[Autophagy 4:5, 717-720; 1 July 2008]; ©2008 Landes Bioscience

Article Addendum

Clearance of a Hirano body-like F-actin aggresome generated by jasplakinolide

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Key words: actin, aggresome, Hirano body, jasplakinolide, microtubules, cytoskeleton, heat-shock proteins, autophagy, lysosomes, proteasomes

We have reported in a variety of mammalian cells the reversible formation of a filamentous actin (F-actin)-enriched aggresome generated by the actin toxin jasplakinolide (Lázaro-Diéguez et al., J Cell Sci 2008; 121:1415–25). Notably, this F-actin aggresome (FAG) resembles in many aspects the pathological Hirano body, which frequently appears in some diseases such as Alzheimer's and alcoholism. Using selective inhibitors, we examined the molecular and subcellular mechanisms that participate in the clearance of the FAG. Chaperones, microtubules, proteasomes and autophagosomes all actively participate to eliminate the FAG. Here we compile and compare these results and discuss the involvement of each process. Because of its simplicity and high reproducibility, our cellular model could help to test pharmacological agents designed to interfere with the mechanisms involved in the clearance of intracellular bodies and, in particular, of those enriched in F-actin.

Alterations in the dynamics and integrity of the cytoskeleton could lead to or facilitate the abnormal aggregation of peptides or proteins in the cytoplasm due to misfolding or inappropriate protein-protein interactions. These aggregates form defined structures that are called inclusion bodies (IBs) and that usually accumulate in the centrosome region of the cell to form aggresomes.[1,2] Filamentous actin (F-actin) accumulates in a cytoplasmic IB or aggresome termed the Hirano body, which is seen in post-mortem histological preparations from patients with Alzheimer's and other neurodegenerative disorders, chronic alcoholism and diabetes.[3-5] The biological significance of Hirano bodies and their precise relation to pathology remain unsolved. Therefore, it is important to have cellular models that reproduce the formation of F-actin enriched IBs. This would help to determine the molecular composition of these bodies as well as the mechanisms involved in their formation, maintenance and, when possible, in their clearance.

In cells cultured under heat shock or osmotic stress, or with ADF/cofilin overexpression, there is an induction in the formation of actin-containing structures called ADF/cofilin-actin rods, which are suggested to be the precursors of Hirano bodies.[6-9] Hirano body-like structures have been generated in mammalian cell cultures by the expression of the carboxy-terminal fragment of the Dictyostelium 34 kDa actin-bundling protein.[10-12] However, the processes involved in the elimination of the generated F-actin-enriched IBs have not been examined in any of these former models, most likely because they result in permanent structures within the cells. This does not mean that cells do not try to eliminate these IBs by a variety of molecular and subcellular processes involving proteasomes, autophagy and/or other non-lysosomal and non-proteasomal proteolytic pathways, but the continuous expression of the altered protein(s) makes it quite difficult to properly address their participation and relative contribution to this degradation. These difficulties increase even more because eventually this continuous protein accumulation could induce a quick cell death.

The cell membrane-permeable toxin jasplakinolide (Jpk) generates F-actin aggregates,[13] which form a reversible F-actin aggresome (FAG).[14] We generated the FAG (Fig. 1A) in a variety of cultured mammalian cell lines, including neurons, by giving to cells either a short 45–60 min pulse of Jpk at high concentration (500 nM), which then is washed-out from the culture medium, or when cells are continuously incubated with Jpk for 6–8 h, but at a lower concentration (50 nM). The shape, size and ultrastructural characteristics of FAGs generated by both protocols are indistinguishable and their formation is always reversible (Fig. 1A–C). In respect to this reversibility, 36 h after the toxin removal, F-actin puncta (FAP) and F-actin amorphous aggregates (FAM) are present in the cytoplasm as remnants of the FAG (Fig. 1B). However, after 48 hours, no sign of the FAG is seen (Fig. 1C). Therefore, we can take advantage of this FAG clearance to examine whether inhibitors or stimulators of cellular components or processes, such as the various intracellular proteolytic pathways

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Submitted: 05/09/08; Revised: 05/26/08; Accepted: 05/27/08

Previously published online as an *Autophagy* E-publication: http://www.landesbioscience.com/journals/autophagy/article/6345

Addendum to: Lázaro-Diéguez F, Aguado C, Mato E, Sánchez-Ruíz Y, Esteban I, Alberch J, Knecht E, Egea G. Dynamics of an F-actin aggresome generated by the actin-stabilizing toxin jasplakinolide. J Cell Sci 2008; 121:1415–25.

(lysosomal and non-lysosomal), accelerate, delay or keep unaltered the life-span of this FAG.

Thus, taking as a reference the remainder of the FAG observed 36 h after removal of the actin toxin (Fig. 1B), cells treated with agents that perturb the dynamics and organization of microtubules such as nocodazole (Fig. 1D) and taxol (not shown), as well as with those of the activity of proteasomes such as lactacystin (Fig. 1E) all inhibited the clearance of the FAG (compare Fig. 1D and E with B). Moreover, the life-span of the FAG was also prolonged with two different treatments, which affect lysosomal function: pepstatin A (an inhibitor of aspartic proteinases such as cathepsin D) (Fig. 1F) or bafilomycin (an inhibitor of the vacuolar H^+-ATPase) (Fig. 1G). Conversely, cells treated with rapamycin or the geldanamycin derivative 17-DMAG, which, respectively, activate autophagy and bind to hsp90, thus activating the expression of other heat-shock proteins, showed no FAG remainder (compare Fig. 1H and I with B), which indicates that both treatments accelerated its disappearance. The involvement of autophagy in the degradation of FAG was further experimentally supported by three observations. First, vesicular structures (autophagosomes/autophagic vacuoles) containing LC3-GFP increased in number in a time-dependent manner in cells containing the FAG (compare Fig. 2A with B). Initially, just after the complete formation of the FAG, LC3-containing structures accumulated near the FAG (Fig. 2A), but later on their number increased and most of them colocalized with F-actin (Fig. 2B). This time-course suggests that the association of LC3 with the FAG is not due to the described artificial association of LC3 with some aggregates.[15] Second, the conversion of LC3-I to LC3-II in cells containing the FAG was significantly higher in comparison to control cells.[14] Third, under the electron microscope, numerous autophagic vacuoles were seen tightly associated with the FAG (Fig. 2C, red asterisks), and ordered F-actin structures were occasionally observed in the lumen of some of them.[14] Consequently, these results strongly support a role of autophagic processes in the clearance of the FAG.

Autophagy is further assisted by proteasomes, chaperones and microtubules in this process. Although it was initially thought that proteasomes and autophagy degraded different sets of proteins (e.g., short-lived vs long-lived proteins), there are recent examples showing that both proteolytic systems collaborate in the degradation of a single protein, as is the case with some mammalian receptors such as the LDL receptor.[16] In addition, there is evidence of a cross-talk among various proteolytic pathways (proteasomes, macroautophagy, chaperone-mediated autophagy and calpains) in such a way that a decreased activity of one can be compensated by activation of the other(s).[17] Therefore, our results provide further evidence for this collaboration, since it appears that proteasomes and autophagy are both involved in the actin degradation of the FAG. In addition, the cooperation of chaperones with the proteolytic pathways in the destruction of misfolded or aggregated proteins is well known.[18] Finally, and concerning microtubules, they are most likely required to support the subcellular rearrangement to the FAG of autophagosomes and/or components of the ubiquitin-proteasome system, as well as to facilitate autophagosome-lysosome fusion.[19,20]

In Figure 3 we depict a model in which we summarize the molecular processes involved in the formation and clearance of the FAG. Jpk is an actin toxin that on one hand stabilizes pre-existing microfilaments (stabilized F-actin) and on the other hand induces

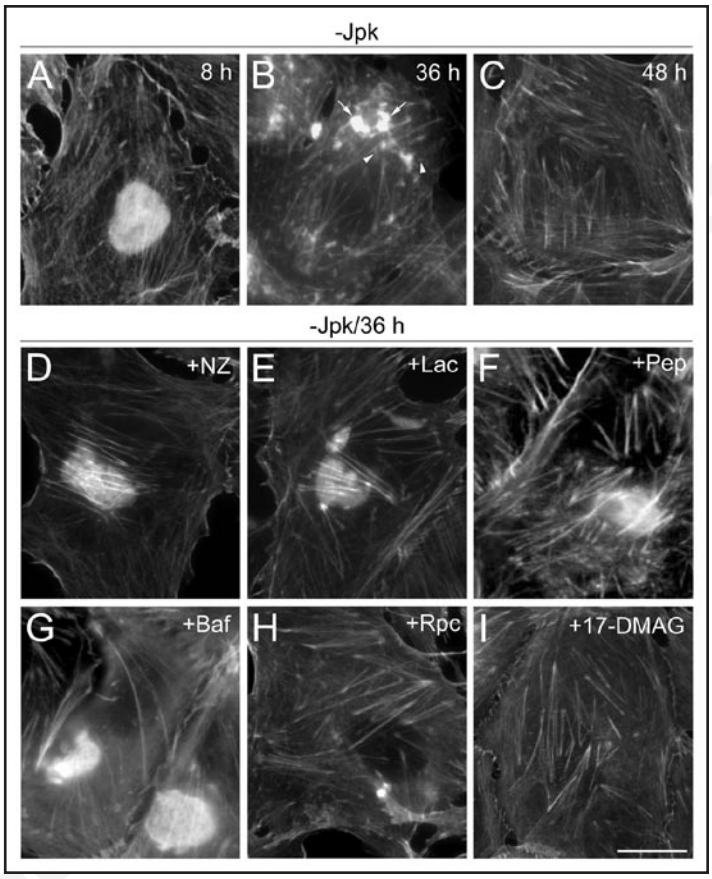

Figure 1. Different effects on the clearance of an F-actin aggresome caused by selective agents that interfere with different proteolytic processes. Vero cells were treated with jasplakinolide (Jpk; 500 nM for 1 h), and subsequently the actin toxin was washed-out from the culture medium. Cells were then fixed and stained with TRITC-phallodin to reveal the F-actin organization. Under these experimental conditions, a single, large F-actin aggresome (FAG) is invariably formed 8 h after Jpk removal (A). Thereafter, the FAG progressively disappears (B and C). As an example of this FAG clearance process, we show the resulting F-actin structures (F-actin puncta, FAP, arrowheads; F-actin amorphous aggregates, FAM, arrows) from the FAG that remains in the cells 36 h after Jpk removal (B). To easily compare the effects of the different treatments, subsequent panels correspond to the results obtained 36 h after Jpk removal (compare D–I with B). Note that in those cells treated with nocodazole (NZ; 30 μM) (D), lactacystin (Lac; 10 μM) (E), pepstatin (Pep; 10 μM (F) or bafilomycin (Baf; 100 nM) (G), which respectively disrupt microtubule integrity, and proteasome and (for the last two) lysosomal functions, the FAG remains virtually unaltered and, therefore, for a longer time in the cytoplasm. In contrast, cells incubated with rapamycin (Rpc; 200 nM) (H) or 17-DMAG (10 μM) (I), which respectively stimulate autophagy and chaperone activities, no FAG remainders are observed. Bar, 10 μm.

aberrant actin polymerization (aberrant F-actin).[21,22] At any rate, Jpk results in the formation of FAP and FAM structures, which are merely distinguished by their size and time of appearance. FAP and FAM are most likely transported by the activity of the minus-end microtubule dynein motor to the centrosomal area where they accumulate,[23] giving rise to the single and large FAG. Note that the absence of a highly ordered structure of the FAG when examined at the ultrastructural level (Fig. 2C) suggests that almost all the F-actin forming the FAG is coming from aberrant F-actin induced by Jpk. Thereafter, FAP and FAM are not formed anymore, the FAG does not grow, and finally it can be efficiently eliminated by

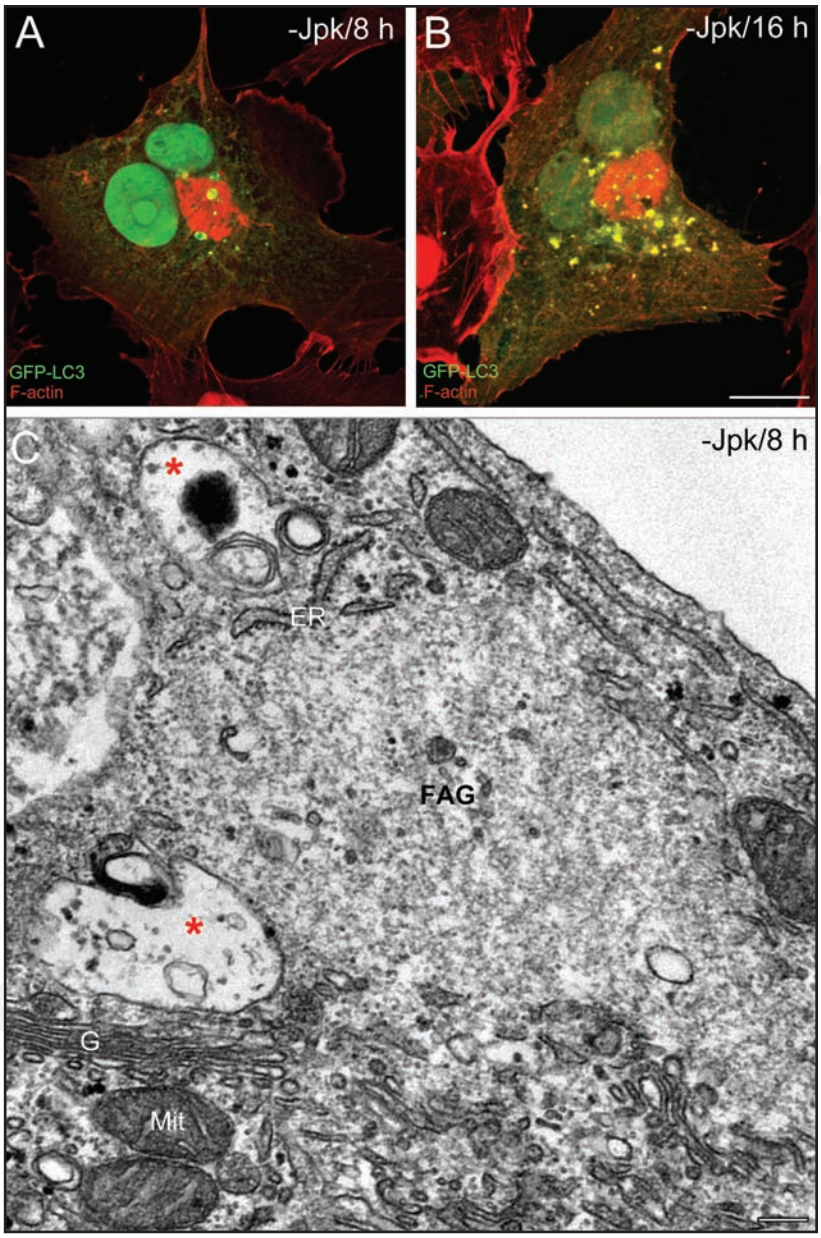

Figure 2. Autophagosomes increase in cells containing the F-actin aggresome. GFP-LC3-expressing Vero cells were treated with Jpk (500 nM for 1 h), the actin toxin was then washed-out, and after 8 h cells were fixed and stained with TRITC-phalloidin. At this time, some GFP-LC3-containing vesicular structures start to accumulate around the FAG (A). Subsequently, the number of GFP-LC3 vesicular structures increased (B), and most of them appeared as yellow due to the presence of LC3 (in green) and F-actin (in red) (B). When cells are examined at the ultrastructural level, numerous autophagic vacuoles (red asterisks) are seen tightly associated with the FAG (C). Abbreviations, Golgi apparatus (G), endoplasmic reticulum (ER), F-actin aggresome (FAG), mitochondria (Mit). Bars, 10 μm (A and B); 200 nm (C).

the combined action of chaperones, proteasomes and autophagy. The fact that FAP and FAM structures are not further formed after a certain time following the Jpk treatment could be attributed either to the catabolism of the actin toxin, or most likely, because the totality of the toxin is bound to stabilized and aberrant F-actin. As soon as no more actin toxin is freely available in the culture medium, actin dynamics proceed normally, and the FAG is slowly cleared by the aforementioned mechanisms. This is demonstrated by the fact that agents that stimulate or inhibit their functions significantly shorten or enhance the life span of the FAG, respectively. At the moment, the reported data do not allow us to establish the relative importance of each proteolytic process or the sequence of degradation events. But in this sense, this cellular model provides a good chance to examine the contribution of the various lysosomal and non-lysosomal pathways of intracellular protein degradation in the clearance of the FAG.[24-29] Moreover, this model could be useful (i) to study the interrelationships occurring among the different proteolytic processes, and (ii) to validate new pharmacological agents designed to stimulate or to inhibit the activity of any of these different processes, proteolytic or not, testing their effects on the life-span of this F-actin aggresome, which in turn could be of potential application to investigate the pathological significance of Hirano bodies.

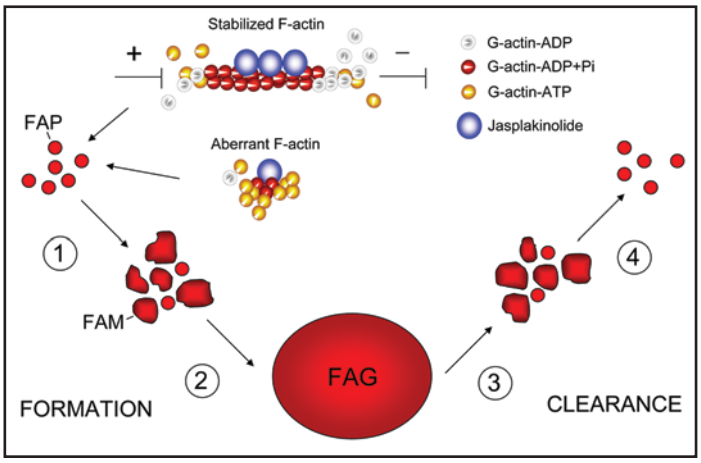

Figure 3. Model that summarizes the molecular mechanisms involved both in the formation and the degradation of the F-actin aggresome produced by jasplakinolide. Briefly, on one hand Jpk stabilizes pre-existing microfilaments (stabilized F-actin), and consequently actin dynamics in the barbed-end (+) and pointed-end (-) are impaired; on the other hand, it also induces an aberrant actin polymerization (aberrant-F-actin). Consequently, both molecular alterations lead to the formation of F-actin puncta (FAP), which in turn aggregate (1) to form F-actin amorphous aggregates (FAM). Both FAP and FAM converge in a microtubule-dependent manner to form the FAG, since nocodazole (NZ) and taxol (TX) treatments prevent its formation (2). In the absence of Jpk, the FAG starts to disappear (3) with the concomitant appearance of FAM and FAP (4). The degradation of the FAG (3) is accelerated by inducers of autophagy (e.g., rapamycin) or by the expression of various heat-shock proteins (e.g., in response to 17-DMAG), and it is delayed by microtubule disrupters (e.g., nocodazole and taxol), and inhibitors of the activities of lysosomes (e.g., bafilomycin and pepstatin) or proteasomes (lactacystin).

Acknowledgements

We thank Susana Granell for comments about the manuscript. This work was supported by grants BFU2006-00867/BMC and Consolider CSD2006-00012 to G.E., and BFU2005-00087 to E.K.

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