP activity in the media than those cells where phospholipid synthesis was not inhibited (Fig. 25A). To ensure that the differences of

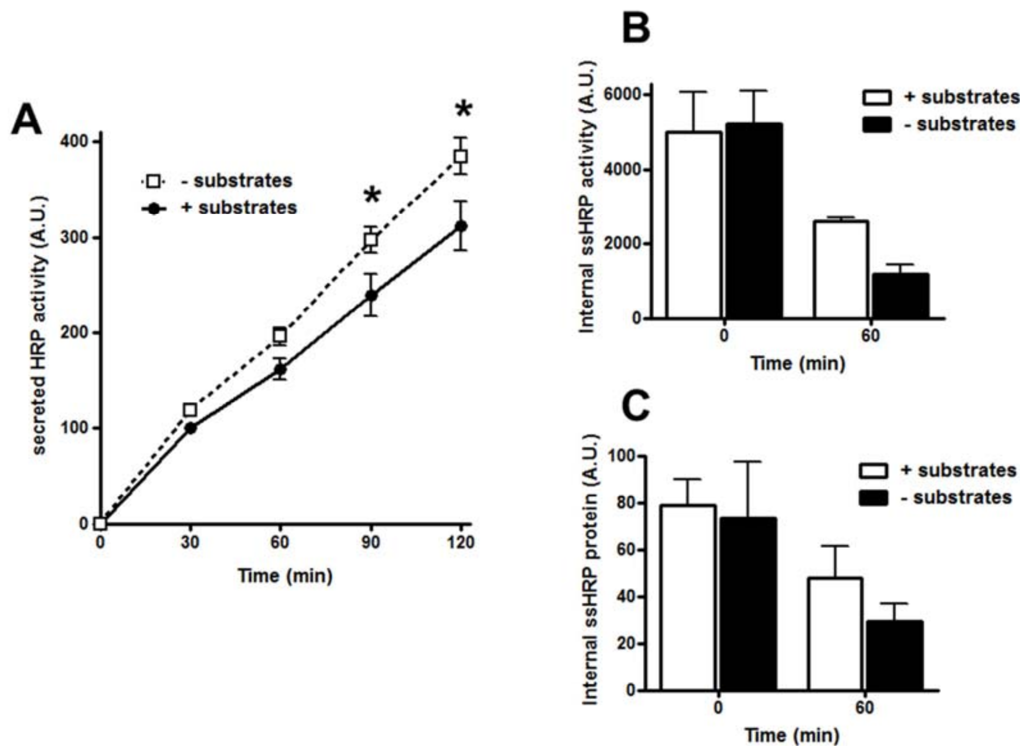

Figure 25. ssHRP secretion is increased by the inhibition of PC and PI synthesis. *CHO-K1 cells with transiently transfected ssHRP were incubated for the times indicated in saline buffer with 200 μM choline and 5 mM inositol (white bars) or without these substrates in the presence of 5 mM LiCl (black bars). ssHRP in the incubation buffer (A) and in cell lysates (B, C) was determined by a luminescent enzymatic assay or Western blot, as indicated. Results are mean ± S.D. of eight experiments performed in duplicate in A and mean ± S.D. of two experiments performed in duplicate in B and C. A.U., arbitrary units.*

HRP activity found in the media reflected changes in the rate of secretion of HRP and not a differential expression, we checked intracellular ssHRP. The amount of HRP measured by both luminescence assay of activity and Western blot, we found that just after changing the media to saline buffer, cells had the same amount of HRP (Figs. 25B, 25C). We also observed that after 60 minutes of incubation, the levels of intracellular ssHRP were slightly higher on cells incubated with choline and inositol, indicating that the differences seen on the media were due to differential secretion in the presence of substrates for phospholipid synthesis. These results confirmed that

soluble protein trafficking, as it happened for transmembrane proteins, can be regulated by modulating the routes of phospholipid synthesis.

Altogether, the results for the trafficking of these three proteins showed that phospholipid synthesis can regulate membrane trafficking at the Golgi complex by controlling DAG levels in this organelle, in both anterograde and retrograde directions and of both transmembrane and soluble proteins.

2.- Role of PLCγ1 in the regulation of DAG required for membrane trafficking at the Golgi complex

2.1.- PLC activity in Golgi-enriched membrane fractions

In order to determine the role of PLC in the Golgi complex, we first studied if there was PLC activity in Golgi-enriched membrane fractions. For this purpose, we incubated Golgi-enriched membrane fractions with radiolabeled [^{32}P]ATP and measured the production of [^{32}P]PA. In this assay, if PLC and DAGK are present and their reactions coupled, then the production of [^{32}P]PA can be a reporter of DAG production. On the other hand, since most of PLC isoforms are found in a soluble form (Gresset, 2012; Kadamur and Ross, 2013), we also added cytosol to the reaction,. Little [^{32}P]PA production was observed in Golgi membranes alone, but this [^{32}P]PA production highly increased after cytosol addition (Fig. 26A). In order to evaluate if this experimental strategy was a good approach to measure PLC activity, we added recombinant PLC to the reaction. In the presence recombinant PLC, [^{32}P]PA production increased almost 2-fold, compared to the condition in the presence of cytosol (Fig.26A). To confirm that cytosol-induced [^{32}P]PA production reflected PLC activity, we tested the effects of the PLC inhibitor U73122 in our experimental conditions. U73122 addition led to a decrease of [^{32}P]PA production (Fig. 26B). U73343, a close analogue of U-73122 that does not suppress PLC activity (Smith, 1990); also decreased [^{32}P]PA production, but its effect was lower than the inhibition by U73122 (Fig. 26B). These results showed that in our experimental strategy [^{32}P]PA production may reflect PLC activity and that this activity could be taking place in Golgi-enriched membrane fractions after cytosol addition.

All previous reactions were performed in the presence of Ca^{2+} and GTPγS. Our next step was to study the effects of Ca^{2+} and GTPγS on the cytosol-induced [^{32}P]PA production. Ca^{2+} is needed for the action of all PLC isoforms (Gresset, 2010; Kadamur and Ross, 2013) and if this [^{32}P]PA production is by the action of PLC we expect that

Ca^{2+} depletion would inhibit it. On the other hand, GTPγS strongly activates the action of G proteins, which are localized at the Golgi complex and activate the PLCβ isoenzymes (Gresset, 2010; Kadamur and Ross, 2013), suggested to be acting at the Golgi complex (Díaz-Añel, 2007). This way, if [^{32}P]PA production is mediated by a PLCβ isoform, this should be sensitive to GTPγS. We found that [^{32}P]PA production was reduced in the absence of Ca^{2+} and also in the absence GTPγS (Fig.27A).

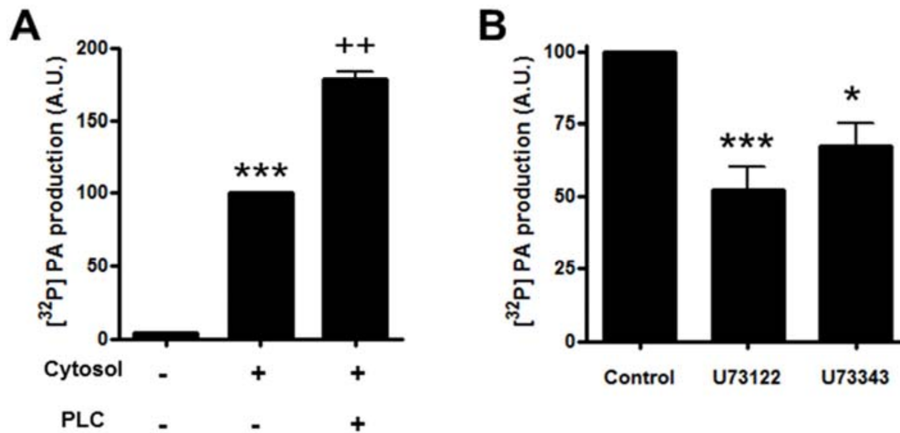

Figure 26. [^{32}P]PA production in Golgi-enriched membrane fractions as a reporter of PLC activity. *A 25 µg of Golgi-enriched membrane fractions from rat liver were incubated with 10 mM Tris/Maleate, 3 mM EGTA, 4 mM $MgCl_2$, 0,62 mM Ca^2+ (1 µM free $Ca^{2+)}$ and 20 µM GTPγS for 30 minutes at 37ºC in the presence of 1µCi of ATP. 60 µg/ml of cytosol and 0,3 U/ml of recombinant PLC were added in the reaction when indicated. B, Golgi-membrane fractions in the presence of 60 µg/ml cytosol were incubated in the presence of 6 µM U73122 or 6 µM U73343. Results are mean ± S.D. of three or more experiments performed in duplicate. Statistical significance according to one-way analysis of variance using Bonferroni's multiple comparison test compared to control (*, $p \leq 0.05$; ***, $p \leq 0.001$) and to Cytosol (++,$p \leq 0.01$) in A.*

Next, we wanted to study the consumption of PIP in Golgi membranes, since this phosphoinositide is abundant in Golgi and can be a substrate for PLC to produce DAG and Inositol (1,4)P_2. We also cheked whether the depletion of Ca^{2+} and GTPγS affect the consumption of PIP. For this, we first incubated the Golgi-enriched membrane fractions with [^{32}P]ATP in order to label [^{32}P]PIP and, after [^{32}P]ATP washout, measure the levels of [^{32}P]PIP after cytosol addition. Cytosol addition led to a decrease of [^{32}P]PIP, compared to those membranes incubated without cytosol (Fig. 27B). Ca^{2+} depletion prevented the cytosol-induced [^{32}P]PIP consumption (Fig. 27B), indicating that this cytosol-induced [^{32}P]PIP consumption was sensitive to Ca^{2+}. However, cytosol-induced [^{32}P]PIP consumption was not altered by the absence of GTPγS (Fig. 27B), suggesting that [^{32}P]PIP consumption was independent of the action of G proteins.

Although not conclusively, these results allow us to hypothezise that PLC can metabolize PIP at the Golgi complex in a manner independent of G proteins. To pursue this hypothesis we then decided to focus our further investigations on the action of PLCγ on Golgi function and structure, since it is sensitive to Ca^{2+}, but not to G proteins.

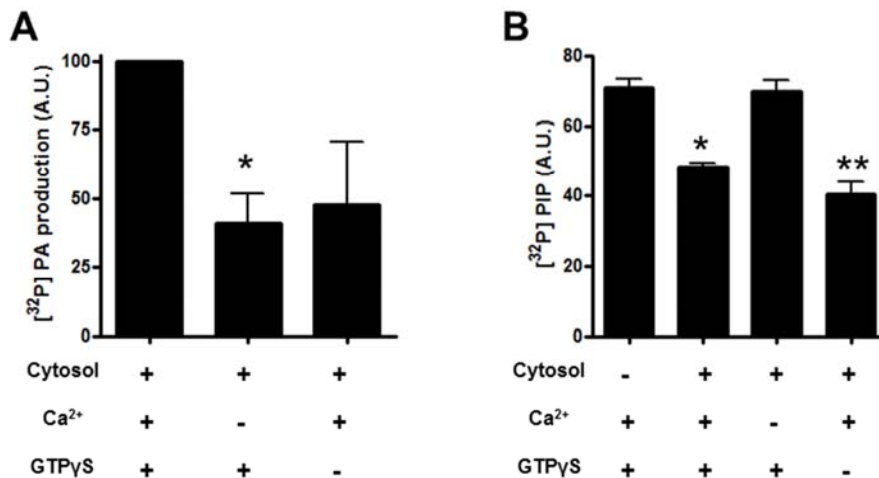

Figure 27. PLC activity in Golgi-enriched membrane fractions requires Ca^{2+} but not GTPγS. *25 µg of Golgi-enriched membrane fractions from rat liver were incubated with 10 mM Tris/Maleate, 3 mM EGTA, 4 mM $MgCl_2$,. 0,62 mM Ca^{2+} (1 µM free $Ca^{2+)}$ and 20 µM GTPγS for 30 minutes at 37ºC in the presence of 1µCi of ATP. 60 µg/ml of cytosol, 0,62 mM Ca^{2+} and 20 and µM GTPγS and were added in the reaction when indicated.. D Golgi-membrane fractions were preincubated for 30 at 37ºC in the presence of 1µCi of ATP, washed and incubated with or without cytosol, Ca^{2+} or GTPγS for 30 minutes at 37ºC, as indicated. Lipids were extracted, separated by TLC, and PA and PIP were identified using lipid standars and counted for radioactivity. Results are mean ± S.D. of three or more experiments performed in duplicate. Statistical significance according to one-way analysis of variance using Bonferroni's multiple comparison test compared to control (*, $p \leq 0.05$; **, $p \leq 0.01$).*

2.2.- Characterization of PLCγ1 tools

For the study of the role of PLCγ in the Golgi complex, we first characterized the PLCγ tools that will allow us these studies in HeLa cells. We first wanted to confirm that HeLa cells express PLCγ1 and that could be recognized by Western Blot and silenced by siRNA. Anti-PLCγ1 antibody worked for WB, recognizing one band around 150 KDa, which fits with the molecular weight of PLCγ1 (Fig. 28A). This band recognized with the anti-PLCγ1 antibody was decreased to less than 10% after 72 hours treatment of siRNA against PLCγ1, but it remained unaltered when incubated against a non-targeting siRNA or with a siRNA against PLCγ2, and it was also decreased after incubation with siRNA against PLCγ1 and PLCγ2 (Fig. 28A).

Moreover, the intensity of anti-PLCγ1 was higher after the overexpression of both wild-type PLCγ1 and a construct of its catalytically inactive form, PLCγ1(H335Q), but not when overexpressing wild-type PLCγ2 or its catalytically inactive form, PLCγ2(H327Q) (Fig. 28B). These results showed that the PLCγ1 antibody specifically recognized

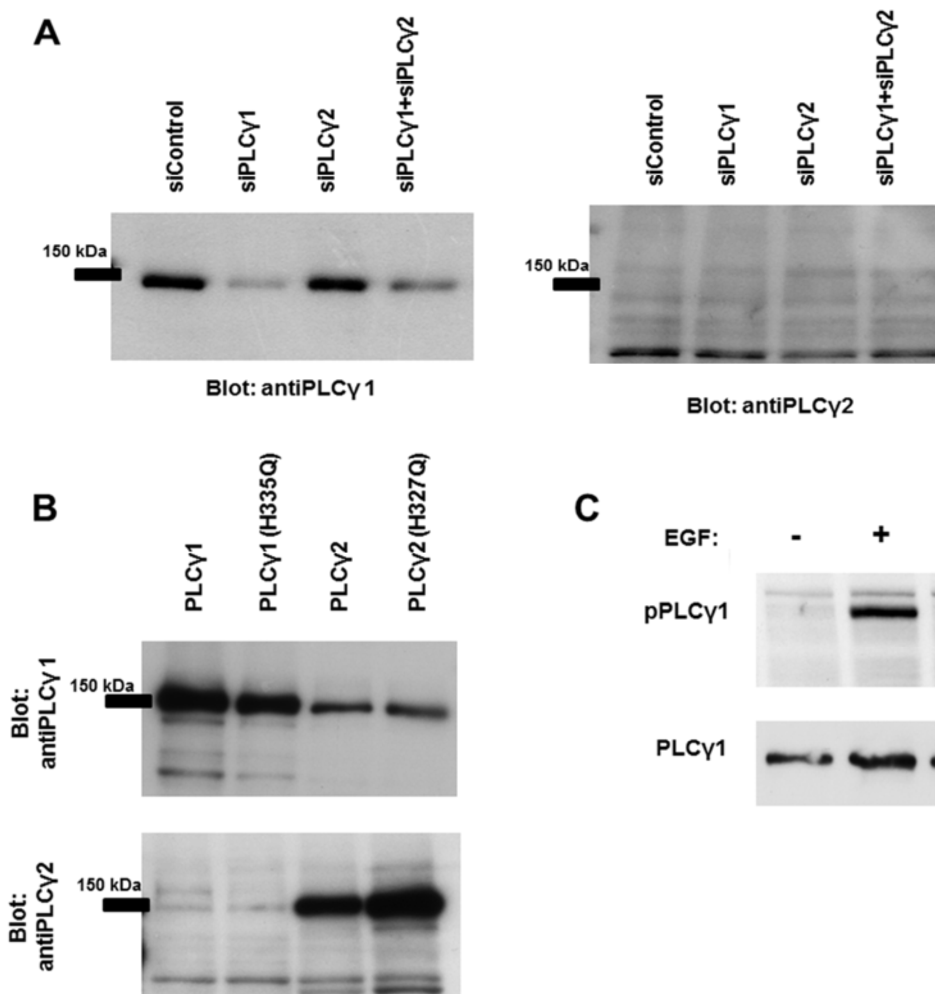

Figure 28. Characterization of PLCγ silencing and antibodies in HeLa cells. *A Western Blot analysis of HeLa lysates transfected with diferent siRNA for 72 hours. B Western Blot analysis of HeLa lysates transfected with diferent PLCγ plasmids for 34 hours. C Western Blot analysis of HeLa cells in control conditions or in the presence of 100 ng/mL EGF.*

PLCγ1 for WB and that the siRNA against PLCγ1 also worked properly. Moreover, the antibody against PLCγ1 phosphorylated in its tyrosine 783 (anti-phosphoPLCγ1), a phosphorylation related to PLCγ1 activation (Gresset, 2010); recognized a band around 150 kDa that increased after treatment with epidermal growth factor (EGF) (Fig. 28C), one known activator of PLCγ1. This result also confirms that the antibody against the activating phosphorylation of PLCγ1 also works properly for WB.

The anti-PLCγ2 antibody did not seem to recognize any specific band around 150 KDa in HeLa cells lysates (Fig. 28A), where its molecular weight would fit. Moreover, treatments with siRNA against PLCγ1, PLCγ2 or both did not modify the pattern of anti-PLCγ2 labeling (Fig.28A). However, anti-PLCγ2 antibody specifically recognized a band around 150 KDA when overexpressing wild-type PLCγ2 or its catalytically inactive form, PLCγ2(H327Q), but it did not recognize overexpression of both PLCγ1 constructs (Fig. 28B). These results showed that anti-PLCγ2 antibody works specifically for WB, since it recognizes the overexpression of its target protein. The lack of signal in lysates of HeLa cells for the anti-PLCγ2 antibody suggests that this cell line does not express this PLC isoform, which is in accordance with the literature, where PLCγ2 expression is described to be restricted to cells of the haematopoietic system (Gresset, 2012, Kadamur and Ross, 2013).

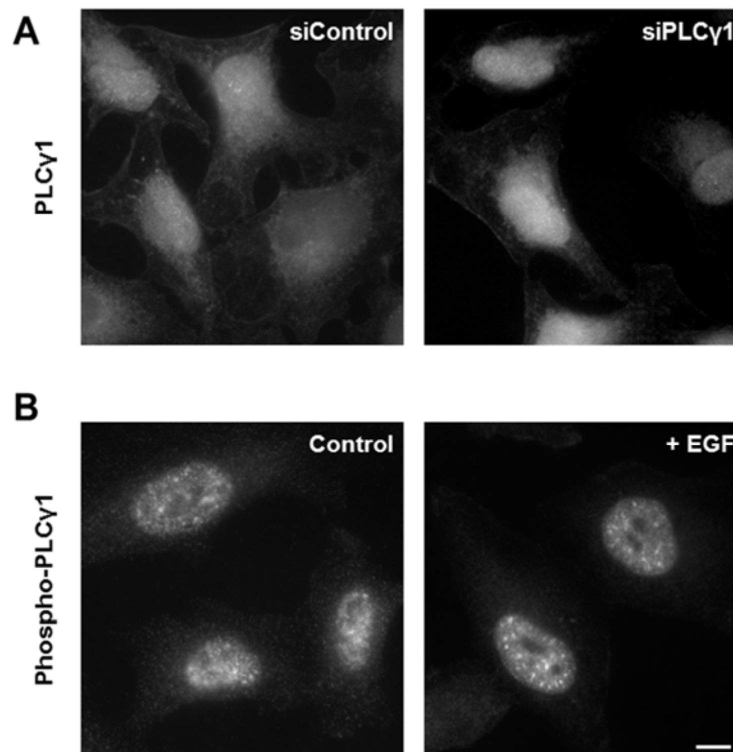

Figure 29. Antibodies against PLCγ1 and pPLCγ1 do not work for immunocytochemistry. *A HeLa cells transfected with control or PLCγ1 siRNA were stained with anti-PLCγ1 antibody. B HeLa cells in control conditions and incubated with EGF 100 ng/mL for 5 min were stained with anti-phospho-tyr783 PLCγ1 antibody. Bar = 10 µm.*

One of our goals was to study the subcellular localization of PLCγ1 and if this protein could be found, or temporally translocated, at the Golgi complex. However, the antibodies against PLCγ1 or phosphoPLCγ1 did not work for immunocytochemistry. Staining of HeLa cells with anit-PLCγ1 did not show any recognizable structure and

treatment with siRNA against PLCγ1, which, according to WB results, worked properly (Fig. 28A), did not change this staining pattern (Fig. 29A). Staining with anti-phospho-PLCγ1 also did not show any recognizable structure, although in some cells it stained the nucleus (Fig. 29B). After stimulation with EGF the staining pattern of anti-phosphoPLCγ1 did not change (Fig. 29B), suggesting that this antibody does not work properly for immunocytochemistry.

Finally, we overexpressed PentaHis-tagged wild type PLCγ1 and its catalytically inactive form PLCγ1(H335Q), to study its subcellular localization and any possible translocation of this protein at the Golgi complex. Both constructs were localized at the cytoplasm, but addition of EGF, a known activator of PLCγ, did not alter this staining pattern (Fig. 30). Since we were unable to see any redistribution after stimulation with EGF, we ruled out the possibility to study any translocation to the Golgi complex by any other stimuli. However, we can identify cells that overexpress PLCγ1 to further analyse the effects of the overexpression of its wild-type or catalytically inactive forms on the Golgi complex.

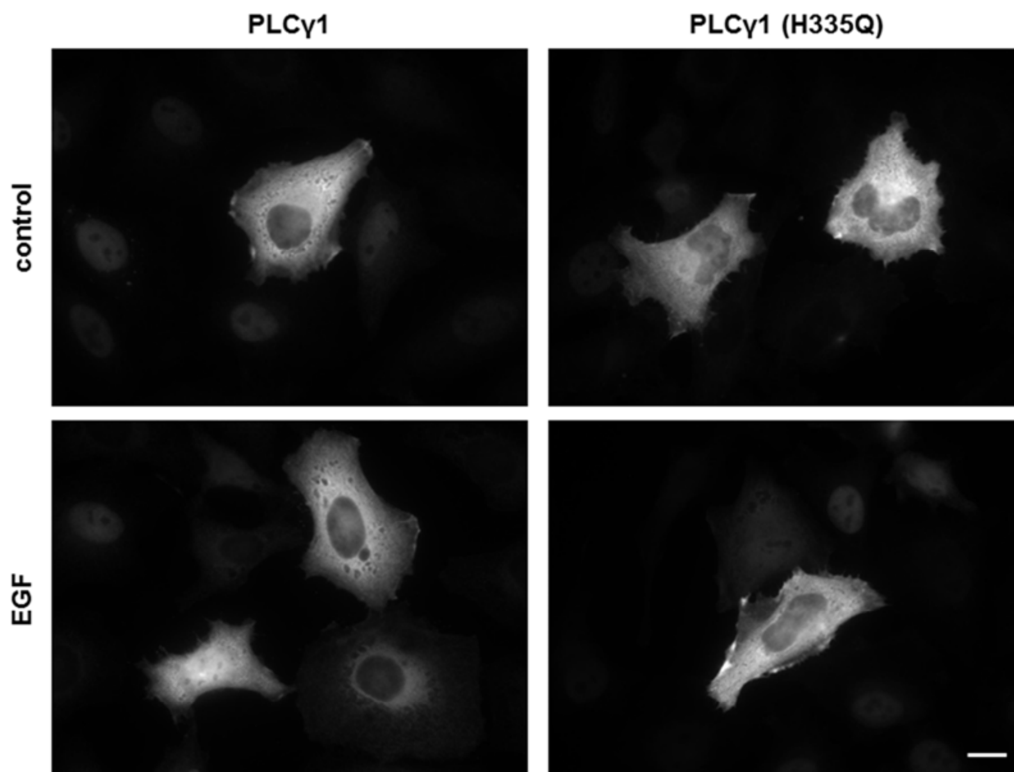

Figure 30. Overexpressed PLCγ1 localyzes at the cytoplasm of HeLa cells. *HeLa cells transiently overexpressing PLCγ1 or its catalitically inactive form PLCγ1(H335Q) were fixed in control conditions or after 5 min incubation of 100 ng/mL and analysed by immunofluorescence. Bar = 10 μm.*

With these tools, and because of the absence of PLC γ2 in our cell system, we decided to focus the study in the role of PLCγ1 at the Golgi complex by using strategies based in PLCγ1 depletion by siRNA, since we could not study any possible localization of PLCγ1 on the Golgi complex or elsewhere by immunofluorescence.

2.3.- Role of PLCγ1 in Golgi function and structure

2.3.1.- Role of PLCγ1 in Golgi secretory function

To study the role of PLCγ1 in the secretory function of the Golgi complex, we analysed the trafficking of VSVG along the secretory pathway. HeLa cells that constitutively express VSVG-GFP were silenced for PLCγ1 using siRNA. First, we studied the trafficking in the early secretory pathway from the ER to the Golgi complex, measuring the acquisition of resistance to the endoglicosylase-H (Endo-H) enzyme. The Endo-H enzyme cuts the glycosyl residues enriched in mannose that proteins possess when they exit the ER, but it cannot process the higher complex N-glican residues that proteins acquire at the mid-Golgi. This way, the acquisition of resistance to Endo-H will represent that proteins, in our case VSVG, have reached the medial region of the Golgi complex. The acquisition of Endo-H resistance is seen by a change in the electrophoretic mobility of the protein, being this lower once it has acquired the resistance (Fig. 31A). HeLa cells transfected with non-targeting (from now on control siRNA) or PLCγ1 siRNA were kept overnight at 40 ºC in order to accumulate VSVG at the ER and then shifted to 32 ºC to allow its exit from the ER to the Golgi complex. The resistance to the Endo-H enzyme at 30 min was the same in cells silenced for siRNA against PLCγ1 and those treated with a control siRNA (Fig. 31A-B), suggesting that PLCγ1 silencing does not alter the transport of VSVG from the ER to the Golgi complex.

Next, we measured the arrival of VSVG to the plasma membrane after PLCγ1 silencing. Cells were kept overnight at 40 ºC to accumulate VSVG at the ER and then shifted to 32 ºC to allow VSVG transport from the ER to the plasma membrane. As seen by immunofluorescence, after 60 minutes at 32 ºC, cells silenced for PLCγ1 presented less amount of VSVG at the plasma membrane compared to those transfected with control siRNA (Fig. 31C). Quantification of VSVG that has reached the plasma membrane with an antibody against the Ecto-Domain of VSVG confirmed that in cells silenced for PLCγ1 the trafficking of VSVG is slower than in control cells (Fig. 31D). Since the transport of VSVG from the ER to the Golgi complex did not seem affected after PLCγ1 silencing, the delay in the arrival of VSVG at the plasma membrane in silenced cells suggests that PLCγ1 may be participating in the late

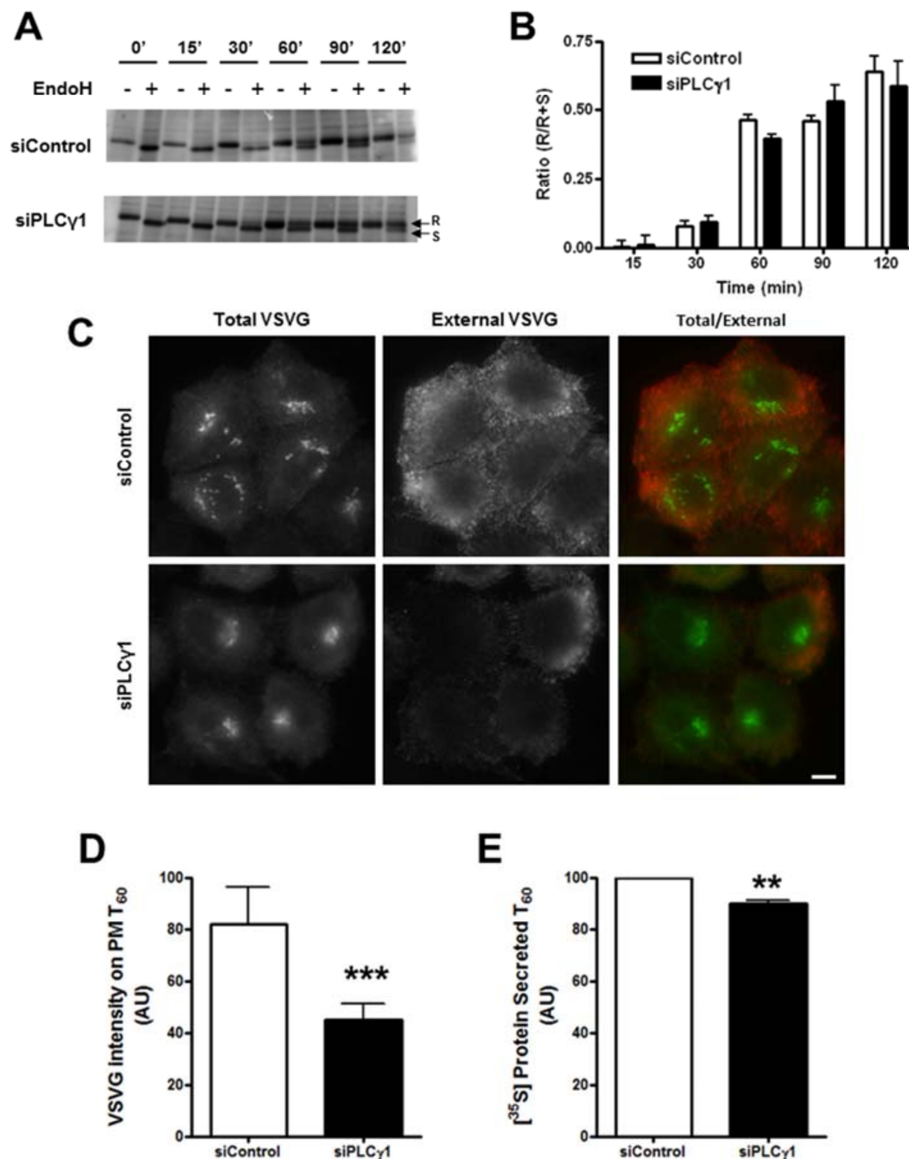

Figure 31. PLCγ1 silencing impairs post-Golgi trafficking. *A-D HeLa cells constitutively expressing VSVG-GFP were transfected for 72h with siRNA control or against PLCγ1 and incubated at 40 °C overnight. 30 minutes after the temperature shift, cyclohexymide was added to inhibit protein synthesis. A-B, Biochemical transport assay for VSV-G-GFP using Endo H. After overnight incubation at 40 ºC and addition of CHX, cells were shifted at 32 °C to induce the transport of VSV-G from the ER, lysed at indicated times, and subjected to Endo H treatment. R and S indicate Endo H-resistant and -sensitive forms, respectively. B The ratio of the amount of Endo H-resistant form to that of total amount is plotted. Values are represented as the mean ± S.D. of three independent experiments.. C Cells were fixed after 60 minutes at 32ºC and stained for VSVG ectodomain antibody without permeabilization. Bar = 10 μm. D Results represent the ratio between the staining of VSV-G at the plasma membrane and the total cellular VSVG-GFP. E HeLa cells were transfected with control or PLCγ1 siRNA. 72 h after the transfection, cells were pulse labeled with [^{35}S]Met/Cys, incubated at 19 °C for 3 h and then shifted to 37 °C. After 60 minutes, proteins in the culture supernatants were precipitated and cell lysates were taken and both quantified by scintillation counting. . Results are mean ± S.D. of four independent experiments. Statistical significance according to Student's t-test (**, $p \leq 0.01$; (***, $p \leq 0.001$).*

secretory pathway. To confirm this result by studying other cargoes, we measured the post-Golgi secretion to the extracellular media of soluble proteins radiolabeled with [^{35}S]cysteine/methionine. HeLa cells were silenced for PLCγ1 or treated with a control siRNA and pulse-labelled with a mixture of [^{35}S]cysteine/ methionine. Then, cells were kept for 3 hours at 19 ºC, in order to accumulate newly synthesised [^{35}S]proteins at the Golgi complex and then shifted to 37 ºC to allow its secretion to the extracellular media. After 60 min at 37 ºC, the amount of [^{35}S]soluble protein secreted in the extracellular media was slightly, but significantly lower in cells silenced for PLCγ1 than in control cells (Fig. 31E), indicating that the post-Golgi trafficking of soluble proteins is slowed by PLCγ1 silencing. This result is in accordance with the delay of VSVG trafficking to plasma membrane (Fig. 31C,D), and confirms that PLCγ1 silencing impairs post-Golgi transport.

We next assesed if PLCγ1 could also contribute to retrograde transport from the Golgi complex to the ER. To this end, we analysed the internalization of the Shiga toxin that contains in its carboxyl terminus the ER retention sequence KDEL conjugated with Cy3 (STxB-KDEL). After binding to the plasma membrane, this toxin is transported to the Golgi complex and to the ER, where it is retained. HeLa cells transfected with control or PLCγ1 siRNA for 72 hours were incubated for STxB-KDEL at 4 ºC for one hour to allow the toxin binding and synchronize its internalization. After that, cells were washed in order to remove the remaining of unbound toxin and then shifted to 37 ºC to start its internalization through the Golgi complex to the ER. After 30 minutes, almost all STxB-KDEL was found at the Golgi complex and progressively reached the ER (Fig. 32). Although the arrival of STxB-KDEL at the ER and its exit from the Golgi complex was apparently delayed in cells silenced for PLCγ1, the quantification of STxB-KDEL dynamics was not significantly different between control cells or cells silenced for PLCγ1 (Fig. 32), suggesting that PLCγ1 is not taking part in the retrograde transport from the ER to the Golgi complex.

2.3.2.- Role of PLCγ1 in the maintenance of Golgi structure

Since PLCγ1 controls post-Golgi membrane trafficking, but not the retrograde transport from the Golgi complex to the ER, we studied if the unbalance of membrane flux at the Golgi complex caused by PLCγ1 silencing could also lead to changes in the structure of this organelle. For this purpose, HeLa cells were transfected with non-targeting or PLCγ1 siRNA for 72 hours. Then, cells were stained for GM130, Golgin97 and TGN46, markers of *cis*-Golgi, *trans*-Golgi and TGN, respectively, in order to analyse the morphology of the different Golgi compartments. Cells silenced for PLCγ1 presented a

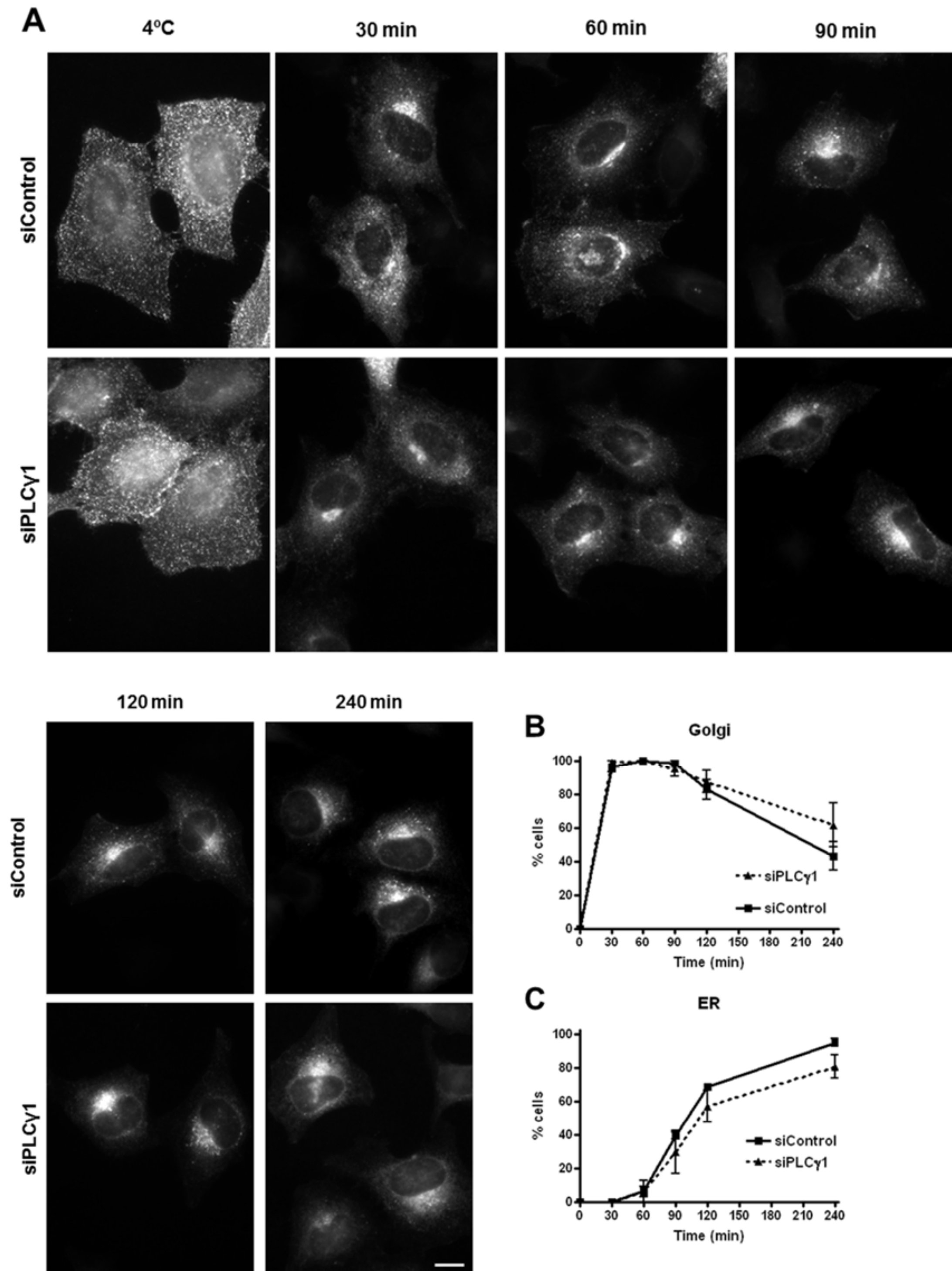

Figure 32. The retrograde transport of STxB-KDEL to the ER is unaltered after PLCγ1 silencing. *A Immunofluorescence microscopy of the retrograde transport kinetics of the cy3-tagged Shiga toxin fragment B containing the ER-retention signal KDEL (STxB-KDEL) in HeLa with control or PLCγ1 siRNA. Plasma membrane internalization of bound STxB-KDEL at 4°C was performed at 37°C for different times until the arrival to the ER (identified by the reticular pattern and nuclear envelope staining), crossing firstly the Golgi (identified by the juxtanuclear staining). Bar = 10 µm.B-C Quantification of cells with STxB-KDEL at the Golgi complex (B) and the ER (C). Values are the mean ± S.D. from three independent experiments.*

more compacted morphology of the Golgi complex, compared to control cells (Fig. 33A). To quantify these morphological differences, we measured the compactness of the Golgi, a dimensionless number that represents circularity, which is a function of the area and perimeter of all Golgi particles (Bard, 2003). For all three Golgi markers, the value of Golgi circularity was higher in cells silenced for PLCγ1 than in control cells (Fig. 33B), which indicates that the Golgi morphology is more compacted in cells

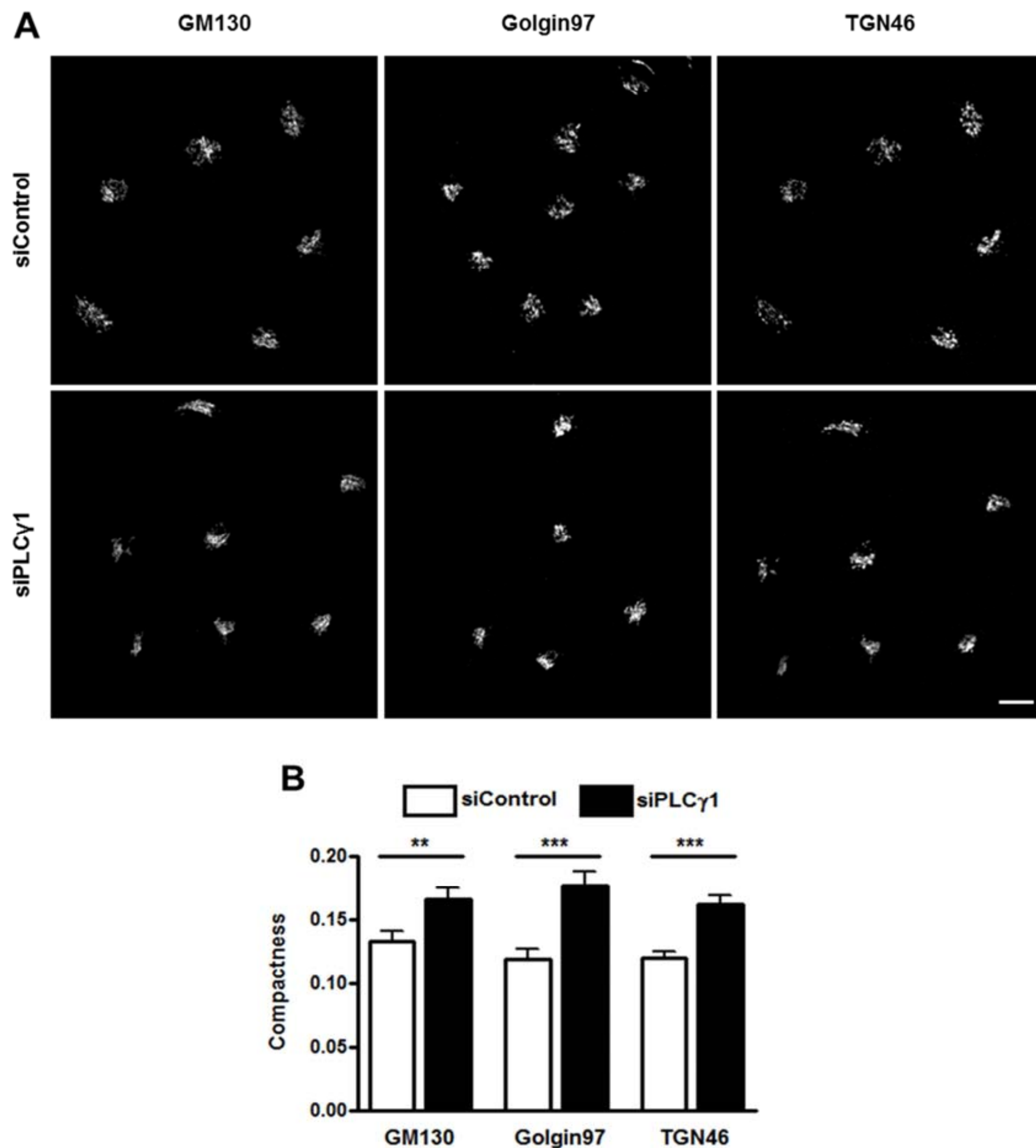

Figure 33. Cells silenced for PLCγ1 present a Golgi-compacted morphology. *A HeLa cells transfected with control or PLCγ1 siRNA were stained with GM130, Golgin97 and TGN46. Bar = 10 μm. B Golgi compactness was quantified as described in methods. More than 150 cells per condition in each experiment were computed and results from from three independent experiments were submitted to statistical analysis. Statistical significance according to Student's t-test (**, p ≤ 0.01, ***, p ≤ 0.001).*

depleted of PLCγ1. Since the morphology of the Golgi complex is highly dependent of alterations of the cytoskeleton (Egea, 2013), we checked if the effects of PLCγ1 silencing on the Golgi morphology could be a consequence of changes in the actin cytoskeleton. Apparently, there were no differences in the actin stress fibers between silenced and control cells (Fig. 34), therefore the cause for changes in the Golgi morphology after PLCγ1 silencing were other than alterations in the actin cytoskeleton.

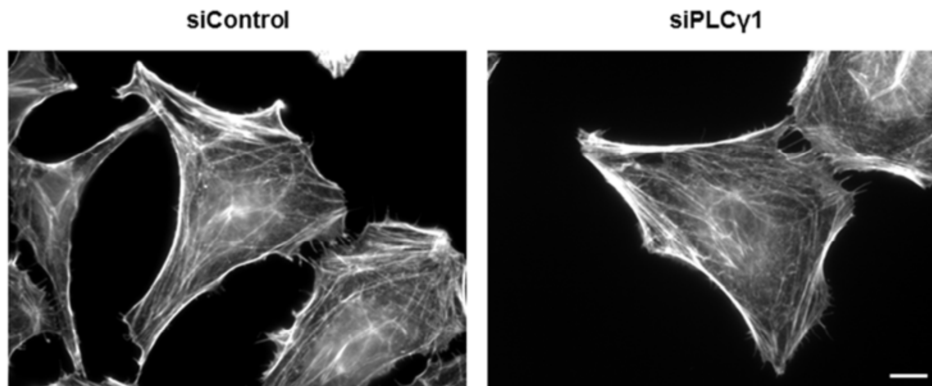

Figure 34. PLCγ1 silencing has no effects on the actin cytoskeleton. *HeLa cells transfected with control or PLCγ1 siRNA for 72 hours were fixed and stress fibers stained with TRITC-phalloidin. Bar = 10 μm.*

To further explore the effect of PLCγ1 on the Golgi structure, we next studied the Golgi morphology after overexpression of wild-type PLCγ1 or its catalytically inactive form PLCγ1(H335Q). After 48 hours of overexpression, cells were stained for GM130 and Golgin97 and Golgi compactness was measured as previously described. Overexpression of PLCγ1, but not PLCγ1(H335), led to a decrease in the compactness of the staining for GM130 (Fig. 35A-B). However, this effect was higher at the *trans*-Golgi, where PLCγ1 overexpression, but not its catalytically inactive form, led to a fragmentation of the structures stained with Golgin97 (Fig. 35C), with the consequent decrease of the value of the Golgi compactness (Fig. 35D). These results confirmed that the catalytic activity of PLCγ1 has a role in the maintenance of the structure of the Golgi complex.

2.4.- Role of PLCγ1 in the regulation of the Golgi-associated DAG pool

All previous results show that PLCγ1 can be participating in different Golgi events, as the post-Golgi transport or in the maintenance of Golgi structure. The changes induced by PLCγ1 silencing or overexpression in Golgi function and structure can be a consequence of at least three different mechanisms. First, PLCγ1 depletion can alter a signalling cascade that could be originated in a location of the cell other than Golgi, as

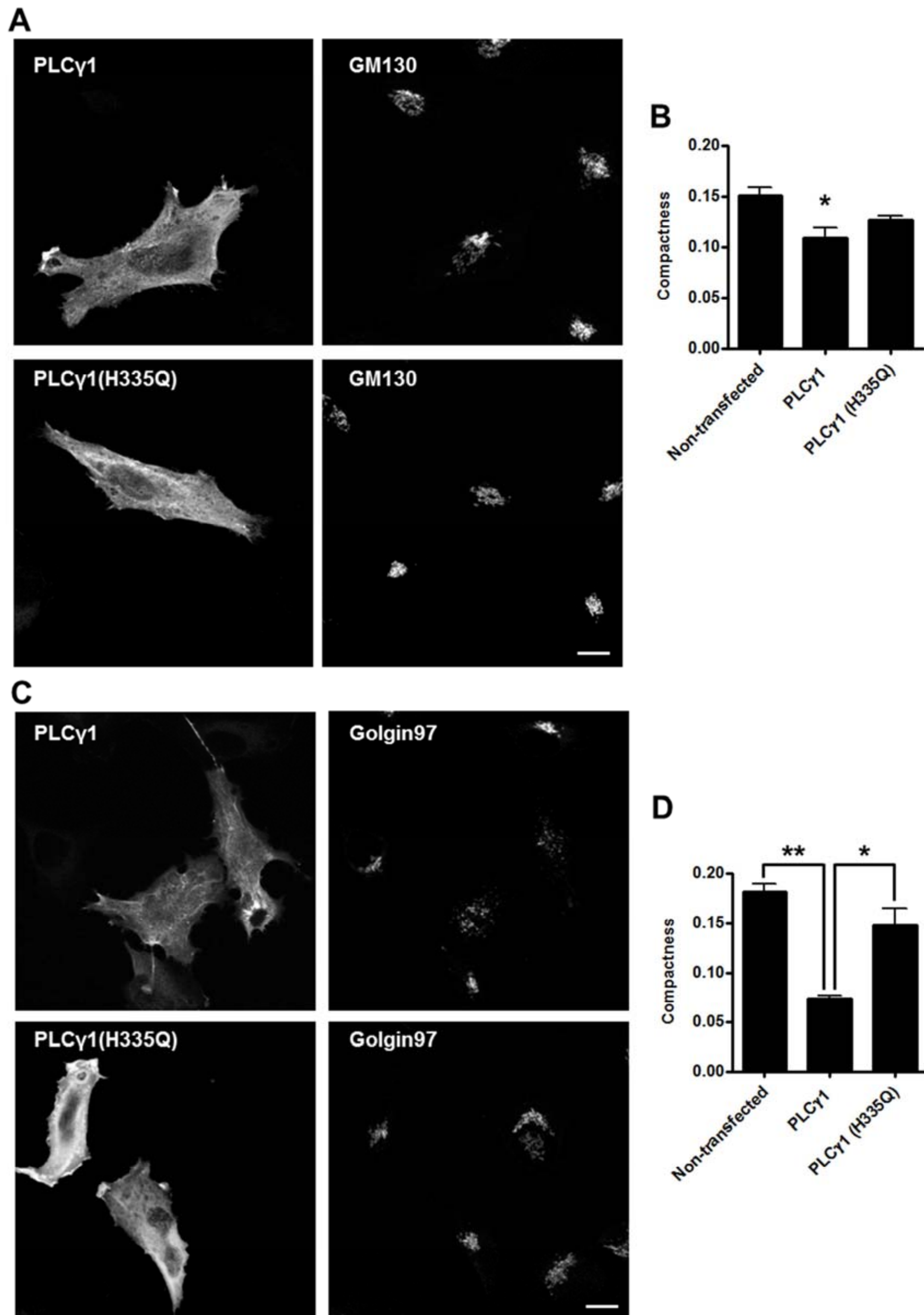

Figure 35. PLCγ1 overexpression leads to fragmentation of the Golgi. *HeLa cells were transfected with PLCγ1 or its catalitically inactive form for 48 hours. Cells were fixed and stained for GM130 and Golgin97, as a marker of cis and trans-Golgi, respectively. Golgi compactness was measured as previously described (Bard et al., 2003). Values are the mean ± S.D. from three independent experiments. Statistical significance according to one-way analysis of variance using Bonferroni's multiple comparison test (*, p ≤ 0.05; **, p ≤ 0.01). Bar = 10 μm.*

for example, the plasma membrane, that could control Golgi structure and function. In this case, as PLCγ1 would not be located at the Golgi complex would affect this organelle in an indirect way. Second, PLCγ1 depletion may lead to an unbalance of the total cell amounts of DAG, PI(4)P and PI(4,5)P_2, its lipid product and substrates, respectively, that could in turn affect the composition of Golgi membranes, leading to the alterations of Golgi function and structure. The third hypothesis is that PLCγ1 is acting on the Golgi complex, where it controls the production of DAG required for membrane traficking, after being stimulated at this organelle.

In order to analyse the mechanism used by PLCγ1 to participate in Golgi function and structure, we first determined if PLCγ1 depletion had an effect on the Golgi associated DAG pool. For this purpose, HeLa cells transfected with control or PLCγ1 siRNA were also transfected with C1-PKCθ-GFP, a construct that acts as a sensor of the Golgi associated DAG pool. In both control and silenced cells, C1-PKCθ-GFP mainly localized at the Golgi complex (Fig. 36A). No significant differences were found in the localization of C1-PKCθ-GFP at this organelle, although the amount present in the Golgi complex was slightly lower in cells silenced for PLCγ1 (Fig. 36A). In order to increase the resolution of this assay, we added PMA for 10 minutes, a compound that induces the recruitment of C1-PKCθ-GFP to the plasma membrane. This way, we could measure the kinetics of the recruitment of C1-PKCθ-GFP, which would depend on the DAG amount present at the Golgi complex. After 10 minutes of PMA incubation, almost all C1-PKCθ-GFP was localized at the plasma membrane in both conditions (Fig. 36A). However, after 5 minutes of PMA incubation, C1-PKCθ-GFP was mainly localized at the Golgi complex in control cells, while in cells silenced for PLCγ1 the presence of C1-PKCθ-GFP in the plasma membrane was more evident (Fig. 36A). Quantification of the amount of C1-PKCθ-GFP at the Golgi complex after 5 minutes of PMA treatment revealed that cells silenced for PLCγ1 had lower percentage of C1-PKCθ-GFP at this organelle than control cells (Fig. 36B). This result suggests that PLCγ1 can control the amount of Golgi associated DAG pool.

Once provided that PLCγ1 can regulate the Golgi associated DAG pool, we wanted to check if PLCγ1 could be involved in DAG production at the Golgi complex and in a manner triggered by cargo arrival. For this purpose, we first studied if the arrival of cargo at the Golgi complex increases the Golgi associated DAG pool, a hypothesis that has not been experimentally addressed before. To this end, HeLa cells were co-transfected with C1-PKCθ-GFP and VSVG and kept overnight at 40 ºC, in order to accumulate VSVG at the ER. Then, cells were shifted to 32 ºC to allow VSVG arrival at the Golgi and fixed at different times. C1-PKCθ-GFP was mainly localized at the Golgi

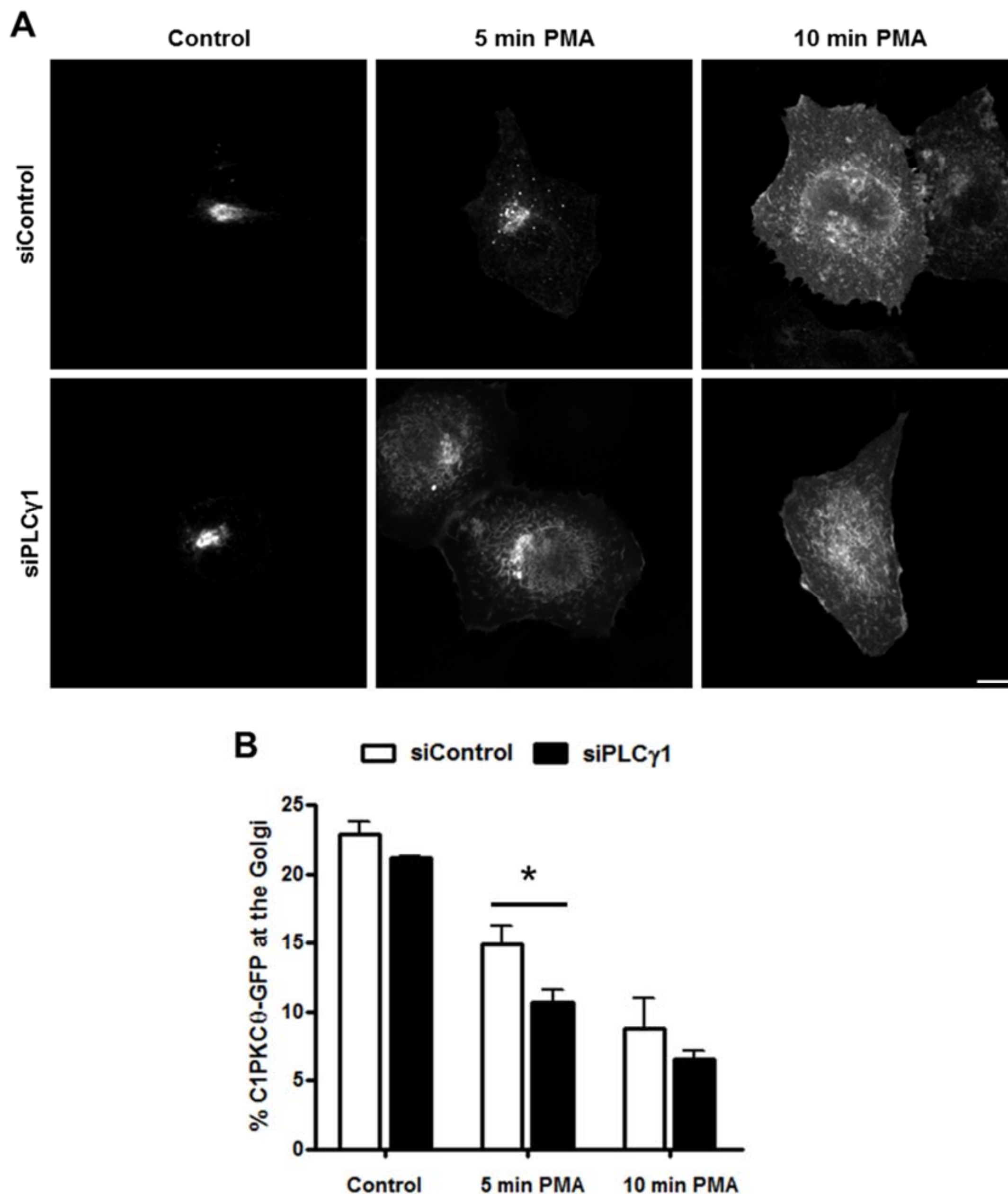

Figure 36. Cells silenced for PLCγ1 have less recruitment of C1-PKCθ-GFP to the Golgi after PMA addition. *HeLa cells transfected with control or PLCγ1 siRNA were also transfected with C1-PKCθ-GFP and incubated in the presence of 100 nM PMA. A, Cells were fixed before and 5 and 10 min after PMA addition and stained for Golgin97 to define Golgi region. B, Quantification of C1-PKCθ-GFP binding at the Golgi complex. Values are the mean ± S.D. from three independent experiments. Statistical significance according to two-way analysis of variance using Bonferroni's multiple comparison test (*, $p \leq 0.05$). Bar = 10 µm.*

complex during all the time, but the GFP fluorescence intensity at this organelle was higher at 30 minutes, when VSVG has reached the Golgi complex (Fig. 37A). Moreover, in the presence of VSVG, the fluorescence intensity of C1-PKCθ-GFP at the Golgi complex constantly increased after the temperature shift, while in the absence of VSVG C1-PKCθ-GFP localization at the Golgi complex remained unaltered from 10 to

30 minutes (Fig. 37B). This result confirms, for the first time, that cargo arrival at the Golgi complex stimulates the production of DAG at this organelle.

Once demonstrated that cargo arrival at the Golgi complex promotes DAG production, we studied if PLCγ1 could be the responsible of the increase of DAG at this organelle.

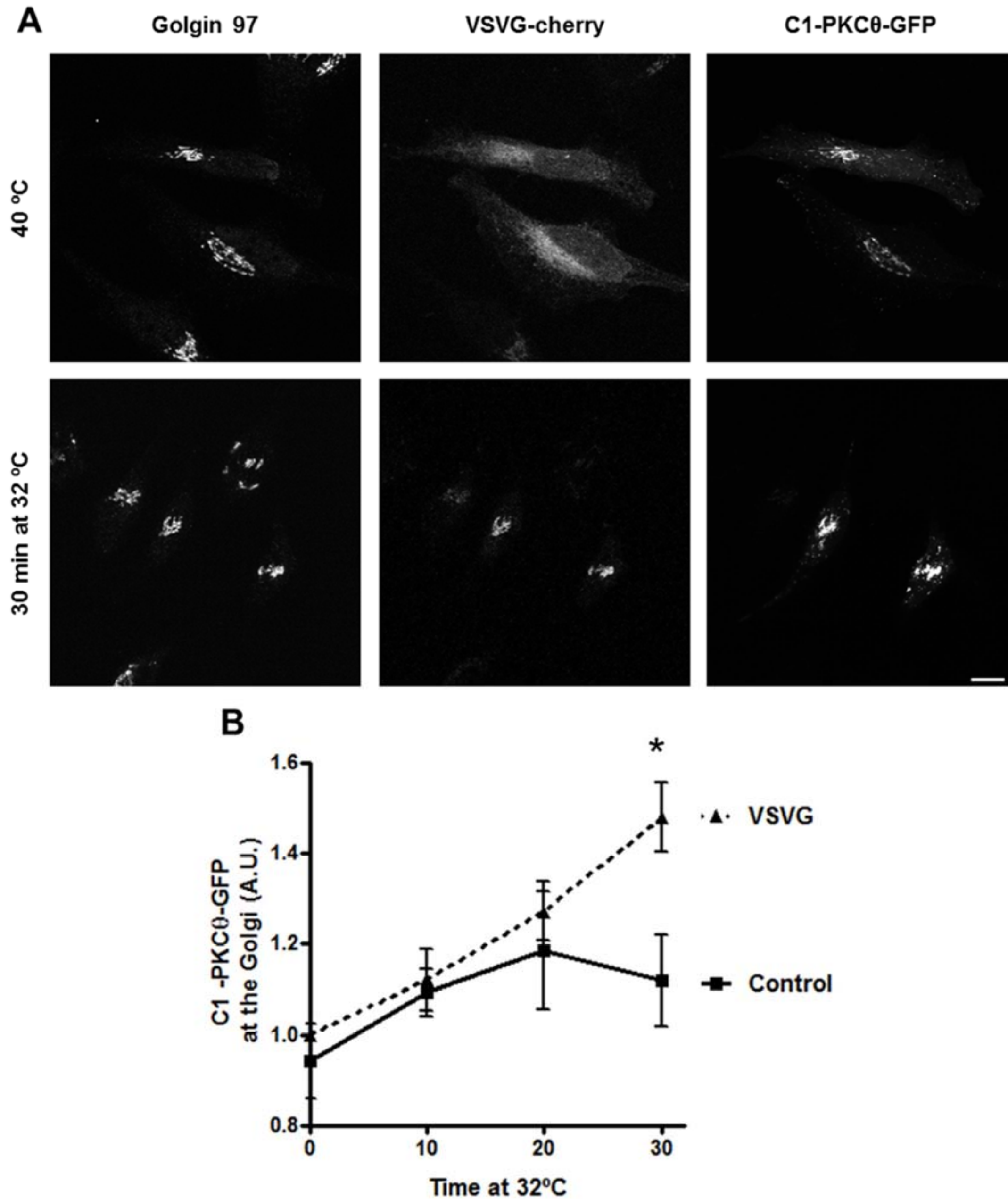

Figure 37. Cargo arrival promotes DAG formation at the Golgi complex. *A Hela cells were transfected with C1-PKCθ-GFP and VSVG-cherry and kept overnight at 40 ºC. After CHX addition, cells were shifted to 32 ºC and cells were fixed at indicated times and stained with Golgin97 to define Golgi area. B Quantification of C1-PKCθ-GFP localizatoin at the Golgi complex. Values are the mean ± S.D. from three independent experiments. Statistical significance according to two-way analysis of variance using Bonferroni's multiple comparison test (*, $p \leq 0.05$). Bar = 10 μm.*

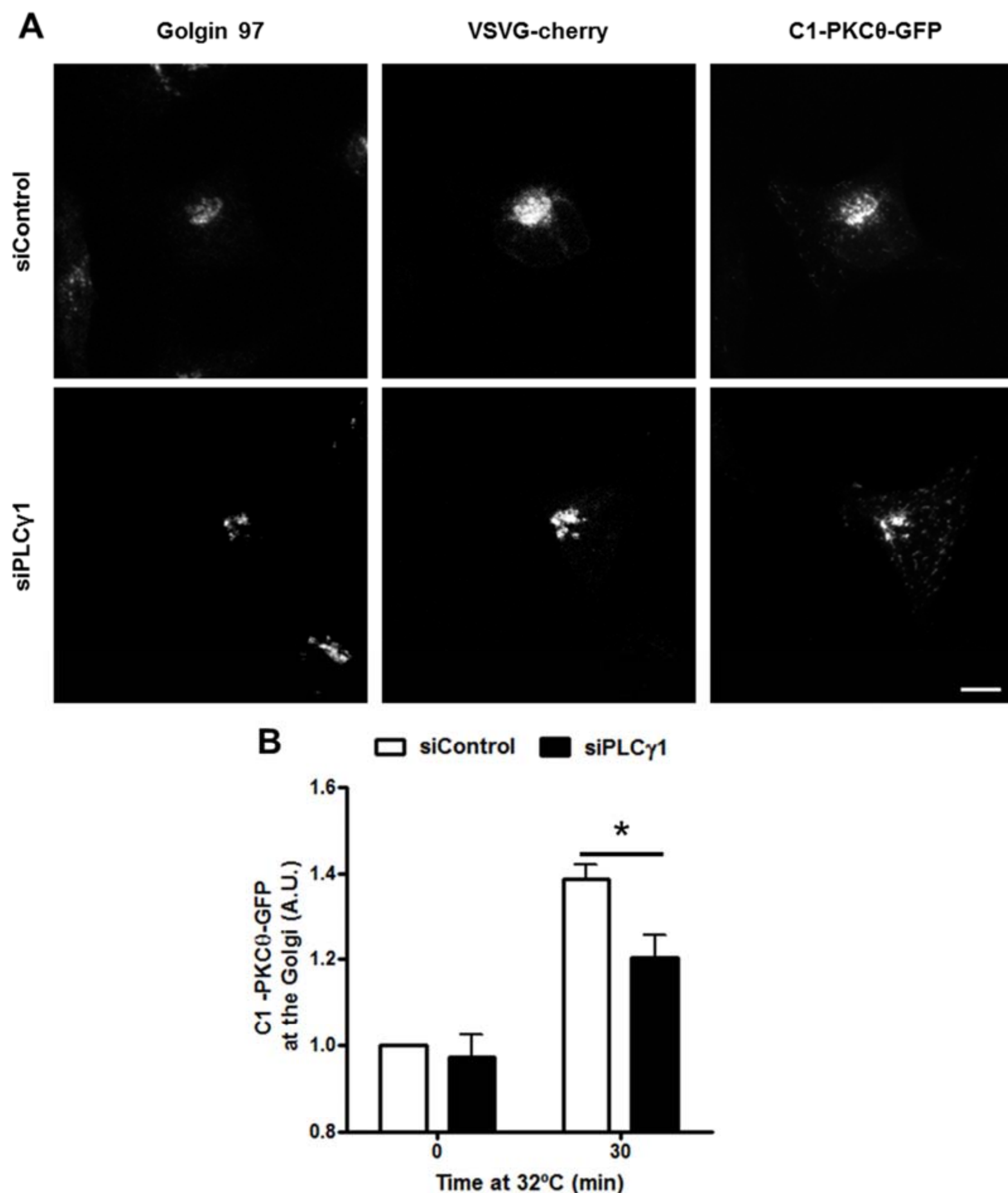

Figure 38. PLCγ1 silencing inhibits the increase of DAG at the Golgi induced by cargo arrival. *A HeLa cells transfected with control or PLCγ1 siRNA were transfected with C1-PKCθ-GFP and VSVG-cherry and kept overnight at 40 ºC. After CHX addition, cells were shifted to 32 ºC and cells were fixed at 0 and 30 minutes after the temperature shift and stained with Golgin97 to define Golgi area. B Quantification of C1-PKCθ-GFP localizatoin at the Golgi complex. Values are the mean ± S.D. from three independent experiments. Statistical significance according to two-way analysis of variance using Bonferroni's multiple comparison test (*, p ≤ 0.05). Bar = 10 μm.*

HeLa cells were transfected with control or PLCγ1 siRNA and co-transfected with C1-PKCθ-GFP and VSVG, in order to measure the localization of C1-PKCθ-GFP at the Golgi complex after VSVG arrival. We found that before the temperature shift, control and silenced cells had the same amount of C1-PKCθ-GFP at the Golgi complex (Fig. 38), but after 30 minutes at 32 ºC, when VSVG has reached the Golgi complex, control

cells showed higher levels of C1-PKCθ-GFP at the Golgi complex than cells silenced for PLCγ1 (Fig. 38). This result suggests that PLCγ1 is responsible, at least in part, of the DAG production at the Golgi complex induced by cargo arrival.

In order to test if PLCγ1 was the responsible for this DAG production by metabolizing the main phosphinositide, PI(4)P, at the Golgi complex, we studied the distribution of PH-OSBP-GFP before and after cargo arrival at the Golgi complex. PH-OSBP-GFP is

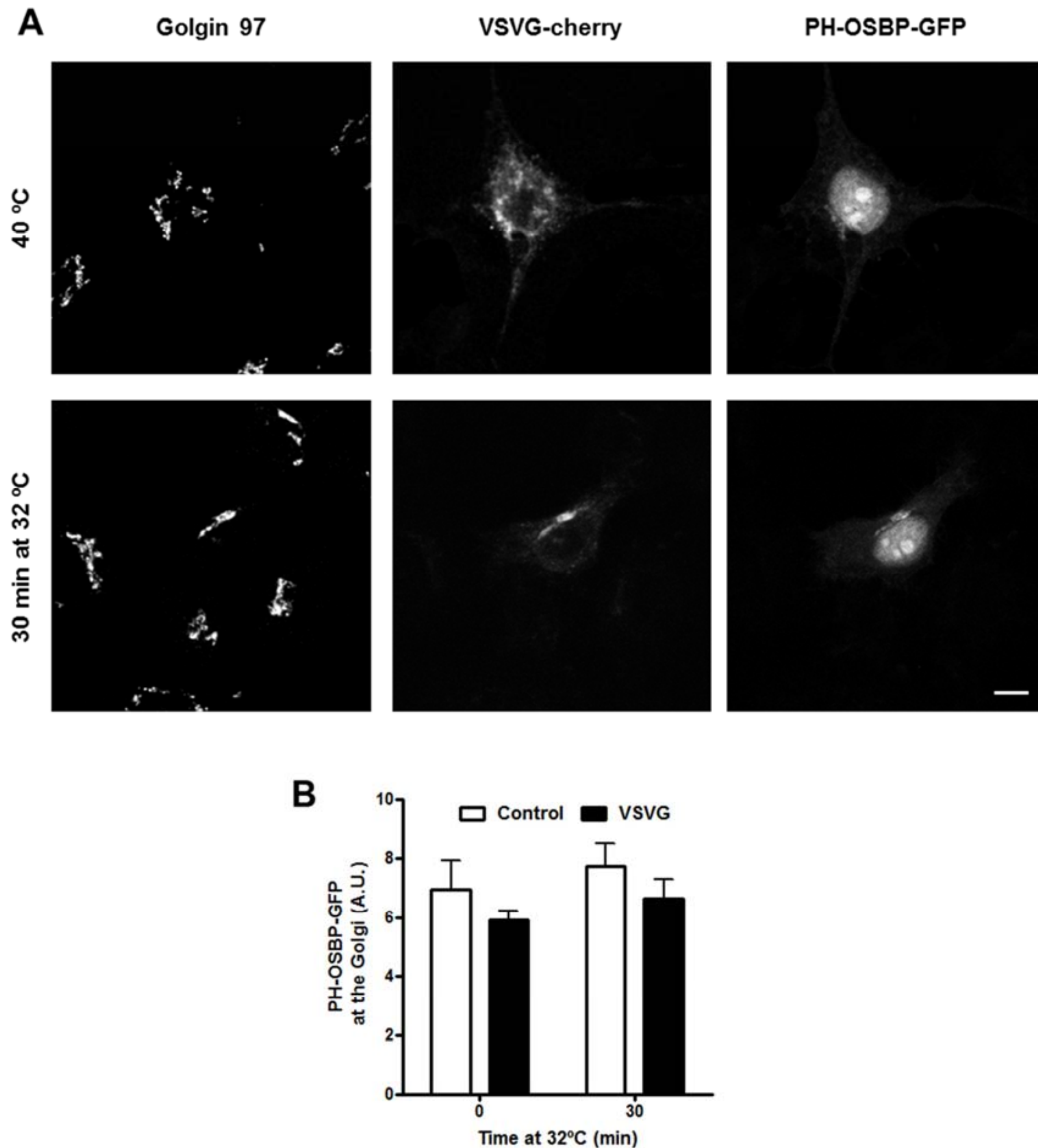

Figure 39. Cargo arrival does not alter the Golgi-associiated PI(4)P pool. *A HeLa cells were transfected with PH-OSBP-GFP and VSVG-cherry and kept overnight at 40 ºC. After CHX addition, cells were shifted to 32 ºC and cells were fixed at indicated times and stained with Golgin97 to define Golgi area. Bar = 10 µm.B Quantification of PH-OSBP-GFP localized at the Golgi complex. Values are the mean ± S.D. from three independent experiments.*

a construct that associates with the PI(4)P pool of the Golgi (Balla, 2005). We analysed PH-OSBP-GFP localization at the Golgi complex, following the same protocol for cargo arrival at the Golgi complex described for C1-PKCθ-GFP measurements. As previously described, we found PH-OSBP-GFP localized at the Golgi complex and also at the nucleus (Fig. 39A; Balla, 2005). Cargo arrival did not alter PH-OSBP-GFP localization at the Golgi complex (Fig. 39B), suggesting that the amount of PI(4)P pool of the Golgi complex is not significantly modified after cargo arrival at this organelle.

Next, we wanted to study the mechanisms underlying PLCγ1 activation after cargo arrival at the Golgi complex. The most known mechanism of PLCγ1 activation is by phosphorylation of its Tyr783 (Gresset, 2010) and it has been suggested that cargo arrival induces an increase of phosphotyrosines at the Golgi complex (Pulvirenti, 2008). For these reasons, we checked if cargo arrival activates PLCγ1 by phosphorylation on its Tyr783. HeLa cells constitutively expressing VSVG were kept at 40 ºC and then shifted to 32 ºC, in order to promote VSVG arrival at the Golgi complex and measure the levels of phosphorylation of PLCγ1 in its Tyr783 (pPLCγ1). As a control, cells were incubated with EGF, to confirm that we are able to measure an increase of pPLCγ1

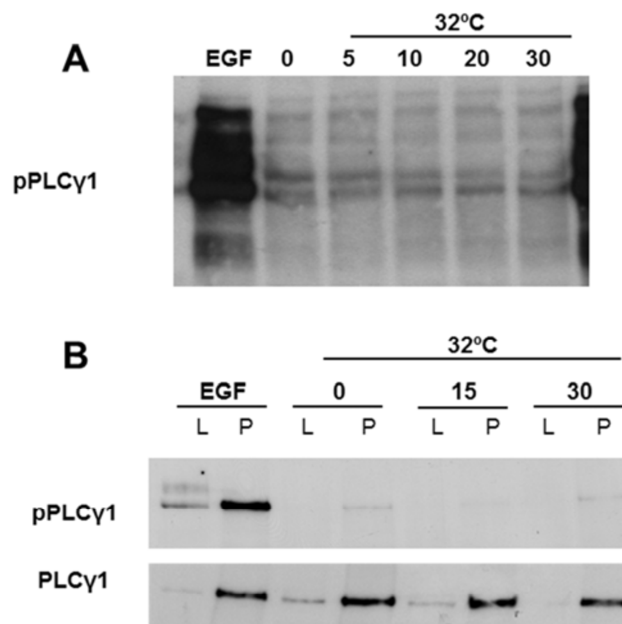

Figure 40. Cargo arrival at the Golgi does not modify the levels of phospho-tyr783 PLCγ1. *HeLa cells constitutively expressing VSVG-GFP were incubated at 40 ºC for 5 hours without FBS and then shifted to 32 ºC. Lysates were taken at indicated times and were processed for WB (A) or immunoprecipitated for PLCγ1 (B) and blotted against phospho-tyr783 PLCγ1 or total PLCγ1 (L= lysates, P=pellet). 100 ng/mL of EGF were added for 5 minutes as a positive control for detection of phospho-tyr783 PLCγ1.*

levels. However, the levels of pPLCγ1 were unaltered after the temperature shift in both cell lysates (Fig. 40A) and immunoprecipitated PLCγ1 (Fig. 40B). This unexpected result suggests that cargo arrival is activating PLCγ1 by another mechanism than phosphorylation on its Tyr783.

V. DISCUSSION

1.- Role of phospholipid synthesis in the regulation of the DAG needed for membrane trafficking at the Golgi complex

In the first part of this work, we postulate that the DAG-consuming steps of phospholipid synthetic pathways present at the Golgi contribute to the regulation of DAG needed for membrane traffic at this organelle.

1.1.- Role of DAG-consuming pathways in the control of cellular DAG

To study the contribution of DAG-consuming pathways in the regulation of DAG levels, we set up two different strategies to inhibit phospholipid synthesis. Our main aim was to design an experimental approach that, by inhibiting phospholipid synthesis, allowed us to change the total amount of DAG, but, at the same, was not inducing changes in other membrane lipids. Since DAG is a metabolic intermediate present in lower amounts than the other phospholipids, we thought that a quick and short tuning of the rate of phospholipid synthesis could be more likely regulating the amount of DAG than the end products of these biosynthetic pathways. We then hypothesised that the cell can take advantage of its own mechanisms to regulate the phospholipid synthetic pathways in order to control the DAG required for membrane trafficking at the Golgi complex. We have proven that short time inhibition of PC and PI synthesis, which was 60 min long in most of our experimental approaches, leads to an increase of cellular DAG. Moreover, as we hypothesized, our experimental design did not significantly affect the total mass of PC and PI, the end products of the metabolic pathways that we were inhibiting. This way, we defined a temporary window where, by inhibiting PC and PI synthesis, the amount of cellular DAG is increased, but PC and PI are not yet altered.

The importance of the duration of the inhibition of phospholipid synthesis to regulate DAG can be seen in other studies, where different results were obtained with similar experimental designs. It has been reported that PC synthesis inhibition in CHO-MT58 cells at the restrictive temperature, the same strategy used in our work, leads to an inhibition of protein transport at the Golgi complex (Testerink, 2009). In that study, the authors conclude that PC is needed for protein secretion at the Golgi complex, while we suggest that an inhibition of PC synthesis increases it. These apparently contradictory results are explained because in that work PC inhibition lasted 24 hours, while in our conditions was just for one hour. After a long time inhibition of PC synthesis not only the amount of PC is affected, but also the amount of other lipids of this synthetic pathway as DAG. In fact, it has also been reported that a 24 hour inhibition of PC synthesis in CHO-MT58 cells not only decrease PC levels, but also

produces a reduction of DAG (Van der Sanden, 2004), which correlates with and highlights our hypothesis that lower amounts of DAG impair protein transport from the Golgi complex. These results also reflect that long-time inhibition of a metabolic pathway can lead to changes not only in its end product, but also can affect its metabolic intermediates, as well as other synthetic pathways. For this reason, in this part of the work, we discarded silencing of regulatory enzymes of PC and PI synthesis. Silencing protocols usually last for a minimum of 72 hours, and along this time the lipid intermediate metabolites, as DAG, can be altered and/or diverted into other metabolic pathways, thus complicating the interpretation of the results obtained.

As described previously, DAG is an intermediate metabolite and its accumulation could lead to changes in the metabolic pathways and alter other classes of membrane lipids. Provided that our experimental conditions do not alter PC and PI, we measured other major phospholipid classes, such as PE, SM, PS, PA and the neutral lipid TAG. We found that at the permissive temperature CHO-MT58 cells present some inhibition of PC synthesis and also have different amounts of phospholipids and TAG than CHO-K1, the wild type control cells, which is in accordance with published results (Caviglia, 2004). We therefore wanted to make sure that our experimental conditions did not significantly alter the amount of phospholipids and TAG of both cell lines. Apart from the basal differences between CHO-K1 and CHO-MT58, we found that our experimental conditions did not modified neither phospholipids nor TAG, indicating that only the amount of cellular DAG is changing. However, we can not rule out that, by inhibiting phospholipid synthesis for a short time, there is a local increase of PA, any other phospholipid or TAG, undetectable by the measurement of the composition of the whole cell lipid extract. So, since DAG is the only measured lipid that is altered by our experimental conditions, we correlated all the changes measured in trafficking to this metabolite. Nonetheless, the results obtained for PA were unexpected. Because PA is also a lipid intermediate metabolite present at much lower levels than other phospholipids and a direct product of DAGK action on DAG, we expected an increase of PA concomitant to the DAG accumulation. However, the amount of PA was unaltered by our conditions, which could mean that PA is rapidly consumed to other metabolic pathways or that the particular DAG accumulated in our strategies is not a good substrate for DAGK.

1.2.- Role of DAG-consuming pathways in the control of the Golgi-associated DAG pool

It is widely accepted that the major site for PC and PI synthesis is the ER, but it is becoming accepted that DAG-consuming steps of phospholipid synthesis are also relevant at the Golgi complex (Chen, 2011). Since we designed our experimental conditions in order to regulate DAG at the Golgi complex, we wanted to confirm that, at least in part, the observed changes in the amount of DAG were taking place at this organelle. However, we were unable to resolve any increase of DAG at the Golgi complex after phospholipid synthesis inhibition. We discarded the option to isolate Golgi-enriched membrane fractions from CHO cells after phospholipid inhibition. The isolation of Golgi-enriched membrane fractions needs several washes and homogenization of both the composition of the buffer and the temperature, the factors that allowed us modify the rate of phospholipid synthesis. And besides, even if we were able to maintain the experimental conditions during the procedure, the time it takes is too long. Thus, this protocol will not ensure the preservation of the previously induced DAG alterations during membrane isolation. We also measured the localization of the DAG sensor C1-PKCθ-GFP at the Golgi complex after phospholipid synthesis inhibition by depletion of its substrates. Inhibition of PC and PI synthesis led to a small increase in C1-PKCθ-GFP localization at the Golgi complex, although this was not significant. One possible explanation is that the changes in DAG at the Golgi induced by phospholipid synthesis inhibition could be small and beyond the resolution of this assay. We ruled out the measurement of localization of C1-PKCθ-GFP in CHO-MT58 cells because temperature shift to 40 ºC reduced its localization to the Golgi complex and thus complicates data interpretation.

Although we could not resolve significant changes in DAG at the Golgi complex induced by the inhibition of phospholipid synthesis, we studied if it could have a functional effect. Our group previously reported that inhibition of DAG production by propanolol, which inhibits LPPs, increased the number of Golgi-associated buds. This effect could be reverted by exogenous addition of 2-dioctanoyl-sn-glycerol (DOG), a short chain DAG that could be incorporated into the Golgi complex, where it compensates the lowered DAG levels induced by propanolol (Fernández-Ulibarri, 2007). Inhibition of PC synthesis in CHO-MT58 cells had the same effect as DOG addition, preventing the increase of the Golgi-associated buds induced by propanolol. This results shows that most likely PC synthesis inhibition is increasing DAG at the Golgi complex, since it is able to compensate the effects produced by the reduction of DAG by propanolol.

This last result shows that phospholipid synthesis inhibition counteracts an effect that was produced by a reduction of DAG at the Golgi complex, but it does not specifically demonstrate that the reactions that consume DAG for phospholipid synthesis are taking place at this organelle. Phospholipid synthesis takes place mainly at the ER and the changes in DAG at the Golgi complex could be a reflex of an increase of DAG that is taking place at the ER and then is transported to the Golgi complex. TAG levels were unaltered after phospholipid synthesis inhibition, indicating that the increase of DAG was not diverted into neutral lipid synthesis. In spite of this, the addition of excess CMP induces a robust increase of DAG in Golgi-enriched fractions, suggesting that reactions of phospholipid synthesis are taking place at this organelle. We think that the chance for DAG to accumulate at the Golgi complex is higher than in the ER, because at the ER DAG can be more easily diverted to other metabolic pathways, like neutral lipid synthesis (Coleman, 2004). However, TAG levels were unaltered after phospholipid synthesis inhibition, indicating that the increase of DAG was not diverted significantly into neutral lipid synthesis in 1 h incubation time. All together these results strongly reinforce the hypothesis that the increase of DAG seen on cellular extracts is taking place at the Golgi complex.

1.3.- Role of DAG-consuming pathways in the regulation of membrane trafficking at the Golgi complex

Our first evidence that DAG-consuming pathways can be involved in membrane trafficking was the alteration that its regulation caused in the subcellular distribution of the KDELR at the ER-Golgi interface, where KDELR is continuously cycling. The reduction of KDELR positive particles cycling between these organelles when DAG was reduced by being consumed for PC and PI synthesis suggests an impairment of its trafficking, that could be caused at the generation of COPI transport carriers at the Golgi complex or at the biogenesis of COPII transport carriers at the ER. Some data support that the alteration in the biogenesis of KDELR-containing transport carriers is localized at the Golgi complex. First, we monitored the trafficking of VSVG from the ER to the Golgi complex in the same experimental conditions and no alteration was found. This result suggests that anterograde transport from the ER to the Golgi complex is not altered in our experimental conditions and, therefore, the impairment in KDELR traffic would likely be localized at the Golgi complex, where we showed that our experimental conditions can modify the amount of DAG. Moreover, the generation of COPI transport carriers has been reported to require DAG, but anterograde transport from the ER to the Golgi complex seems to be unaffected by DAG depletion (Fernández-Ulibarri, 2007).

Moreover, we found that the inhibition of phospholipid synthesis increased the post-Golgi trafficking of two different cargoes, VSVG and ssHRP; confirming the functional effects of DAG alterations in membrane trafficking by our experimental conditions at the Golgi complex. We observed that VSVG transport to the plasma membrane was blocked at the Golgi complex; but we did not know the precise subcellular location along the secretory pathway where the delay of ssHRP secretion took place. However, the transport of VSVG indicates that DAG regulated by phospholipid synthesis affects the exit from the Golgi but not from the ER, and this could also be suggested for ssHRP secretion.

The cycling of KDELR at the ER-Golgi interface allowed us to establish a correlation between the amount of cellular DAG and the number of KDELR transport carriers. We found that the correlation between these two parameters followed a hyperbola-shaped function. In this approach we measured cellular DAG, while it would have been more appropiated to measure DAG at the Golgi complex, since is at this organelle where DAG regulates the formation of KDELR transport carriers. However, since this organelle has abundant DAG content compared to the total cellular membranes, we suggest that changes in total cell DAG can well reflex changes in DAG at the Golgi complex. We counted the total of KDELR containing transport carriers, because we were unable to distinguish those trafficking from the ER to the Golgi complex from those who were trafficking retrogradely. In spite of these limitations, we found that the number of KDELR particles increased with the DAG content and the data matched with a hyperbola-shaped correlation. This correlation indicates that the number of KDELR transport carriers depends on the amount of DAG that can be consumed in phospholipid synthesis, in a manner that fits with an interaction of DAG with an effector molecule, as it could be for PKD for TGN-to-plasma membrane transport carriers (Baron and Malhotra, 2002). Such result would also be in accordance with the requirement of DAG as a membrane component that facilitates the curvature needed for the generation of transport carriers (Semesh, 2003).

1.4.- Physiological implications of the regulation of DAG at the Golgi complex by DAG-consuming pathways

Our results highlight the role of DAG-consuming pathways in the regulation of DAG at the Golgi complex. Most studies focus in the regulation of this metabolite by metabolic pathways that produce it (Baron and Malhotra, 2002; Fernández-Ulibarri, 2007; Subathra, 2011). Here, we have shown that the amount of DAG at the Golgi complex, and membrane trafficking at this organelle, can be also regulated by DAG-consuming

pathways. At the Golgi complex, a local inhibition of PC and PI synthesis can produce an increase of DAG at this organelle in order to promote membrane trafficking.

Interestingly, one of the regulatory mechanisms of CCT, the regulatory enzyme of the CDP-choline pathway, relies on its ability to sense the elastic stress of a membrane. CCT increases its activity on membranes with relatively low PC and enriched with DAG (Fagone and Jackowski, 2013). This regulatory mechanism suggests that the action of the CCT at the Golgi complex could be the resetting of the elastic stress of the membrane, by consuming DAG and restoring basal DAG levels, which could have been increased for transport carrier formation. CCT would produce again PC, which could by consumed for DAG production by the SMS at the *trans*-Golgi, thereby producing a "wave"' of DAG all through the Golgi complex. Moreover, it has been described that an increase of Ca^{2+} promotes CCT ubiquitinization at the Golgi complex (Chen, 2011) that promotes CCT degradation and therefore inhibit PC synthesis, a process previously seen and proposed to maintain high DAG levels in pancreatic acini (Matozaki, 1991). Cargo arrival at the Golgi complex triggers an increase of Ca^{2+} (Micaroni, 2010). Thus, a Ca^{2+} increase induced by cargo arrival would inhibit PC synthesis in order to maintain a temporary increase of DAG, which in turn would promote the generation of transport carriers to facilitate cargo exit at the Golgi complex.

2.- Role of PLC in the regulation of the DAG needed for membrane trafficking at the Golgi complex

2.1.- PLC activity in Golgi-enriched membrane fractions

As a first screening for the study of the role of PLC in the Golgi complex, we tried to measure PLC activity in Golgi-enriched membrane fractions. For this purpose, we measured the production of [^{32}P]PA in the presence of [^{32}P]ATP in Golgi-enriched membrane fractions, which reflects the rate of its production by DAGK from DAG, together with the changes of [^{32}P]PIP. In this context, the amount of [^{32}P]PIP depends on the activity of the enzymes that first incorporate the radiolabeled [^{32}P]ATP to [^{32}P]PIP, as the PI(4)K, and the enzymes that could metabolise [^{32}P]PIP, such as PLC or the phosphatase Sac1.

In these experiments, [^{32}P]PA production is in part indicative of DAG formation, since DAG is rapidly converted into PA by the action of DAGK. Production of [^{32}P]PA was dependent on the presence of cytosol. This could be explained because DAGKs, the enzymes that incorporate [^{32}P]ATP to [^{32}P]PA, are cytosolic. To confirm that this experimental design allowed the measurement of PLC activity coupled to DAGK, we

added recombinant PLC from *B.cereus* as a positive control. We observed that the addition of recombinant PLC led to a high increase of the labelling of [^{32}P]PA, confirming that in these experimental conditions the measurement of [^{32}P]PA could be a reporter of PLC activity. However, in these experiments we could not specify if any regulated increase of [^{32}P]PA production induced by cytosol addition is caused by DAGK alone or by PLC coupled to DAGK. To discriminate the action of PLC and DAGK in [^{32}P]PA production in Golgi-enriched membrane fractions with added cytosol, we used the general PLC inhibitor U73122 (Bleasdale, 1989). U73122 had an inhibitory effect on [^{32}P]PA production, although we could not absolutely trust the specificity of this compound for PLC, since a study published while we were realizing this work showed that U73122 had non-specific effects *in vitro*, where it can even increase PLC activity instead of inhibit it (Klein, 2011).

To further analyse the possibility that PLC could be acting on Golgi-enriched membrane fractions we analysed the dependence of [^{32}P]PA production in the presence of Ca^{2+} and GTPγS. Ca^{2+} is required for all PLCs to be active, while we expect that GTPγS stimulates the activity of PLCβ isoforms by the action of G proteins. In parallel, we analysed the consumption of [^{32}P]PIP in the same conditions. We observed that Ca^{2+} depletion impaired [^{32}P]PA production and [^{32}P]PIP consumption, while GTPγS depletion only impaired [^{32}P]PA production. These results are not conclusive, but allow us to suggest that if there is any PIP consuming PLC activity after the addition of cytosol to Golgi-enriched membrane fractions this PLC would be sensitive to Ca^{2+}, but independent of G protein activation.

However, the interpretation of the results from Golgi-enriched membrane fractions is not straightforward. Of note, another study used the same experimental conditions to measure [^{32}P]PA production in Golgi membranes by Arf stimulation, and concluded that ARF-stimulated [^{32}P]PA production was a result of the action of PLD (Godi, 1999). Although this was their interpretation, the incorporation of [^{32}P]Pi to [^{32}P]PA requires the action of DAGK, since PLD conversion of PC to PA does not incorporate a phosphate group of [^{32}P]ATP to [^{32}P]PA. Moreover, the inhibition of [^{32}P]PA production and of [^{32}P]PIP consumption caused by Ca^{2+} depletion that we hypothesise that could be PLC activity, could also be mixed up with the direct effect of Ca^{2+} on DAGKs or on the Sac1 phosphatase.

In spite of all these limitations, we felt courageous to focus in the possible action of PLCγ1 on the Golgi complex, rather PLCβ isoforms, which are activated by G proteins.

2.2.- Role of PLCγ1 in the regulation of Golgi secretory function and structure

Some previous studies involved PLCγ1 in Golgi events. First, it was reported that PLCγ1 is required for Ras activation at the Golgi complex (Bivona, 2003). More recently, it has been described that in keratinocytes PLCγ1 participates in Ca^{2+} uptake at this organelle (Tu, 2007). We found that PLCγ1 silencing led to an inhibition of the post-Golgi membrane trafficking of both soluble and transmembrane cargo, and led to a reduction of the Golgi-associated DAG pool. Since DAG is required for the formation of transport carriers at this organelle (Baron and Malhotra, 2002) and PLCγ1 silencing reduces it, with this data, we can suggest that PLCγ1 is involved in anterograde membrane trafficking at the Golgi complex by regulating DAG levels at this organelle. Whether the reduction of Golgi DAG reflects the reduction of DAG elsewhere in the cell after 72 h of PLCγ1 silencing or it is a consequence of a reduced PLCγ1 activity directly at the Golgi complex was still not resolved at this point.

PLCγ1 silencing or overexpression led to changes in the Golgi complex structure. PLCγ1 silencing led to a slight, but significant compactation of all Golgi compartments, from *cis*-Golgi to the TGN. Overexpression of PLCγ1, but not its inactive form, had the contrary effect, leading to a fragmentation of the *trans*-Golgi and to a less extent of the *cis*-Golgi. Interestingly, the Golgi fragmentation induced by PLCγ1 overexpression is in accordance with other studies that by overactivating the fission machinery also produce a fragmentation of this organelle. This effect was firstly seen by the action of IQ, which led to the fragmentation of the *trans*-compartments of the Golgi complex (Takizawa, 1993). The overexpression of a constitutively active form of PKD also promotes vesiculation of the Golgi complex (Bossard, 2007). The similarity of these effects to the fragmentation of the Golgi complex seen after PLCγ1 overexpression suggests that PLCγ1 could be part of the machinery for transport carrier formation at the Golgi complex. In this context, activation of the Src kinase also promoted fragmentation of the Golgi, which was shown to be dependent of dynamin 2 phosphorylation by Src (Weller, 2010). Taking into account that first: PLCγ1 could be activated by phosphorylation of its tyrosine 783; second: Src could act as an activator of PLCγ1 (Bivona, 2003), and third: the phosphorylation and dephosphorylation cycle in tyrosines can regulate the structure of the *trans*-Golgi with an increase in phospho-tyrosines leading to a fragmentation of the *trans*-Golgi, but not of the *cis* compartments (Webb, 2005), it is tempting to propose that PLCγ1 could act downstream of the Golgi fragmentation induced by Src (Weller, 2010). To confirm this, it would be interesting to check the phosphorylation state of PLCγ1 in cells with an overactivated Src. However It is not known why the *trans*-Golgi is more sensible to fragmentation than the *cis*-Golgi

under these conditions. One possible explanation relies on our results concerning the role of PLCγ1 in membrane trafficking, which affected the anterograde transport, but not the retrograde. Since the generation of transport carriers for anterograde transport, where we have observed that PLCγ1 participates, takes place at the TGN, which is in close proximity to the *trans*-Golgi, the overactivation of the fission machinery is more likely to alter the *trans*-Golgi rather than the *cis*-Golgi. Moreover, PLCγ1 has been localized at the TGN, where it is found to be part of a complex with TGN38, SPCA and CaR in keratinocytes (Tu, 2007), which could also explain the higher fragmentation of the TGN and the *trans*-Golgi in cells that overexpress PLCγ1 or present higher levels of phosphotyrosines.

2.3.- Role of PLCγ1 in the production of DAG after cargo arrival at the Golgi complex

The main physiological relevance of the finding that PLCγ1 could be regulating membrane trafficking at the Golgi complex is that PLCγ1 activation could be coupled to a potential signal triggered earlier in the secretory pathway. As previously proposed, but not addressed, the arrival of cargo at the Golgi could be the signal that triggers a signalling cascade at the Golgi complex to produce DAG for transport carrier formation (Bard and Malhotra, 2006). Recently, the basis of this hypothesis was strengthened by the finding that cargo arrival is triggering a signalling circuit at the Golgi complex that involves the KDELR and Src (Pulvirenti, 2008). To further investigate this, we studied if this signalling cascade triggered by cargo arrival could be promoting DAG production. We demonstrated for the first time that cargo arrival at the Golgi complex promotes DAG production. This finding shows that some of the metabolic pathways that control the amount of DAG at the Golgi complex are responding to a stimulus, the presence of cargo, in order to promote an increase of DAG at this organelle, probably for the generation of transport carries. Thus, it is tempting to propose that one goal of the signalling pathway involving the KDELR and Src at the Golgi complex, is the production of DAG, since inhibition of Src leads to an inhibition of the exit of cargo from this organelle (Pulvirenti, 2008).

Once found that DAG production at the Golgi complex is stimulated by cargo arrival, we assessed the role of PLCγ1 in this process. Silencing of PLCγ1 impaired the increase of DAG at the Golgi complex triggered by cargo arrival. This result not only involves PLCγ1 in the signalling cascade triggered by cargo at this organelle, but also highlights its role in the regulated DAG production for transport carrier formation. To date, some studies showed that a reduction of DAG production led to an inhibition of membrane trafficking at the Golgi complex, but none showed that the DAG-producing

pathway that was inhibited was the one stimulated by the cell in the need of transport carrier formation at the trans-Golgi (Baron and Malhotra, 2002; Fernández-Ulibarri, 2007; Subathra, 2011; Gutiérrez-Martinez, 2013). Thus, cargo-triggered PLCγ1-dependent production of DAG that we describe here is the first evidence of a regulated DAG production at the Golgi complex for the generation of transport carriers.

Other enzymes than PLCγ1 should be involved in this event. If DAG production for transport carrier formation was only dependent on PLCγ1, we would expect a complete inhibition of transport at the Golgi complex after PLCγ1 silencing. However, in our trafficking assays after PLCγ1 silencing we observe an inhibition, but not a complete block of the anterograde trafficking. This effect suggests that PLCγ1 is required for a quick and efficient production of DAG for transport carrier formation, but other DAG producing mechanisms can also contribute to this process. Another interpretation of our data could be that PLCγ1 responds to cargo arrival producing DAG, which recruits PKD, and then PKD activates a signalling pathway to efficiently produce DAG by SMS to generate transport carriers, as was previously suggested (Malhotra and Campelo, 2011). In the absence of PLCγ1, the generation of the DAG that recruits PKD could be diminished, thus reducing but not totally impairing the PKD localization at the Golgi complex. However, as said before, DAG generated by other mechanisms, either regulated or not, could also promote PKD recruitment and DAG formation downstream of PKD. The idea that the DAG produced by PLCγ1 have a signalling role to recruit PKD and trigger the machinery required for transport carrier formation downstream of PKD is reinforced by the results of our studies with the StxBKDEL, where we could not observe any significant change in the retrograde trafficking of StxBKDEL from the Golgi complex to the ER after PLCγ1 silencing. If the role for DAG produced by PLCγ1 at the Golgi complex is to recruit PKD, it could explain why PLCγ1 silencing has no effect on retrograde trafficking, since this transport pathway is independent of PKD. To confirm this hypothesis would be really interesting to measure PKD recruitment at the Golgi complex after cargo arrival and the involvement of PLCγ1 in this process.

Strikingly, the levels of phosphoPLCγ1 were unaltered after cargo arrival at the Golgi complex. Phosphorylation of the 783 tyrosine is the most common mechanism to activate PLCγ1 (Gresset, 2010). At the plasma membrane, PLCγ1 can be activated by Src (Bivona, 2002), which is activated after cargo arrival at the Golgi complex (Pulvirenti, 2008). Since we have observed a DAG production at the Golgi complex after cargo arrival that was dependent on PLCγ1, we expected that PLCγ1 could be activated by phosphorylation after cargo arrival. However, we did not observe changes in the amount of phosphorylation of the 783 tyrosine of PLCγ1, and therefore we could

not confirm the PLC γ1 activation by its classical way. But still, the action of PLCγ1 in DAG production after cargo arrival at the Golgi complex is clear. We have no result-based rationale for this observation, but we attempt some explanations, although speculative, for this finding. One possibility is that the changes of the amount of phosphorylated PLCγ1 were smaller than the resolution of the assay. Another explanation is based in the possibility that other mechanisms, different from phosphorylation, could be activating PLCγ1 after cargo arrival at the Golgi complex. In this context, it has been reported that an increase of Ca^{2+} is sufficient to activate both PLCγ1 and PLCγ2, independently of its phosphorylation state (Piechulek, 2005). After cargo arrival, the peri-Golgi Ca^{2+} concentration increased (Micaroni, 2010) and can affect the activities of Golgi-associated proteins (Micaroni, 2012), as could be happening with PLCγ1. We propose that the increase of the peri-Golgi Ca^{2+} concentration triggered by cargo arrival activates PLCγ1, promoting DAG formation for the generation of transport carriers at this organelle. To test this hypothesis, it would have been interesting to study the effects of Ca^{2+} chelation by BAPTA on DAG production after cargo arrival at the Golgi complex, but we ruled out the possibility to test this, since it has been previously described that BAPTA impairs protein transport from the ERGIC to the Golgi complex (Chen, 2002), making incompatible the arrival of cargo at the Golgi complex with Ca^{2+} chelation. Interestingly, BAPTA also impairs the exit of cargo from the Golgi complex (Chen, 2002). This effect of Ca^{2+} chelation on the post-Golgi transport would fit with a reduction of PLCγ1 activity and DAG production required for membrane trafficking at the Golgi complex. Moreover, it has been described that an increase of Ca^{2+} promotes DAG production at the Golgi complex and enhances PKD recruitment to this organelle (Kunkel and Newton, 2010). In this study, the authors rule out that the increase of DAG seen after Ca^{2+} stimulation could be produced by the action of PLC, since edelfosine does not inhibit DAG production at the Golgi complex, but inhibits DAG accumulation at the plasma membrane. However, the precise mechanism of action of edelfosine it is not known. It has been shown that after interaction with $PI(4,5)P_2$ at the plasma membrane, the substrate of PLC, it interferes with PLC activity (Powis, 1992). But, to date, it is not known if edelfosine can inhibit PLC activity by interfering on phosphoinositides found in endomembras, such as PI(4)P at the Golgi complex. So, if edelfosine were not interacting with PI(4)P still PLC could be the responsible for the described DAG production at the Golgi stimulated by Ca^{2+}.

We then measured the levels of PI(4)P at the Golgi complex after cargo arrival with the localization of PH-OSBP-GFP at this organelle. PI(4)P is the principal phosphoinositide at the Golgi complex and can also be metabolised by PLC (Claro, 1993). We did not

find a decrease on PI(4)P that matched with the observed increase of DAG. This apparently contradictory observation has some possible explanations. The first one is based on the resolution of the assay. Although we have seen changes in DAG by using the localization of C1-PKCθ-GFP at the Golgi complex, the resolution of these two constructs may be different. Thus, a significant increase of DAG by PLCγ1 activity by measurement of C1-PKCθ-GFP localisation has not necessarily be paralleled with a significant decrease in the localisation of PH-OSBP-GFP. Moreover, as we propose, DAG production after cargo arrival could not be only from PLC activity and other mechanisms could act downstream of PLCγ1, thus increasing the difference between the relative changes of DAG and PI(4)P at the Golgi complex. Another possibility is that we do not observe changes in the localisation of PH-OSBP-GFP because PLCγ1 hydrolyses PI(4,5)P_2, its most common substrate, which is proposed to be also found at the Golgi complex (Godi, 1999). It would be interesting to determine which phosphoinostide is the substrate of PLCγ1 at the Golgi complex to know how this can affect Ca^{2+} signalling. Hydrolysis of PI(4,5)P_2 generates DAG and IP_3, which promotes the release of Ca^{2+} from cellular stores as the Golgi complex and the ER. However, if PLCγ1 hydrolyses PI(4)P the products of the reaction will be DAG and IP_2, which does not affect Ca^{2+} release. Since Ca^{2+} may be playing a critical role in PLCγ1 activation at the Golgi complex, it would be important to know if PLCγ1 is coupled to an increase of Ca^{2+}, which would feedforward the signal to sustain PLCγ1 activation. If PI(4)P is the substrate in PLCγ1 at the Golgi complex, this Ca^{2+} would not take place and the physiological relevance of PLCγ1 action would only relay on DAG production.

3.- Summary

In this work, we show that DAG-consuming pathways and PLCγ1 can regulate the DAG required for membrane trafficking at the Golgi complex, but with different relevance for anterograde and retrograde transport. An integration model of the action of both processes could be as follows. For the generation of post-Golgi transport carriers, cargo arrival, by increasing the peri-Golgi Ca^{2+} concentration, activates PLCγ1 which produces a signalling DAG that recruits PKD. Then, PKD promotes DAG formation by indirect stimulation of SMS, which could facilitate the fission of transport carriers. DAG produced by this with pathway could be accumulated because, at the same time, Ca^{2+} inhibits PC synthesis by promoting CCT degradation, thus inhibiting DAG consumption. After the Ca^{2+} increase and cargo passage through the Golgi, the remaining CCT at the Golgi complex could be activated by sensing the increase of DAG on Golgi membranes, thus restoring DAG and PC levels at the Golgi complex, leaving the system ready for another round of cargo arrival (Fig.41).

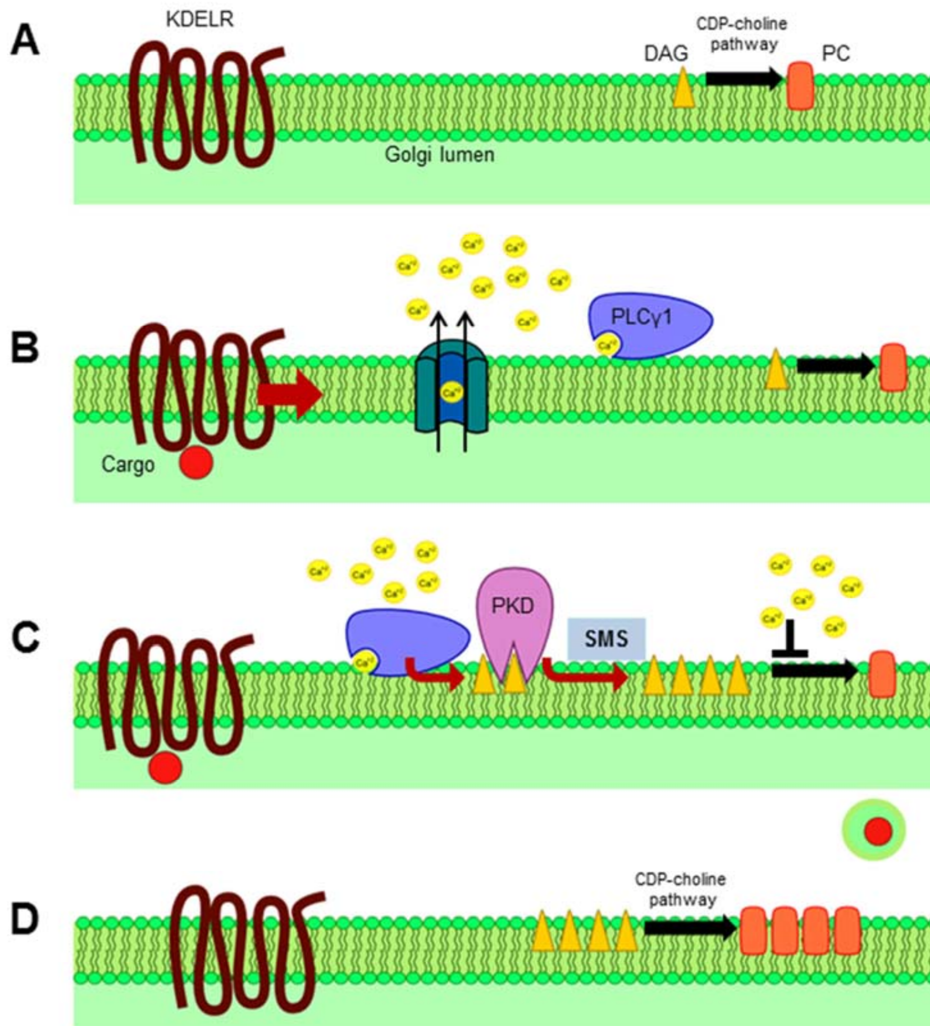

Figure 41. Proposed model for the generation of post-Golgi transport carriers. *A) In the absence of cargo, KDLER remains unactivated and the CDP-choline pathway maintain the levels of DAG. B) Cargo arirval triggers the activation of the KDELR, which, by unknown mechanisms, promotes Ca^{2+} release from the Golgi Complex, thus increasing the peri-Golgi Ca2+ concentration. This increase of Ca2+ activates PLCγ1 at the Golgi complex. C) PLCγ1 produces DAG, which recruits and activates PKD at the Golgi complex, stimulating DAG production by SMS. At the same time, the CDP-choline pathway is inhibited by the increase of the Ca2+ concentration, allowing the accumulation of DAG necessary for the formation of transport carriers. D) After fission of the transport carrier and passage of cargo, Ca2+ concentrarion is restored and the CDP-choline pathway consumes the excess DAG in order to reset the Golgi membrane compostion for another round of transport carrier formation.*

On the other hand, retrograde membrane trafficking could be also regulated by an increase of Ca^{2+} triggered by cargo arrival at the Golgi complex. This increase of Ca^{2+} would inhibit the CDP-choline pathway as described before, but would have no effect on the activity of PLCγ1 or one of its downstream targets. LPP3 could be producing DAG and its accumulation by the inhibition of PC synthesis would be sufficient to promote the formation of transport carriers that travel from the Golgi complex to the

ER. After fission of the transport carrier and the decrease of Ca^{2+}, the system could be restored by the activation of CCT by sensing the increase of DAG on Golgi membranes, as proposed for post-Golgi trafficking (Fig.42).

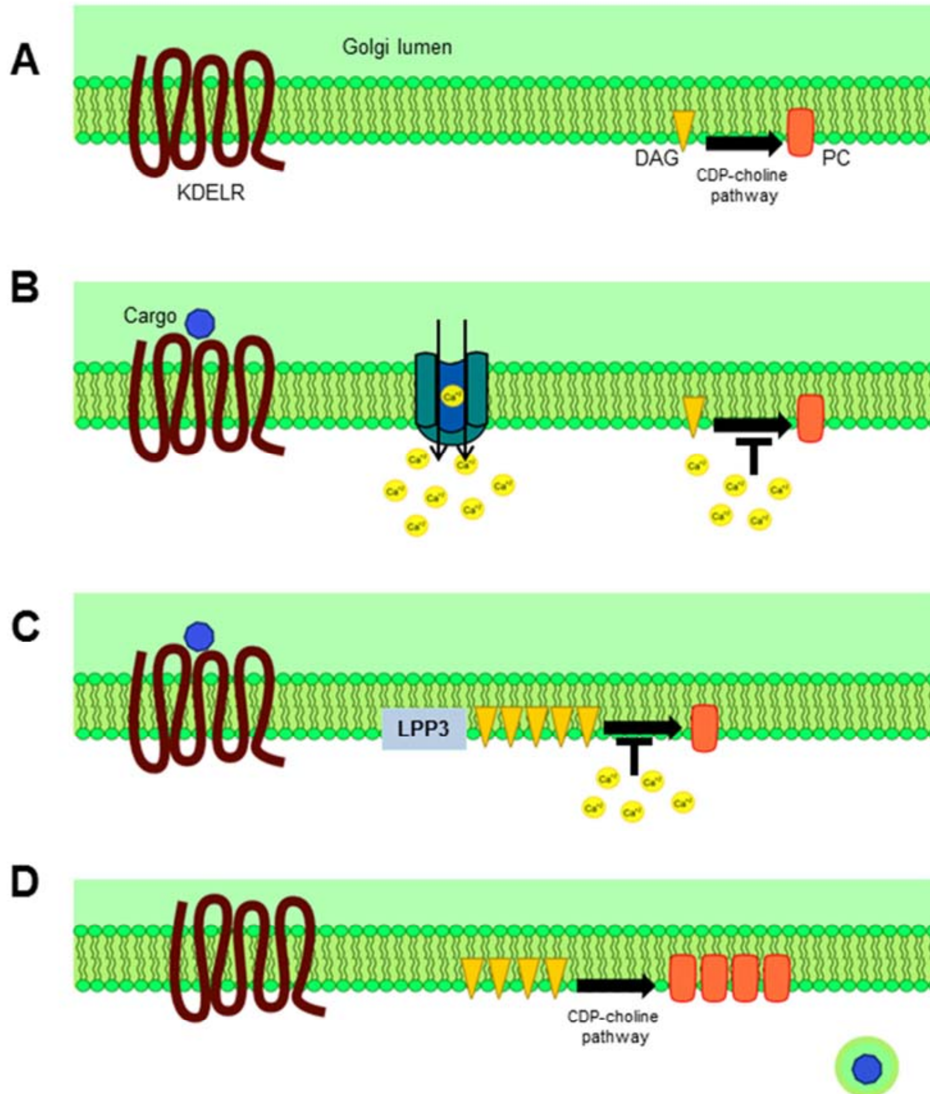

Figure 42. Proposed model for the generation of retrograde transport carriers. *A) In the absence of cargo, KDLER remains unactivated and the CDP-choline pathway maintain the levels of DAG. B) Cargo arirval triggers the activation of the KDELR, which, by unknown mechanisms, promotes Ca^{2+} that inhibits the CDP-choline pathway. C) The inhibition of the CDP-choline pathway leads to DAG accumulation, which can be produced by LPP3, that promotes the formation of transport carriers from the Golgi complex to the ER. D) After fission of the transport carrier and passage of cargo, Ca2+ concentrarion is restored and the CDP-choline pathway consumes the excess DAG in order to reset the Golgi membrane compostion for another round of transport carrier formation.*

VI. CONCLUSIONS

1.- Metabolic pathways for the synthesis of phospholipids that consume DAG regulate its levels at the Golgi complex.

2.- Phospholipid synthesis controls the levels of DAG needed for both retrograde and anterograde trafficking at the Golgi complex.

3.- Cargo arrival at the Golgi complex promotes DAG production.

4.- PLCγ1 is involved in the production of DAG triggered by cargo arrival at the Golgi complex.

5.- PLCγ1 is needed for post-Golgi transport and maintenance of the structure of the Golgi complex.

VII. REFERENCES

Ahyayauch H, Villar AV, Alonso A, Goñi FM (2005) Modulation of PI-specific phospholipase C by membrane curvature and molecular order. Biochemistry. 44:11592-11600.

Alderton F, Darroch P, Sambi B, McKie A, Ahmed IS, Pyne N, Pyne S (2001) G-protein-coupled receptor stimulation of the p42/p44 mitogen-activated protein kinase pathway is attenuated by lipid phosphate phosphatases 1, 1a, and 2 in human embryonic kidney 293 cells. J Biol Chem. 276:13452-13460.

Allen V, Swigart P, Cheung R, Cockcroft S, Katan M (1997) Regulation of inositol lipid-specific phospholipase cdelta by changes in Ca2+ ion concentrations. Biochem J. 327:545-52.

Antonny B, Huber I, Paris S, Chabre M, Cassel D (1997) Activation of ADP-ribosylation factor 1 GTPase-activating protein by phosphatidylcholine-derived diacylglycerols. J Biol Chem. 272:30848-30851.

Antonsson B (1997) Phosphatidylinositol synthase from mammalian tissues. Biochim Biophys Acta. 19971348:179-186

Asp L, Kartberg F, Fernandez-Rodriguez J, Smedh M, Elsner M, Laporte F, Bárcena M, Jansen KA, Valentijn JA, Koster AJ, Bergeron JJ, Nilsson T (2009) Early stages of Golgi vesicle and tubule formation require diacylglycerol. Mol Biol Cell. 20:780-790

Balch WE, Dunphy WG, Braell WA, Rothman JE (1984) Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. Cell. 39:405-16.

Balla A, Tuymetova G, Tsiomenko A, Várnai P, Balla T (2005) A plasma membrane pool of phosphatidylinositol 4-phosphate is generated by phosphatidylinositol 4-kinase type-III alpha: studies with the PH domains of the oxysterol binding protein and FAPP1. Mol Biol Cell. 16:1282-1295

Bankaitis VA, Malehorn DE, Emr SD, Greene R (1989) The Saccharomyces cerevisiae SEC14 gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. J Cell Biol. 108:1271-1281.

Bannykh SI, Balch WE (1997) Membrane dynamics at the endoplasmic reticulum-Golgi interface. J Cell Biol. 1997 138:1-4

Bannykh SI, Nishimura N, Balch WE (1998) Getting into the Golgi. Trends Cell Biol. 8:21-25.

Bard F, Malhotra V (2006) The formation of TGN-to-plasma-membrane transport carriers. Annu Rev Cell Dev Biol. 22:439-455

Bard F, Mazelin L, Péchoux-Longin C, Malhotra V, Jurdic P (2003) Src regulates Golgi structure and KDEL receptor-dependent retrograde transport to the endoplasmic reticulum. J Biol Chem. 2003 Nov 21;278(47):46601-46606.

Barlowe C, Orci L, Yeung T, Hosobuchi M, Hamamoto S, Salama N, Rexach MF, Ravazzola M, Amherdt M, Schekman R (1994) COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell. 77:895-907.

Baron CL, Malhotra V (2002) Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane. Science. 295:325-328

Bartke N, Hannun YA (2009) Bioactive sphingolipids: metabolism and function. J Lipid Res. 50 Suppl:S91-96.

Bell RM, Ballas LM, Coleman RA (1981) Lipid topogenesis. J Lipid Res. 22:391-403.

Bethune J, Kol M, Hoffmann J, Recjmann I, Brugger B, Wieland F (2006) Coatomer, the coat protein of COPI transport vesicles, discriminates endoplasmic reticulum residents from p24 proteins. Mol Cell Biol 26:8011:8021.

Bi K, Roth MG, Ktistakis NT (1997) Phosphatidic acid formation by phospholipase D is required for transport from the endoplasmic reticulum to the Golgi complex. Curr Biol. 7:301-307.

Biddlecome GH, Berstein G, Ross EM (1996) Regulation of phospholipase C-beta1 by Gq and m1 muscarinic cholinergic receptor. Steady-state balance of receptor-mediated activation and GTPase-activating protein-promoted deactivation. J Biol Chem. 271:7999-8007.

Bigay J, Casella JF, Drin G, Mesmin B, Antonny B (2005) ArfGAP1 responds to membrane curvature through the folding of a lipid packing sensor motif. EMBO J. 24:2244-2253.

Bigay J, Gounon P, Robineau S, Antonny B (2003) Lipid packing sensed by ArfGAP1 couples COPI coat disassembly to membrane bilayer curvature. Nature. 426:563-566.

Bivona TG, Pérez De Castro I, Ahearn IM, Grana TM, Chiu VK, Lockyer PJ, Cullen PJ, Pellicer A, Cox AD, Philips MR (2003) Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasGRP1. Nature. 424:694-698.

Bivona TG, Philips MR (2003) Ras pathway signaling on endomembranes. Curr Opin Cell Biol. 15:136-142.

Blagoveshchenskaya A, Cheong FY, Rohde HM, Glover G, Knödler A, Nicolson T, Boehmelt G, Mayinger P (2008) Integration of Golgi trafficking and growth factor signaling by the lipid phosphatase SAC1.J Cell Biol. 180:803-812

Blázquez M, Shennan KI (2000) Basic mechanisms of secretion: sorting into the regulated secretory pathway. Biochem Cell Biol. 78:181-191.

Bleasdale JE, Bundy GL, Bunting S, Fitzpatrick FA, Huff RM, Sun FF, Pike JE (1989) Inhibition of phospholipase C dependent processes by U-73, 122. Adv Prostaglandin Thromboxane Leukot Res. 19:590-593.

Bonifacino JS, Glick BS (2004) The mechanisms of vesicle budding and fusion. Cell. 116:153-166.

Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem. 72:395-447

Bossard C, Bresson D, Polishchuk RS, Malhotra V (2007) Dimeric PKD regulates membrane fission to form transport carriers at the TGN. J Cell Biol. 179:1123-1131

Brandizzi F, Barlowe C (2013) Organization of the ER-Golgi interface for membrane traffic control. Nat Rev Mol Cell Biol 14:382-392.

Bremser M, Nickel W, Schweikert M, Ravazzola M, Amherdt M, Hughes CA, Söllner TH, Rothman JE, Wieland FT (1999) Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. Cell. 1999 96:495-506.

Brindley DN, Pilquil C, Sariahmetoglu M, Reue K (2009) Phosphatidate degradation: phosphatidate phosphatases (lipins) and lipid phosphate phosphatases. Biochim Biophys Acta. 1791:956-961.

Brown HA, Gutowski S, Moomaw CR, Slaughter C, Sternweis PC (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. Cell. 75:1137-1144.

Bunney TD, Katan M (2011) PLC regulation: emerging pictures for molecular mechanisms. Trends Biochem Sci. 36:88-96.

Cabrera M, Muñiz M, Hidalgo J, Vega L, Martín ME, Velasco A (2003) The retrieval function of the KDEL receptor requires PKA phosphorylation of its C-terminus. Mol Biol Cell. 14:4114-4125.

Campelo F, Malhotra V (2012) Membrane fission: the biogenesis of transport carriers. Annu Rev Biochem. 81:407-427

Cancino J, Jung JE, Luini A (2013) Regulation of Golgi signaling and trafficking by the KDEL receptor. Histochem Cell Biol. 140:395-405.

Cancino J, Luini A (2013) Signaling circuits on the Golgi complex. Traffic 14:121-134.

Carrasco S, Merida I (2004) Diacylglycerol-dependent binding recruits PKCtheta and RasGRP1 C1 domains to specific subcellular localizations in living T lymphocytes. Mol Biol Cell. 15:2932-2942

Carrasco S, Mérida I (2007) Diacylglycerol, when simplicity becomes complex. Trends Biochem Sci. 32:27-36.

Carvou N, Holic R, Li M, Futter C, Skippen A, Cockcroft S (2010) Phosphatidylinositol- and phosphatidylcholine-transfer activity of PITPbeta is essential for COPI-mediated retrograde transport from the Golgi to the endoplasmic reticulum. J Cell Sci. 123:1262-1267

Caviglia JM, De Gómez Dumm IN, Coleman RA, Igal RA (2004) Phosphatidylcholine deficiency upregulates enzymes of triacylglycerol metabolism in CHO cells. J Lipid Res. 45:1500-9.

Chen BB, Coon TA, Glasser JR, Mallampalli RK (2011) Calmodulin antagonizes a calcium-activated SCF ubiquitin E3 ligase subunit, FBXL2, to regulate surfactant homeostasis. Mol Cell Biol. 31:1905-1920.

Chen JL, Ahluwalia JP, Stamnes M (2002) Selective effects of calcium chelators on anterograde and retrograde protein transport in the cell. J Biol Chem. 277:35682-35687

Chia J, Goh G, Racine V, Ng S, Kumar P, Bard F (2012) RNAi screening reveals a large signaling network controlling the Golgi apparatus in human cells. Mol Syst Biol. 8:629.

Claro E, Sarri E, Picatoste F (1993) Endogenous phosphoinositide precursors of inositol phosphates in rat brain cortical membranes. Biochem Biophys Res Commun. 193:1061-1067.

Cleves A, McGee T, Bankaitis V (1991) Phospholipid transfer proteins: a biological debut. Trends Cell Biol. 1:30-34.

Cockcroft S, Thomas GM, Fensome A, Geny B, Cunningham E, Gout I, Hiles I, Totty NF, Truong O, Hsuan JJ (1994) Phospholipase D: a downstream effector of ARF in granulocytes. Science. 263:523-526.

Coleman RA, Lee DP (2004) Enzymes of triacylglycerol synthesis and their regulation. Prog Lipid Res. 43:134-176.

Conner SD, Schmid SL (2004) Regulated portals of entry into the cell. Nature. 422:37-44.

Cukierman E, Huber I, Rotman M, Cassel D (1995) The ARF1 GTPase-activating protein: zinc finger motif and Golgi complex localization. Science. 270:1999-2002.

D'Angelo G, Polishchuk E, Di Tullio G, Santoro M, Di Campli A, Godi A, West G, Bielawski J, Chuang CC, van der Spoel AC, Platt FM, Hannun YA, Polishchuk R, Mattjus P, De Matteis MA (2007) Glycosphingolipid synthesis requires FAPP2 transfer of glucosylceramide. Nature. 449:62-67

De Matteis MA, Luini A (2008) Exiting the Golgi complex. Nat Rev Mol Cell Biol. 9:273-284

De Matteis MA, Santini G, Kahn RA, Di Tullio G, Luini A (1993) Receptor and protein kinase C-mediated regulation of ARF binding to the Golgi complex. Nature. 364:818-821.

Denker SP, McCaffery JM, Palade GE, Insel PA, Farquhar MG (1996) Differential distribution of alpha subunits and beta gamma subunits of heterotrimeric G proteins on Golgi membranes of the exocrine pancreas. J Cell Biol. 133:1027-1040.

Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics.Nature. 443:651-657

Díaz Añel AM (2007) Phospholipase C beta3 is a key component in the Gbetagamma/PKCeta/PKD-mediated regulation of trans-Golgi network to plasma membrane transport. Biochem J. 406:157-165

Díaz Añel AM, Malhotra V (2005) PKCeta is required for beta1gamma2/beta3gamma2- and PKD-mediated transport to the cell surface and the organization of the Golgi apparatus. J Cell Biol. 169:83-91.

Doherty GJ, McMahon HT (2009) Mechanisms of endocytosis. Annu Rev Biochem. 78:857-902.

Donaldson JG, Kahn RA, Lippincott-Schwartz J, Klausner RD (1991) Binding of ARF and beta-COP to Golgi membranes: possible regulation by a trimeric G protein. Science. 254:1197-1199.

Du G, Huang P, Liang BT, Frohman MA (2004) Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis. Mol Biol Cell. 15:1024-1030.

Duden R, Griffiths G, Frank R, Argos P, Kreis TE (1991) Beta-COP, a 110 kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to beta-adaptin. Cell 64:649-665.

Duran JM, Campelo F, van Galen J, Sachsenheimer T, Sot J, Egorov MV, Rentero C, Enrich C, Polishchuk RS, Goñi FM, Brügger B, Wieland F, Malhotra V (2012) Sphingomyelin organization is required for vesicle biogenesis at the Golgi complex. EMBO J. 31:4535-4546.

Edelman AM, Blumenthal DK, Krebs EG (1987) Protein serine/threonine kinases. Annu Rev Biochem. 56:567-613

Egea G (2001) Train trip into the cell: the Golgi Apparatus as the central station of the intracellular membrane traffic. Ciencia al día Internacional 4:1-20.

Egea G, Ríos RM (2008) The role of the cytoskeleton in the structure and function of the Golgi apparatus. The Golgi Apparatus – State of the art 110 after camillo's Golgi discovery, 270-300.

Egea G, Serra-Peinado C, Salcedo-Sicilia L, Gutiérrez-Martínez E (2013) Actin acting at the Golgi. Histochem Cell Biol. 140:347-360

Esko JD, Wermuth MM, Raetz CR (1981) Thermolabile CDP-choline synthetase in an animal cell mutant defective in lecithin formation. J Biol Chem. 256:7388-7393.

Eugster A, Frigerio G, Dale M, Duden R (2000) COP I domains required for coatomer integrity, and novel interactions with ARF and ARF-GAP. EMBO J. 19:3905-3917.

Fagone P, Jackowski S (2013) Phosphatidylcholine and the CDP-choline cycle. Biochim Biophys Acta. 1831:523-532

Farhan H, Rabouille C (2011) Signalling to and from the secretory pathway. J Cell Sci. 124:171-178

Farhan H, Wendeler MW, Mitrovic S, Fava E, Silberberg Y, Sharan R, Zerial M, Hauri HP (2010) MAPK signaling to the early secretory pathway revealed by kinase/phosphatase functional screening. J Cell Biol. 189:997-1011

Fernández-Ulibarri I, Vilella M, Lázaro-Diéguez F, Sarri E, Martínez SE, Jiménez N, Claro E, Mérida I, Burger KN, Egea G (2007) Diacylglycerol is required for the formation of COPI vesicles in the Golgi-to-ER transport pathway. Mol Biol Cell. 18:3250-3263.

Foti M, Audhya A, Emr SD (2001) Sac1 lipid phosphatase and Stt4 phosphatidylinositol 4-kinase regulate a pool of phosphatidylinositol 4-phosphate that functions in the control of the actin cytoskeleton and vacuole morphology. Mol Biol Cell. 12:2396-2411.

Freyberg Z, Siddhanta A, Shields D (2003) "Slip, sliding away": phospholipase D and the Golgi apparatus.Trends Cell Biol. 13:540-546.

Freyberg Z, Sweeney D, Siddhanta A, Bourgoin S, Frohman M, Shields D (2001) Intracellular localization of phospholipase D1 in mammalian cells. Mol Biol Cell. 12:943-955.

Gasull T, Sarri E, DeGregorio-Rocasolano N, Trullas R (2003) NMDA receptor overactivation inhibits phospholipid synthesis by decreasing choline-ethanolamine phosphotransferase activity. J Neurosci. 23:4100-4107.

Giannotta M, Ruggiero C, Grossi M, Cancino J, Capitani M, Pulvirenti T, Consoli GM, Geraci C, Fanelli F, Luini A, Sallese M (2012) The KDEL receptor couples to Gαq/11 to activate Src kinases and regulate transport through the Golgi. EMBO J. 31:2869-2881.

Girod A, Storrie B, Simpson JC, Johannes L, Goud B, Roberts LM, Lord JM, Nilsson T, Pepperkok R (1999) Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. Nat Cell Biol. 7:423-430.

Glick BS, Luini A (2011) Models for Golgi traffic: a critical assessment. Cold Spring Harb Perspect Biol. 3:a005215

Glick BS, Malhotra V (1998) The curious status of the Golgi apparatus. Cell. 95:883-889

Godi A, Pertile P, Meyers R, Marra P, Di Tullio G, Iurisci C, Luini A, Corda D, De Matteis MA (1999) ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex. Nat Cell Biol. 1:280-287.

Gommel DU, Memon AR, Heiss A, Lottspeich F, Pfannstiel J, Lechner J, Reinhard C, Helms JB, Nickel W, Wieland FT (2001) Recruitment to Golgi membranes of ADP-ribosylation factor 1 is mediated by the cytoplasmic domain of p23. EMBO J. 20:6751-6760.

Goñi FM, Alonso A (1999) Structure and functional properties of diacylglycerols in membranes. Prog Lipid Res. 38:1-48

Gresset A, Hicks SN, Harden TK, Sondek J (2010) Mechanism of phosphorylation-induced activation of phospholipase C-gamma isozymes. J Biol Chem. 285:35836-35847.

Gresset A, Sondek J, Harden TK (2012) The phospholipase C isozymes and their regulation. Subcell Biochem. 58:61-94.

Gutiérrez-Martínez E, Fernández-Ulibarri I, Lázaro-Diéguez F, Johannes L, Pyne S, Sarri E, Egea G (2013) Lipid phosphate phosphatase 3 participates in transport carrier formation and protein trafficking in the early secretory pathway. J Cell Sci. 2013 126:2641-2655.

Hanada K, Kumagai K, Tomishige N, Yamaji T (2009) CERT-mediated trafficking of ceramide. Biochim Biophys Acta. 1791:684-691

Hanada K, Kumagai K, Yasuda S, Miura Y, Kawano M, Fukasawa M, Nishijima M (2003) Molecular machinery for non-vesicular trafficking of ceramide. Nature. 426:803-809.

Hannich JT, Umebayashi K, Riezman H. (2011) Distribution and functions of sterols and sphingolipids. Cold Spring Harb Perspect Biol. 3: a004762.

Hara-Kuge S, Kuge O, Orci L, Amherdt M, Ravazzola M, Wieland FT, Rothman JE (1994) En bloc incorporation of coatomer subunits during the assembly of COP-coated vesicles. J Cell Biol. 124:883-889

Harter C, Wieland FT (1998) A single binding site for dilysine retrieval motifs and p23 within the gamma subunit of coatomer. Proc Natl Acad Sci U S A. 1998 95:11649-11654

Hauri HP, Schweizer A (1992) The endoplasmic reticulum-Golgi intermediate compartment. Curr Opin Cell Biol. 4:600-608.

Hausser A, Storz P, Märtens S, Link G, Toker A, Pfizenmaier K (2005) Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIbeta at the Golgi complex. Nat Cell Biol. 7:880-886

Heacock AM, Agranoff BW (1997) CDP-diacylglycerol synthase from mammalian tissues. Biochim Biophys Acta. 1997 1348:166-172.

Henneberry AL, Wright MM, McMaster CR (2002) The major sites of cellular phospholipid synthesis and molecular determinants of Fatty Acid and lipid head group specificity. Mol Biol Cell. 13:3148-3161.

Hicks SN, Jezyk MR, Gershburg S, Seifert JP, Harden TK, Sondek J (2008) General and versatile autoinhibition of PLC isozymes. Mol Cell. 31:383-394.

Hille-Rehfeld A (1995) Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. Biochim Biophys Acta. 1241:177-194.

Holthuis JC, Levine TP (2005) Lipid traffic: floppy drives and a superhighway. Nat Rev Mol Cell Biol. 6:209-220.

Holthuis JC, Luberto C (2010) Tales and mysteries of the enigmatic sphingomyelin synthase family. Adv Exp Med Biol. 688:72-85.

Horowitz LF, Hirdes W, Suh BC, Hilgemann DW, Mackie K, Hille B (2005) Phospholipase C in living cells: activation, inhibition, Ca2+ requirement, and regulation of M current. J Gen Physiol. 126:243-262.

Huitema K, van den Dikkenberg J, Brouwers JF, Holthuis JC (2004) Identification of a family of animal sphingomyelin synthases. EMBO J. 23:33-44

Irannejad R, Wedegaertner PB (2010) Regulation of constitutive cargo transport from the trans-Golgi network to plasma membrane by Golgi-localized G protein betagamma subunits. J Biol Chem. 285:32393-32404.

Jamora C, Takizawa PA, Zaarour RF, Denesvre C, Faulkner DJ, Malhotra V (1997) Regulation of Golgi structure through heterotrimeric G proteins. Cell. 91:617-626.

Jamora C, Yamanouye N, Van Lint J, Laudenslager J, Vandenheede JR, Faulkner DJ, Malhotra V (1999) Gbetagamma-mediated regulation of Golgi organization is through the direct activation of protein kinase D. Cell. 98:59-68.

Jansen SM, Groener JE, Bax W, Suter A, Saftig P, Somerharju P, Poorthuis BJ (2001) Biosynthesis of phosphatidylcholine from a phosphocholine precursor pool derived from the late endosomal/lysosomal degradation of sphingomyelin. Biol Chem. 276:18722-18727.

Jelsema CL, Morré DJ (1978) Distribution of phospholipid biosynthetic enzymes among cell components of rat liver.J Biol Chem. 253:7960-7971.

Jezyk MR, Snyder JT, Gershberg S, Worthylake DK, Harden TK, Sondek J (2006) Crystal structure of Rac1 bound to its effector phospholipase C-beta2. Nat Struct Mol Biol. 13:1135-1140.

Johnson JE, Rao NM, Hui SW, Cornell RB (1998) Conformation and lipid binding properties of four peptides derived from the membrane-binding domain of CTP:phosphocholine cytidylyltransferase. Biochemistry. 37:9509-9519.

Jones AT (2007) Macropinocytosis: searching for an endocytic identity and role in the uptake of cell penetrating peptides. J Cell Mol Med 11: 670-694.

Kadamur G, Ross EM (2013) Mammalian phospholipase C. Annu Rev Physiol. 75:127-154.

Katan M, Williams RL (1997) Phosphoinositide-specific phospholipase C: structural basis for catalysis and regulatory interactions. Semin Cell Dev Biol. 8:287-296.

Kazanietz MG, Caloca MJ, Eroles P, Fujii T, García-Bermejo ML, Reilly M, Wang H (2000) Pharmacology of the receptors for the phorbol ester tumor promoters: multiple receptors with different biochemical properties. Biochem Pharmacol. 60:1417-1424.

Kearns BG, McGee TP, Mayinger P, Gedvilaite A, Phillips SE, Kagiwada S, Bankaitis VA (1997) Essential role for diacylglycerol in protein transport from the yeast Golgi complex. Nature. 387:101-105.

Kim YH, Park TJ, Lee YH, Baek KJ, Suh PG, Ryu SH, Kim KT (1999) Phospholipase C-delta1 is activated by capacitative calcium entry that follows phospholipase C-beta activation upon bradykinin stimulation. J Biol Chem. 274:26127-26134.

Kim YJ, Guzman-Hernandez ML, Balla T (2011) A highly dynamic ER-derived phosphatidylinositol-synthesizing organelle supplies phosphoinositides to cellular membranes. Dev Cell. 21:813-824.

Kirchhausen T (2007) Making COPII coats. Cell 129: 1251-1252.

Klein RR, Bourdon DM, Costales CL, Wagner CD, White WL, Williams JD, Hicks SN, Sondek J, Thakker DR (2011) Direct activation of human phospholipase C by its well known inhibitor U73122. J Biol Chem. 286:12407-12416.

Kooijman EE, Chupin V, Fuller NL, Kozlov MM, de Kruijff B, Burger KN, Rand PR (2005) Spontaneous curvature of phosphatidic acid and lysophosphatidic acid. Biochemistry. 44:2097-2102.

Kozlov MM, McMahon HT, Chernomordik LV (2010) Protein-driven membrane stresses in fusion and fission. Trends Biochem Sci. 35:699-706

Kozlovsky Y, Kozlov MM (2003) Membrane fission: model for intermediate structures. Biophys J. 85:85-96.

Kreis TE, Pepperkok R (1994) Coat proteins in intracellular membrane transport. Curr Opin Cell Biol 6:533-537.

Ktistakis NT, Brown HA, Waters MG, Sternweis PC, Roth MG (1996) Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. J Cell Biol. 134:295-306.

Kunkel MT, Newton AC (2010) Calcium transduces plasma membrane receptor signals to produce diacylglycerol at Golgi membranes. J Biol Chem. 285:22748-22752.

Langer JD, Roth CM, Béthune J, Stoops EH, Brügger B, Herten DP, Wieland FT (2008) A conformational change in the alpha-subunit of coatomer induced by ligand binding to gamma-COP revealed by single-pair FRET. Traffic. 9:597-607

Lee AG (2004) How lipids affect the activities of integral membrane proteins Biochim. Biophys. Acta, 1666:62–87

Levine TP, Munro S (2002) Targeting of Golgi-specific pleckstrin homology domains involves both PtdIns 4-kinase-dependent and -independent components. Curr Biol. 12:695-704.

Lewis MJ, Pelham HR (1990) A human homologue of the yeast HDEL receptor. Nature. 348:162-163.

Liljedahl M, Maeda Y, Colanzi A, Ayala I, Van Lint J, Malhotra V. (2001) Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network. Cell. 104:409-420.

Lippincott-Schwartz J, Liu W (2006) Insights into COPI coat assembly and function in living cells. Trends Cell Biol 16 e1-e4

Lippincott-Schwartz J, Phair RD (2010) Lipids and cholesterol as regulators of traffic in the endomembrane system. Annu Rev Biophys. 39:559-578

Litvak V, Dahan N, Ramachandran S, Sabanay H, Lev S (2005) Maintenance of the diacylglycerol level in the Golgi apparatus by the Nir2 protein is critical for Golgi secretory function. Nat Cell Biol. 7:225-234

Liu W, Duden R, Phair RD, Lippincott-Schwartz J (2005) ArfGAP1 dynamics and its role in COPI coat assembly on Golgi membranes of living cells. J Cell Biol. 2005 168:1053-1063.

Lowe M (2011) Structural organization of the Golgi apparatus. Curr Opin Cell Biol. 23:85-93.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Maeda Y, Beznoussenko GV, Van Lint J, Mironov AA, Malhotra V (2001) Recruitment of protein kinase D to the trans-Golgi network via the first cysteine-rich domain. EMBO J. 20:5982-5990.

Majoul I, Sohn K, Wieland FT, Pepperkok R, Pizza M, Hillemann J, Söling HD (1998) KDEL receptor (Erd2p)-mediated retrograde transport of the cholera toxin A subunit from the Golgi involves COPI, p23, and the COOH terminus of Erd2p. J Cell Biol. 143:601-612.

Malhotra V, Campelo F (2011) PKD regulates membrane fission to generate TGN to cell surface transport carriers. Cold Spring Harb Perspect Biol 1;3(2)

Malhotra V, Serafini T, Orci L, Shepherd JC, Rothman JE (1989) Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. Cell. 58:329-336.

Marsh BJ, Howell KE (2002) The mammalian Golgi—complex debates. Nat Rev Mol Cell Biol, 3:789–795

Marsh BJ, Volkmann N, McIntosh JR, Howell KE (2004) Direct continuities between cisternae at different levels of the Golgi complex in glucose-stimulated mouse islet beta cells. Proc Natl Acad Sci U S A. 101:5565-5570

Martín ME, Hidalgo J, Vega FM, Velasco A (1999) Trimeric G proteins modulate the dynamic interaction of PKAII with the Golgi complex. J Cell Sci. 112:3869-3878.

Martinez O, Antony C, Pehau-Arnaudet G, Berger EG, Salamero J, Goud B (1997) GTP-bound forms of rab6 induce the redistribution of Golgi proteins into the endoplasmic reticulum. Proc Natl Acad Sci U S A. 94:1828-1833.

Matozaki T, Sakamoto C, Nishisaki H, Suzuki T, Wada K, Matsuda K, Nakano O, Konda Y, Nagao M, Kasuga M (1991) Cholecystokinin inhibits phosphatidylcholine synthesis via a Ca(2+)-calmodulin-dependent pathway in isolated rat pancreatic acini. A possible mechanism for diacylglycerol accumulation. J Biol Chem. 266:22246-22253.

Mavillard F, Hidalgo J, Megias D, Levitsky KL, Velasco A (2010) PKA-mediated Golgi remodeling during cAMP signal transmission. Traffic. 11:90-109.

Maxfield FR, McGraw TE (2004) Endocytic recycling. Nat Rev Mol Cell Biol 5: 121-132.

McGee TP, Skinner HB, Whitters EA, Henry SA, Bankaitis VA (1994) A phosphatidylinositol transfer protein controls the phosphatidylcholine content of yeast Golgi membranes. J Cell Biol. 124:273-287.

McMahon HT, Gallop JL (2005) Membrane curvature and mechanisms of dynamic cell membrane remodelling. Nature. 2005438:590-596

Mérida I, Avila-Flores A, Merino E (2008) Diacylglycerol kinases: at the hub of cell signalling. Biochem J. 409:1-18.

Micaroni M (2012) Calcium around the Golgi apparatus: implications for intracellular membrane trafficking. Adv Exp Med Biol. 740:439-60.

Micaroni M, Perinetti G, Di Giandomenico D, Bianchi K, Spaar A, Mironov AA (2010) Synchronous intra-Golgi transport induces the release of Ca2+ from the Golgi apparatus. Exp Cell Res. 316:2071-2086.

Mironov AA, Beznoussenko GV, Nicoziani P, Martella O, Trucco A, Kweon HS, Di Giandomenico D, Polishchuk RS, Fusella A, Lupetti P, Berger EG, Geerts WJ, Koster AJ, Burger KN, Luini A (2001) Small cargo proteins and large aggregates can traverse the Golgi by a common mechanism without leaving the lumen of cisternae. J Cell Biol. 155:1225-12238.

Mironov AA, Mironov AA Jr, Beznoussenko GV, Trucco A, Lupetti P, Smith JD, Geerts WJ, Koster AJ, Burger KN, Martone ME, Deerinck TJ, Ellisman MH, Luini A (2003) ER-to-Golgi carriers arise through direct en bloc protrusion and multistage maturation of specialized ER exit domains. Dev Cell. (4):583-594

Missiaen L, Dode L, Vanoevelen J, Raeymaekers L, Wuytack F (2007) Calcium in the Golgi apparatus. Cell Calcium. 41:405-416

Muñiz M, Alonso M, Hidalgo J, Velasco A (1996) A regulatory role for cAMP-dependent protein kinase in protein traffic along the exocytic route. J Biol Chem. 271:30935-30941.

Muñiz M, Martín ME, Hidalgo J, Velasco A (1997) Protein kinase A activity is required for the budding of constitutive transport vesicles from the trans-Golgi network. Proc Natl Acad Sci U S A. 94:14461-14466.

Nilsson T, Jackson M, Peterson PA (1989) Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. Cell 58):707-718.

Nishikawa K, Toker A, Wong K, Marignani PA, Johannes FJ, Cantley LC (1998) Association of protein kinase Cmu with type II phosphatidylinositol 4-kinase and type I phosphatidylinositol-4-phosphate 5-kinase. J Biol Chem. 273:23126-23133.

Oldham WM, Hamm HE (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. Nat Rev Mol Cell Biol. 9:60-71

Orci L, Ravazzola M, Amherd T, Perrelet A, Sollner TH, Rothman JE (1997) Bidirectional transport by distinct populations of COPI-coated vesicles. Cell 90:335-349.

Palmer DJ, Helms JB, Beckers CJ, Orci L, Rothman JE (1993) Binding of coatomer to Golgi membranes requires ADP-ribosylation factor. J Biol Chem. 268:12083-12089.

Patterson GH, Hirschberg K, Polishchuk RS, Gerlich D, Phair RD, Lippincott-Schwartz J (2008) Transport through the Golgi apparatus by rapid partitioning within a two-phase membrane system. Cell. 133:1055-1067

Pelham HR (1991) Recycling of proteins between the endoplasmic reticulum and Golgi complex. Curr Opin Cell Biol. 3:585-591

Pettitt TR, Wakelam MJ (1993) Bombesin stimulates distinct time-dependent changes in the sn-1,2-diradylglycerol molecular species profile from Swiss 3T3 fibroblasts as analysed by 3,5-dinitrobenzoyl derivatization and h.p.l.c. separation. Biochem J. 289:487-495.

Pfeffer SR (2010) How the Golgi works: a cisternal progenitor model. Proc Natl Acad Sci U S A. 107:19614-19618

Piechulek T, Rehlen T, Walliser C, Vatter P, Moepps B, Gierschik P (2005) Isozyme-specific stimulation of phospholipase C-gamma2 by Rac GTPases. J Biol Chem. 280:38923-38931.

Pimplikar SW, Simons K (1993) Regulation of apical transport in epithelial cells by a Gs class of heterotrimeric G protein. Nature. 362:456-458.

Pinton P, Pozzan T, Rizzuto R. (1998) The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca2+ store, with functional properties distinct from those of the endoplasmic reticulum.EMBO J. 17:5298-5308.

Ponnambalam S, Baldwin SA (2003) Constitutive protein secretion from the trans-Golgi network to the plasma membrane. Mol Membr Biol. 20:129-139

Popoff V, Adolf F, Brügger B, Wieland F (2011) COPI budding within the Golgi stack. Cold Spring Harb Perspect Biol. 3:a005231

Potter BA, Ihrke G, Bruns JR, Weixel KM, Weisz OA (2004) Specific N-glycans direct apical delivery of transmembrane, but not soluble or glycosylphosphatidylinositol-

anchored forms of endolyn in Madin-Darby canine kidney cells. Mol Biol Cell. 15:1407-1416.

Powis G, Seewald MJ, Gratas C, Melder D, Riebow J, Modest EJ (1992) Selective inhibition of phosphatidylinositol phospholipase C by cytotoxic ether lipid analogues. Cancer Res. 52:2835-2840.

Pruett ST, Bushnev A, Hagedorn K, Adiga M, Haynes CA, Sullards MC, Liotta DC, Merrill AH Jr (2008) Biodiversity of sphingoid bases ("sphingosines") and related amino alcohols. J Lipid Res. 49:1621-1639.

Pulvirenti T, Giannotta M, Capestrano M, Capitani M, Pisanu A, Polishchuk RS, San Pietro E, Beznoussenko GV, Mironov AA, Turacchio G, Hsu VW, Sallese M, Luini A (2008) A traffic-activated Golgi-based signalling circuit coordinates the secretory pathway. Nat Cell Biol. 10:912-922

Rabouille C, Klumperman J (2005) Opinion: The maturing role of COPI vesicles in intra-Golgi transport.Nat Rev Mol Cell Biol. 6:812-817.

Rambourg A, Clermont Y (1990) Three-dimensional electron microscopy: structure of the Golgi apparatus. Eur J Cell Biol. 51:189-200

Randazzo PA, Yang YC, Rulka C, Kahn RA (1993) Activation of ADP-ribosylation factor by Golgi membranes. Evidence for a brefeldin A- and protease-sensitive activating factor on Golgi membranes. J Biol Chem. 268:9555-9563.

Reinhard C, Harter C, Bremser M, Brügger B, Sohn K, Helms JB, Wieland F (1999) Receptor-induced polymerization of coatomer. Proc Natl Acad Sci U S A. 96:1224-1228.

Ríos RM, Bornens M (2003) The Golgi apparatus at the cell centre. Curr Opin Cell Biol 15:60-66.

Riske KA, Döbereiner HG (2003) Diacylglycerol-rich domain formation in giant stearoyl-oleoyl phosphatidylcholine vesicles driven by phospholipase C activity. Biophys J. 85:2351-2362.

Robinson MS, Bonifacino JS (2001) Adaptor-related proteins. Curr Opin Cell Biol. 444-453.

Rohde HM, Cheong FY, Konrad G, Paiha K, Mayinger P, Boehmelt G (2003) The human phosphatidylinositol phosphatase SAC1 interacts with the coatomer I complex. J Biol Chem. 278:52689-52699.

Ross EM (2008) Coordinating speed and amplitude in G-protein signaling. Curr Biol. 18:R777-R783.

Roth MG (2008) Molecular mechanisms of PLD function in membrane traffic. Traffic. 9:1233-1239

Rothman JE, Wieland FT (1996) Protein sorting by transport vesicles. Science. 272:227-234

Saini DK, Karunarathne WK, Angaswamy N, Saini D, Cho JH, Kalyanaraman V, Gautam N (2010) Regulation of Golgi structure and secretion by receptor-induced G protein βγ complex translocation. Proc Natl Acad Sci U S A. 107:11417-11422

San Pietro E, Capestrano M, Polishchuk EV, DiPentima A, Trucco A, Zizza P, Mariggiò S, Pulvirenti T, Sallese M, Tete S, Mironov AA, Leslie CC, Corda D, Luini A, Polishchuk RS (2009) Group IV phospholipase A(2)alpha controls the formation of inter-cisternal continuities involved in intra-Golgi transport. PLoS Biol.;7:e1000194.

Sandvig K, Pust S, Skotland T, van Deurs B (2011) Clathrin-independent endocytosis: mechanisms and function. Curr Opin Cell Biol. 23:413-420.

Sarri E, Garcia-Dorado D, Abellan A, Soler-Soler J (2006) Effects of hypoxia, glucose deprivation and acidosis on phosphatidylcholine synthesis in HL-1 cardiomyocytes. CTP:phosphocholine cytidylyltransferase activity correlates with sarcolemmal disruption. Biochem J. 394:325-334.

Sarri E, Pardo R, Fensome-Green A, Cockcroft S (2003) Endogenous phospholipase D2 localizes to the plasma membrane of RBL-2H3 mast cells and can be distinguished from ADP ribosylation factor-stimulated phospholipase D1 activity by its specific sensitivity to oleic acid. Biochem J. 369:319-329.

Schorr M, Then A, Tahirovic S, Hug N, Mayinger P (2001) The phosphoinositide phosphatase Sac1p controls trafficking of the yeast Chs3p chitin synthase. Curr Biol. 11:1421-1426.

Schuck S, Simons K (2004) Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. J Cell Sci. 117:5955-5964

Sciorra VA, Morris AJ (1999) Sequential actions of phospholipase D and phosphatidic acid phosphohydrolase 2b generate diglyceride in mammalian cells. Mol Biol Cell. 10:3863-3876.

Serafini T, Orci L, Amherdt M, Brunner M, Kahn RA, Rothman JE (1991) ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. Cell. 67:239-253.

Serafini T, Stenbeck G, Brecht A, Lottspeich F, Orci L, Rothman JE, Wieland FT (1991) A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein beta-adaptin. Nature. 349:215-220.

Shemesh T, Luini A, Malhotra V, Burger KN, Kozlov MM (2003) Prefission constriction of Golgi tubular carriers driven by local lipid metabolism: a theoretical model. Biophys J. 85:3813-3827.

Siddhanta A, Backer JM, Shields D (2000) Inhibition of phosphatidic acid synthesis alters the structure of the Golgi apparatus and inhibits secretion in endocrine cells. J Biol Chem. 275:12023-12031.

Simon JP, Morimoto T, Bankaitis VA, Gottlieb TA, Ivanov IE, Adesnik M, Sabatini DD (1998) An essential role for the phosphatidylinositol transfer protein in the scission of

coatomer-coated vesicles from the trans-Golgi network. Proc Natl Acad Sci U S A. 95:11181-6.

Simpson JC, Joggerst B, Laketa V, Verissimo F, Cetin C, Erfle H, Bexiga MG, Singan VR, Hériché JK, Neumann B, Mateos A, Blake J, Bechtel S, Benes V, Wiemann S, Ellenberg J, Pepperkok R (2012) Genome-wide RNAi screening identifies human proteins with a regulatory function in the early secretory pathway. Nat Cell Biol. 2012 14:764-774

Smith RJ, Sam LM, Justen JM, Bundy GL, Bala GA, Bleasdale JE (1990) Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. J Pharmacol Exp Ther. 253:688-697.

Spang A, Matsuoka K, Hamamoto S, Schekman R, Orci L (1998) Coatomer, Arf1p, and nucleotide are required to bud coat protein complex I-coated vesicles from large synthetic liposomes. Proc Natl Acad Sci U S A. 95:11199-11204.

Stow JL, de Almeida JB, Narula N, Holtzman EJ, Ercolani L, Ausiello DA (1991) A heterotrimeric G protein, G alpha i-3, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK1 epithelial cells. J Cell Biol. 114:1113-1124.

Stow JL, Heimann K (1998) Vesicle budding on Golgi membranes: regulation by G proteins and myosin motors. Biochim Biophys Acta. 1404:161-171.

Subathra M, Qureshi A, Luberto C (2011) Sphingomyelin synthases regulate protein trafficking and secretion. PLoS One. 2011;6:e23644

Sumara G, Formentini I, Collins S, Sumara I, Windak R, Bodenmiller B, Ramracheya R, Caille D, Jiang H, Platt KA, Meda P, Aebersold R, Rorsman P, Ricci R (2009) Regulation of PKD by the MAPK p38delta in insulin secretion and glucose homeostasis. Cell. 136:235-248.

Szule JA, Fuller NL, Rand RP (2002) The effects of acyl chain length and saturation of diacylglycerols and phosphatidylcholines on membrane monolayer curvature. Biophys J. 83:977-984.

Takizawa PA, Yucel JK, Veit B, Faulkner DJ, Deerinck T, Soto G, Ellisman M, Malhotra V (1993) Complete vesiculation of Golgi membranes and inhibition of protein transport by a novel sea sponge metabolite, ilimaquinone. Cell. 73:1079-1090.

Tanigawa G, Orci L, Amherdt M, Ravazzola M, Helms JB, Rothman JE (1993) Hydrolysis of bound GTP by ARF protein triggers uncoating of Golgi-derived COP-coated vesicles. J Cell Biol. 1123:1365-1371.

Testerink N, van der Sanden MH, Houweling M, Helms JB, Vaandrager AB (2009) Depletion of phosphatidylcholine affects endoplasmic reticulum morphology and protein traffic at the Golgi complex. J Lipid Res. 50:2182-2192.

Tillmann KD, Millarte V, Farhan H (2013) Regulation of traffic and organelle architecture of the ER-Golgi interface by signal transduction. Histochem Cell Biol. 140:297-306.

Trucco A, Polishchuk RS, Martella O, Di Pentima A, Fusella A, Di Giandomenico D, San Pietro E, Beznoussenko GV, Polishchuk EV, Baldassarre M, Buccione R, Geerts WJ, Koster AJ, Burger KN, Mironov AA, Luini A (2004) Secretory traffic triggers the formation of tubular continuities across Golgi sub-compartments. Nat Cell Biol. 6:1071-1081.

Tu CL, Chang W, Bikle DD. (2007) The role of the calcium sensing receptor in regulating intracellular calcium handling in human epidermal keratinocytes. J Invest Dermatol. 127:1074-1083.

van der Sanden MH, Houweling M, Duijsings D, Vaandrager AB, van Golde LM (2004) Inhibition of phosphatidylcholine synthesis is not the primary pathway in hexadecylphosphocholine-induced apoptosis. Biochim Biophys Acta. 1636:99-107

Van Deurs B, Roepstorff K, Hommelgaard AM, Sandvig K (2003) Caveolae: anchored, multifunctional platforms in the lipid ocean. Trens Cell Biol 13: 92-100.

van Meer G (2010) Membranes in motion. EMBO Rep. 11:331-333.

Van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9:112-124.

Villani M, Subathra M, Im YB, Choi Y, Signorelli P, Del Poeta M, Luberto C (2008) Sphingomyelin synthases regulate production of diacylglycerol at the Golgi. Biochem J. 414:31-41

Waldo GL, Ricks TK, Hicks SN, Cheever ML, Kawano T, Tsuboi K, Wang X, Montell C, Kozasa T, Sondek J, Harden TK (2010) Kinetic scaffolding mediated by a phospholipase C-beta and Gq signaling complex. Science330:974-980.

Ward DT (2004) Calcium receptor-mediated intracellular signalling.Cell Calcium. 35:217-228.

Waters MG, Serafini T, Rothman JE (1991) 'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. Nature. 349:248-251.

Webb RJ, Judah JD, Lo LC, Thomas GM (2005) Constitutive secretion of serum albumin requires reversible protein tyrosine phosphorylation events in trans-Golgi. Am J Physiol Cell Physiol. 289:748-756.

Weller SG, Capitani M, Cao H, Micaroni M, Luini A, Sallese M, McNiven MA (2010) Src kinase regulates the integrity and function of the Golgi apparatus via activation of dynamin 2. Proc Natl Acad Sci U S A. 107:5863-5868.

Wilson C, Venditti R, Rega LR, Colanzi A, D'Angelo G, De Matteis MA (2010) The Golgi apparatus: an organelle with multiple complex functions. Biochem J. 433:1-9

Yang C, Kazanietz MG (2003) Divergence and complexities in DAG signaling: looking beyond PKC. Trends Pharmacol Sci. 24:602-608

Yang JS, Gad H, Lee SY, Mironov A, Zhang L, Beznoussenko GV, Valente C, Turacchio G, Bonsra AN, Du G, Baldanzi G, Graziani A, Bourgoin S, Frohman MA, Luini A, Hsu VW (2008) A role for phosphatidic acid in COPI vesicle fission yields insights into Golgi maintenance.Nat Cell Biol. 10:1146-1153.

Yang W, Boggs KP, Jackowski S (1995) The association of lipid activators with the amphipathic helical domain of CTP:phosphocholine cytidylyltransferase accelerates catalysis by increasing the affinity of the enzyme for CTP. J Biol Chem. 270:23951-23957.

Zhang L, Malik S, Pang J, Wang H, Park KM, Yule DI, Blaxall BC, Smrcka AV (2013) Phospholipase Cε hydrolyzes perinuclear phosphatidylinositol 4-phosphate to regulate cardiac hypertrophy. Cell. 153:216-227.

Zierath JR, Lendahl U (2013) The Nobel Prize in Physiology or Medicine 2013. Scientific background: machinery regulating vesicle traffic, a major transport system in our cells.

VIII. ANNEX

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Phospholipid Synthesis Participates in the Regulation of Diacylglycerol Required for Membrane Trafficking at the Golgi Complex*

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The lipid metabolite diacylglycerol (DAG) is required for transport carrier biogenesis at the Golgi, although how cells regulate its levels is not well understood. Phospholipid synthesis involves highly regulated pathways that consume DAG and can contribute to its regulation. Here we altered phosphatidylcholine (PC) and phosphatidylinositol synthesis for a short period of time in CHO cells to evaluate the changes in DAG and its effects in membrane trafficking at the Golgi. We found that cellular DAG rapidly increased when PC synthesis was inhibited at the non-permissive temperature for the rate-limiting step of PC synthesis in CHO-MT58 cells. DAG also increased when choline and inositol were not supplied. The major phospholipid classes and triacylglycerol remained unaltered for both experimental approaches. The analysis of Golgi ultrastructure and membrane trafficking showed that 1) the accumulation of the budding vesicular profiles induced by propanolol was prevented by inhibition of PC synthesis, 2) the density of KDEL receptor-containing punctated structures at the endoplasmic reticulum-Golgi interface correlated with the amount of DAG, and 3) the post-Golgi transport of the yellow fluorescent temperature-sensitive G protein of stomatitis virus and the secretion of a secretory form of HRP were both reduced when DAG was lowered. We confirmed that DAG-consuming reactions of lipid synthesis were present in Golgi-enriched fractions. We conclude that phospholipid synthesis pathways play a significant role to regulate the DAG required in Golgi-dependent membrane trafficking.

Processes such as membrane budding, cargo selection, and vesicle fission constitute the basis for the formation of transport carriers (vesicles and tubules) at the Golgi. A plethora of proteins, such as coat-forming, signaling, and cytoskeleton components, ensure the specificity of the process and the cellular destination of the vesicles (1, 2). The lipid composition of the membrane, including the amount of metabolic intermediates, is also involved in this process (3–6).

DAG[5] is needed at the *trans*-Golgi network for the recruitment of protein kinase D and post-Golgi protein transport (7) and at the *cis*-Golgi for the biogenesis of coat protein complex I-coated vesicles (8, 9). Several pharmacological agents were used to show this requirement of DAG and also to examine its source. First, the effects of propanolol, a phosphatidic acid (PA) phosphatase inhibitor (10), suggested a role for PA-derived DAG (8). In this context, the PA produced by phospholipase D1 (PLD1) could be the source of DAG because PLD1 is a candidate to mediate the formation of vesicles stimulated by ARF1 at the Golgi (11). Second, the results of experiments with U73122, an inhibitor of agonist-induced phospholipase C activation (12), suggested a contribution of phospholipase C to the production of the DAG required at the *cis*-Golgi (8). Third, the results obtained with fumonisin B1, an inhibitor of ceramide synthase (13), suggested a role for the DAG produced by sphingomyelin synthase at the *trans*-Golgi network (7). Of these three lipid-metabolizing enzymes, only sphingomyelin synthase is widely believed to localize to the Golgi. PLD localization at this organelle is controversial (14–16), and phospholipase C action has not been widely studied (17).

Phospholipid transfer proteins have also been implicated in vesicular transport at the Golgi. The PI transfer protein (PITP) Nir2 participates in the secretory pathway at the Golgi in mammalian cells (18), as demonstrated previously for the PITP Sec14 in yeast (19). In both studies, the silencing of PC synthesis precluded the blockage of secretion produced by the silencing of their respective PITPs, which suggested that PITPs maintain partial inhibition of PC synthesis and regulate the homeostasis of DAG required for the production of secretory transport carriers at the Golgi. Recently, it has been shown that PITPβ is needed for the vesicular transport at the Golgi (20). In this case, the authors suggested that PITPβ is needed to bring PI to the Golgi for the synthesis of phosphatidylinositol 4-phosphate, which is involved in vesicular trafficking at the Golgi (21).

Pharmacological and silencing experimental strategies have concluded that the metabolic intermediate DAG is required for the transport carrier biogenesis at the Golgi. However, the par-

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[5] The abbreviations used are: DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; PS, phosphatidylserine; ssHRP, secretory form of horseradish peroxidase; YFP-ts045VSV-G, yellow fluorescent temperature-sensitive G protein of stomatitis virus; ER, endoplasmic reticulum; PITP, PI transfer protein; KDELr, KDEL receptor; NRK, normal rat kidney.

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βIII Spectrin Regulates the Structural Integrity and the Secretory Protein Transport of the Golgi Complex*[S]

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Background: βIII spectrin function at the Golgi remains unclear.
Results: βIII spectrin is enriched in distal Golgi compartments and supports anterograde transport. PI4P is determinant for the βIII spectrin association with Golgi membranes.
Conclusion: βIII spectrin is necessary for the structural and functional organization of the Golgi.
Significance: We provide new *in vivo* insights of the role of βIII spectrin at the Golgi.

A spectrin-based cytoskeleton is associated with endomembranes, including the Golgi complex and cytoplasmic vesicles, but its role remains poorly understood. Using new generated antibodies to specific peptide sequences of the human βIII spectrin, we here show its distribution in the Golgi complex, where it is enriched in the *trans*-Golgi and *trans*-Golgi network. The use of a drug-inducible enzymatic assay that depletes the Golgi-associated pool of PI4P as well as the expression of PH domains of Golgi proteins that specifically recognize this phosphoinositide both displaced βIII spectrin from the Golgi. However, the interference with actin dynamics using actin toxins did not affect the localization of βIII spectrin to Golgi membranes. Depletion of βIII spectrin using siRNA technology and the microinjection of anti-βIII spectrin antibodies into the cytoplasm lead to the fragmentation of the Golgi. At ultrastructural level, Golgi fragments showed swollen distal Golgi cisternae and vesicular structures. Using a variety of protein transport assays, we show that the endoplasmic reticulum-to-Golgi and post-Golgi protein transports were impaired in βIII spectrin-depleted cells. However, the internalization of the Shiga toxin subunit B to the endoplasmic reticulum was unaffected. We state that βIII spectrin constitutes a major skeletal component of distal Golgi compartments, where it is necessary to maintain its structural integrity and secretory activity, and unlike actin, PI4P appears to be highly relevant for the association of βIII spectrin the Golgi complex.

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In erythrocytes and nucleated cells, there is a spectrin-based skeleton that supports cell shape and maintains the organization and stability of the plasma membrane and its mechanical properties (1–3). Spectrin is a cytoskeletal protein that binds simultaneously to integral membrane proteins, cytosolic proteins, and certain phospholipids to create a multifunctional scaffold. Spectrins are composed of α and β subunits, which are assembled side by side to form rod-like αβ dimers, which in turn self-associate to form tetramers. However, β spectrins can also exist as homopolymeric complexes. Although mammalian red blood cells contain only one type of spectrin tetramer (αIβI subunits), nucleated cells contain numerous isoforms of both subunits, which are located in diverse endomembrane systems. The existence of a Golgi-localized, spectrin membrane skeleton was reported almost 20 years ago (4). Similarly to what occurs in erythrocytes and other cell types, such as epithelial cells (5, 6), Golgi-localized spectrin could act as an extensive, two-dimensional interactive platform on the cytoplasmic surface of Golgi cisternae regulating its shape and transport functions (7–9). First evidences of spectrin on Golgi and cytoplasm vesicles were based on the reactivity of erythroid βI spectrin-specific antibodies with a single polypeptide in purified rat liver Golgi membranes with a similar molecular mass to erythroid β spectrin (≈220 kDa), and on the ability of βI spectrin peptides to compete with the function of Golgi spectrin (10, 11). Due to the problems with its precise molecular identification, this band was initially referred to as $\beta I\Sigma^*$ spectrin, but after definite identification, it was named βIII spectrin (12). Over the years, specific isoforms of spectrin-binding partners typically present in the plasma membrane of red blood cells have been localized in the Golgi, such as ankyrin (Ank_{G119} and Ank_{195}) (13, 14), protein 4.1 (4.1B) (15), anion exchanger (AE2) (16), and tropomyosin (17).