Dynamics of an F-actin aggresome generated by the actin-stabilizing toxin jasplakinolide

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Summary

In this study, we report the formation of several cytoplasmic inclusion bodies composed of filamentous actin (F-actin) and generated by experimental treatments using depolymerizing or stabilizing actin toxins in neuronal and non-neuronal mammalian cell lines. The actin-stabilizing toxin jasplakinolide (Jpk) induced, in a microtubule-dependent manner, a single, large F-actin aggregate, which contained β- and γ-actin, ADF/cofilin, cortactin, and the actin-nucleator Arp2/3. This aggregate was tightly associated with the Golgi complex and mitochondria, and was surrounded by vimentin intermediate filaments, microtubules and MAP4. Therefore, the Jpk-induced single, large F-actin aggregate fits the established criteria for being considered an aggresome. Lysosomes and/or autophagic vacuoles, proteasomes and microtubules were found to directly participate in the dissolution of this F-actin aggresome. Finally, the model reported here is simple, highly reproducible and reversible, and it provides an opportunity to test pharmacological agents that interfere with the formation, maintenance and/or disappearance of F-actin-enriched pathological inclusion bodies.

Introduction

Alterations in the structure and dynamics of the cytoskeleton have been reported in many diseases, such as cancer (Suresh, 2007) and neurodegenerative disorders (Weissmann and Brandt, 2007). One of their common characteristics is that cytoskeleton disruption usually leads to peptide or protein deposits that aggregate abnormally owing to misfolding or abnormal protein-protein interactions. These aggregates form cytoplasmic and non-membranous structures called inclusion bodies (IBs). One type of IBs (generically named aggresomes) are composed of misfolded or mutant proteins that are aggregated in a single structure in the centrosome region (Garcia-Mata et al., 1999; Johnston et al., 1998; Kopito, 2000). Several cytoskeleton components, such as vimentin intermediate filaments (Kopito, 2000) and the minus-end-directed microtubular dynein motor (Johnston et al., 2002), are involved in the formation of IBs, regardless of their molecular composition. Filamentous actin (F-actin) is of particular interest because it interacts with the amyloid precursor, presenilin and Tau proteins of IBs in Alzheimer disease (Santa-Maria et al., 2007; Maloney et al., 2005), and it strongly accumulates in other IBs, termed Hirano bodies, seen in post-mortal neurological histological preparations as indicators of Alzheimer disease (and other neurodegenerative disorders) (Hirano, 1994) and of chronic alcoholism (Laas and Hagel, 1994). Aggregation of F-actin has also been described in some myopathies (Schroder, 1990; Podlubaia and Nowak, 2006). The biological significance and molecular mechanisms involved in the formation, composition and proteolysis of these F-actin-enriched IBs, and their precise relation to (neuro)diseases, are unknown. Cellular models that reproduce the formation and composition of the F-actin-enriched IBs are required in order to perform detailed examinations of the molecular mechanisms involved in their structural and functional dynamics. In this regard, cells cultured under heat-shock, osmotic-stress or cofilin-overexpression experimental conditions induce the formation of actin-containing structures called ADF/cofilin-actin rods (Nishida et al., 1987; Minamide et al., 2000; Bernstein et al., 2006; Jang et al., 2005). A model for the formation of Hirano bodies in mammalian cell cultures by expression of a fragment of a 34-kDa actin-bundling protein has recently been reported (Davis et al., 2008).

One of the fastest and most effective methods of perturbing actin dynamics involves the use of actin toxins that induce the dissolution of the actin cytoskeleton. Most natural actin toxins – such as cytochalasins, latrunculins and some botulinum toxins – depolymerize F-actin. Far fewer F-actin stabilizers are known and the most commonly used is the cell membrane-permeable toxin jasplakinolide (Spector et al., 1999; Holzinger, 2001; Fenteany and Zhu, 2003; Allingham et al., 2006). Cytochalasins and latrunculins are widely used to study the involvement of actin in cellular events (for example, in endo/exocytosis, cell motility and migration, cell polarity and differentiation, and axonal transport and neuritogenesis). Therefore, actin toxins could also be useful in determining the molecular processes impaired in neurological and non-neurological disorders in which actin is thought to play an essential role.

In this study, we perturbed the actin-cytoskeleton dynamics in a variety of mammalian cells using actin toxins. We report the formation of F-actin IBs, in which we highlight the single, large F-actin aggregate induced by jasplakinolide. This IB fits the criteria...
of an aggresome, and we provide evidence of the molecular mechanisms involved in its formation and clearance.

Results

Structural dynamics (formation, maintenance and clearance) of a large F-actin aggregate induced by jasplakinolide

We examined the way in which the actin cytoskeleton is perturbed by actin toxins. Vero cells were treated with actin-depolymerizing toxins such as cytochalasin D (CyD), latrunculin B (LtB) or mycalolide B (MyB), or with the actin-stabilizing toxin jasplakinolide (Jpk) (supplementary material Table S1). They all induced the disruption of the actin cytoskeleton visualized by the loss of actin stress fibers and the rounding-up of cells (Fig. 1B-D). NRK and HeLa cells also showed highly similar alterations (not shown). Next, we studied the reversibility of these impairments following toxin washout (supplementary material Table S1). The early removal of LtB or CyD led to the formation of numerous F-actin punctae (arrowheads and inset in Fig. 1E). The cell shape and the actin-cytoskeleton organization were completely restored at 60 and 90 minutes (Fig. 1F). At 48 (Fig. 1H) or 32 (Fig. 1J) hours after MyB or Jpk removal, respectively, the normal cell shape and actin-stress-fiber organization were seen. MyB-washout cells showed juxtanuclear accumulations of F-actin punctae after 24 and 32 hours (arrowheads and inset in Fig. 1G). Interestingly, at 6 or 8 hours after Jpk removal, cells contained a single large F-actin aggregate (FAG) (Fig. 1I and inset), which localized in the centrosomal region (identified with anti-\(\gamma\)-tubulin antibodies; green in the inset of Fig. 1I). Notably, a FAG was also generated when cells were incubated in the continuous presence of a lower concentration of Jpk. Using this other strategy, no cellular rounding-up was observed and, moreover, we could follow in detail the kinetics of the formation and clearance of the FAG. Thus, in the continuous presence of Jpk, the coalescence of F-actin punctae (arrowheads in Fig. 2B-D) and F-actin amorphous aggregates (arrows in Fig. 2B-E) to the centrosomal region formed the FAG (Fig. 2F). Over time, the FAG fragmented into multiple F-actin amorphous aggregates (arrows in Fig. 2G-I) and F-actin punctae, which were dispersed throughout the cytoplasm (arrowheads in Fig. 2G-K) and finally disappeared (Fig. 2L). The larger size of the FAG was maintained, and even increased, when the culture medium was replaced every 12 hours by other medium containing new Jpk. In this case, cells showed a much larger FAG, which sometimes completely enclosed the nucleus (supplementary material Fig. S1).

Taking into account their morphology and F-actin content, F-actin punctae and/or F-actin amorphous aggregates and the FAG resemble, in some degree, rod-shaped actin bundles and Hirano bodies (Minamide et al., 2000; Hirano, 1994). Therefore, we next studied the potential formation of a FAG in neural cells, which included mouse c17.2 neural stem cells (NSCs) and primary cultures of rat astrocytes and mouse hippocampal neurons. The majority of NSCs, astrocytes and, to a lesser extent, hippocampal neurons also showed a FAG. Strikingly, neurons associated (Fig. 1G,I). As soon as the FAG disappeared, binucleate cells were no longer observed (Fig. 1H,J). We tested whether the FAG impaired the viability of Jpk-washout Vero cells using either Trypan Blue exclusion (not shown) or MTT reduction assays (supplementary material Fig. S3A). There were no significant differences between control cells and either those that contained a FAG (±Jpk for 6 hours or 24 hours, and –Jpk for 8 hours and 24 hours) or those in which the FAG had already disappeared (±Jpk for 48 hours and –Jpk for 48 hours). Strikingly, no binucleate neural cells were seen at any point but, unlike in non-neural cells, apoptotic nuclei appeared in hippocampal neurons at 24 and 48 hours after the Jpk washout (supplementary material Fig. S3B).

Cell viability is not compromised by the presence of the F-actin aggregate

Interestingly, most Vero cells were binucleate after either MyB or Jpk withdrawal, which indicates that cells were transiently arrested in cytokinesis. The two nuclei and FAG were always tightly associated (Fig. 1G,J). As soon as the FAG disappeared, binucleate cells were no longer observed (Fig. 1H,J). We tested whether the FAG impaired the viability of Jpk-washout Vero cells using either Trypan Blue exclusion (not shown) or MTT reduction assays (supplementary material Fig. S3A). There were no significant differences between control cells and either those that contained a FAG (±Jpk for 6 hours or 24 hours, and –Jpk for 8 hours and 24 hours) or those in which the FAG had already disappeared (±Jpk for 48 hours and –Jpk for 48 hours). Strikingly, no binucleate neural cells were seen at any point but, unlike in non-neural cells, apoptotic nuclei appeared in hippocampal neurons at 24 and 48 hours after the Jpk washout (supplementary material Fig. S3B).

Organelle association and ultrastructure of the F-actin aggregate

Next, we analyzed the morphology of the FAG. We first obtained a 3D model from confocal serial images of Jpk-washout Vero cells.
In most cases, the FAG was slightly ellipsoid and, curiously, was invariably housed by the Golgi complex (Fig. 3A). A large accumulation of mitochondria around the FAG was also observed (Fig. 3B). Under the electron microscope, the FAG was seen as a large electron-lucent body (Fig. 3C). In some regions, a more electron-dense material was occasionally seen (asterisks in Fig. 3D), which, in some cases, when examined at higher magnification, showed a regular structure made up of aligned and tightly compacted F-actin or actin bundles (Fig. 3E,F). The FAG lacked a membrane, but intermediate filaments accumulated at its periphery (arrowheads in Fig. 3G). As already observed under the fluorescence microscope, numerous mitochondria (Fig. 3C,D), autophagic vacuoles and lysosomes (Fig. 3D,G), endoplasmic reticulum, and the Golgi complex (Fig. 3D,G) with peri-Golgi coat protein I (COPI)-coated vesicles (arrows in Fig. 3G) accumulated around the FAG. The use of brefeldin A and the steady-state distribution of the KDEL receptor (KDELr) in FAG-containing cells showed that the tight morphological association between the Golgi complex and the FAG did not lead to any alteration in the ER-Golgi-interface membrane trafficking (not shown).

**Molecular composition of the F-actin aggregate**

In Vero cells, FAG contained β- and γ-actin isoforms (not shown), ADF/cofilin (Fig. 4A), the p21-Arc subunit of the actin nucleator multiprotein complex Arp2/3 (Fig. 4B), and cortactin (Fig. 4C). However, it lacked N-WASP, the small Rho GTPases Cdc42, RhoA and Rac (not shown), and myosins IIA (Fig. 4D) and IIB (not shown). The FAG was surrounded by spectrin (not shown), vimentin intermediate filaments (Fig. 4E) and microtubules (MTs) (Fig. 4F). The MT-associated protein MAP4 was only present at the periphery of the FAG (not shown). In hippocampal neurons, neither MAP4 (Fig. 4H), Tau (not shown) nor synapsin (Fig. 4I) were seen in FAGs. By contrast, we observed a significant accumulation of cortactin (Fig. 4G) and spinophilin (Fig. 4J).

We also determined the distribution of several proteins in nuclei-free homogenates from Vero cells containing a FAG. Cell homogenates were separated by centrifugation in discontinuous sucrose-density gradients, and fractions were subsequently...
immunoblotted for β-actin, the Arp3 component of the Arp2/3 complex, ADF/cofilin and the myosin IIA isoform. In untreated cells, the immunoreactivity of these proteins was broadly distributed along the gradient (supplementary material Fig. S4A, Control). However, in Jpk-treated cells, β-actin, Arp3 and ADF/cofilin were redistributed to the densest membrane fractions along the gradient (supplementary material Fig. S4A, +Jpk). By contrast, myosin IIA maintained a broader distribution in the density profile. These results indicate a restricted distribution of endogenous actin and some actin-binding and/or actin-associated proteins in subcellular structures, which is consistent with the results from confocal microscopy (Fig. 4). We also used immunofluorescence microscopy to assess F-actin reactivity in the sucrose fractions (supplementary material Fig. S4B). Unlike low- (1-3 fractions; left panel, Fig. 4) and mid- (4-6 fractions; middle panel, Fig. 4) density fractions, those with higher densities (7-10 fractions; right panel, Fig. 4) were enriched with FAGs with an altered and heterogeneous morphology as a consequence of the mechanical stress produced during its isolation.

Actin dynamics in the F-actin aggregate

We first examined the dynamics of actin inside the FAG. Vero cells transiently expressing YFP-actin were treated with Jpk to induce the formation of the FAG, and then equal areas of cytoplasm and FAG were photobleached. The fluorescence emitted by the YFP-actin from the cytoplasm area was rapidly recovered but not that from the FAG (supplementary material Fig. S5A, control and +Jpk, respectively; also see Movie 1 in the supplementary material). Next, we examined whether the F-actin from the FAG was sensitive to LtB treatment. As expected, LtB induced the shrinkage of cells, and actin stress fibers completely disappeared in the control cells and Jpk-treated cells, but the FAG remained apparently unaltered (supplementary material Fig. S5B, +LtB). Next, we examined whether the presence of the FAG perturbs the restoration of the actin-cytoskeleton organization, which takes place after removal of LtB. In these conditions, a normal cell shape and actin-stress-fiber organization was observed both in control cells and in cells containing a FAG (supplementary material Fig. S5B, –LtB). Finally, control and FAG-containing cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) without fetal bovine serum (FBS) and stimulated with phorbol myristate acetate or lysophosphatidic acid quickly formed lamellipodia and stress fibers, respectively (supplementary material Fig. S6) (Ridley et al., 1992; Ridley and Hall, 1992).

Fig. 4. Molecular composition of the FAG in different cell types assessed by immunofluorescence. (A-F) Vero cells (A-F) and mouse hippocampal neurons (G-J) were treated with Jpk (50 nM for 6 hours and 500 nM for 24 hours, respectively), fixed and double-stained with TRITC-phalloidin (red) and with a variety of antibodies against different cytoskeleton and/or cytoskeleton-associated proteins and neuronal markers (indicated in each panel; green). Scale bar: 10 μm.

Fig. 5. Microtubule involvement in the formation and location of the FAG. (A-C) Vero cells (A-F) and mouse hippocampal neurons (G-J) were treated with Jpk (50 nM for 6 hours and 500 nM for 24 hours, respectively), fixed and double-stained with TRITC-phalloidin (red) and with a variety of antibodies against different cytoskeleton and/or cytoskeleton-associated proteins and neuronal markers (indicated in each panel; green). Scale bar: 10 μm.
Microtubules are essential for the formation and clearance of the F-actin aggregate

Next, we assessed the degree of MT dependence of the formation and clearance of FAG in Vero cells. To this end, we used nocodazole (NZ) to depolymerize MTs or taxol (TX) to stabilize them (Fig. 5A). In cells treated with NZ and then co-incubated with Jpk, instead of a FAG, a large number of F-actin punctae and F-actin amorphous aggregate structures were formed throughout the cytoplasm (Fig. 5B and inset). When NZ was removed in the continuous presence of Jpk, F-actin punctae and F-actin amorphous aggregates repositioned to the centrosomal region producing the FAG (Fig. 5D and inset). Similar results were obtained when cells were treated with TX (Fig. 5C,E and respective insets). Therefore, results indicate that the formation and positioning of the FAG is MT-dependent. However, when cells were incubated with Jpk and then with NZ, the FAG remained unaltered despite the disruption of MTs (Fig. 5F). This indicates that FAG integrity is MT-independent. We then tested the role of MTs in the clearance of the FAG. As indicated above, it is not a permanent structure and it disappeared in Vero cells at 32 and 48 hours after its generation (Figs 1, 2). Cells were incubated with Jpk so that a FAG was formed and were then treated with either NZ or TX for 48 hours in the continuous presence of Jpk. At this time, and unlike cells treated with Jpk alone, co-incubation with NZ or TX did not alter FAG structure and increased its life-span (supplementary material Fig. S7). Therefore, this indicates that intact MTs are necessary for the dissolution of the FAG.

Fig. 6. Lysosomal distribution and function during the formation and/or clearance of the FAG. (A-C) Vero cells were treated with Jpk (+Jpk, 50 nM) for different time periods, as indicated in the panels. Cells were double-stained with TRITC-phalloidin (red) and anti-LAMP2 antibodies (green). Lysosomes in cells containing a FAG show either a uniform distribution or accumulate around the FAG (arrow in B). At 48 hours after Jpk treatment, an increase in lysosomal staining was observed despite the absence of a FAG. (D-F) Vero cells were treated with Jpk (+Jpk, 50 nM for 6 hours) and then co-incubated with bafilomycin A1 (Baf, 100 nM; +Jpk +Baf; D,E) or pepstatin A (Pep, 10 μM; +Jpk +Pep; F). Notice that the dysfunction of the lysosomal activity prolonged the lifespan of the FAG. (E) Enlargement of the boxed area in D, in which the co-staining with TRITC-phalloidin and anti-LAMP2 antibodies revealed that Baf treatment traps lysosomes in an F-actin net (asterisks) that is structurally different from the FAG (arrowhead). Scale bars: 10 μm.
during its clearance, there was a tight association and partial colocalization of F-actin punctae with lysosomes and/or MDC-containing vesicles (inset in Fig. 7H). The ultrastructural analysis of FAG clearance showed proliferation of autophagic vacuoles (Fig. 7J), which, in some cases, contained a highly ordered filamentous structure resembling F-actin together with glycogen particles (Fig. 7K-L). Therefore, altogether, these results strongly suggest that autophagy is induced in cells containing a FAG and that this lysosomal process participates in its clearance.

We next checked whether chaperones are also involved in the dissolution of the FAG, because the association of heat-shock proteins (HSPs) with IBs could represent in our particular case an attempt of chaperons to favor FAG dissolution. To this end, Vero cells containing FAG were treated with the geldanamycin derivate 17-DMAG. This reagent binds to the ATPase site of human HSP90 and thus prevents its interaction with Hs factor 1, which in turn results in the activation and synthesis of HSPs (Muchowski and Wacker, 2005; Chiosis et al., 2006; Herbst and Wanker, 2007). 17-DMAG accelerated the fragmentation of the FAG and its subsequent clearance (compare panels in supplementary material Fig. S8A-D).

Next, we examined the involvement of the UPS, which plays an important role in the degradation of certain neurodegenerative IBs (Hol et al., 2006; Rubinsztein, 2006). Double immunolabeling (Fig. 8A) and western blot analysis (not shown) showed that the FAG did not contain polyubiquitylated proteins. By contrast, the FAG contained proteasomes (Fig. 8B). In addition, in cells treated with the proteasome-inhibitor lactacystin, the FAG remained for longer in the cytoplasm (compare panels in Fig. 8C-F). Moreover, lactacystin delayed the effect of 17-DMAG and therefore normalized FAG-clearance kinetics (supplementary material Fig. S8E,F). To assess the functionality of proteasomes in cells containing a FAG, we measured the chymotrypsin activity of proteasomes in the supernatant of lysates from cells treated with Jpk for different periods of time (Fig. 8G), because the proteasome activity of pellets (which contained FAG) could not be reliably measured because of their incomplete solubilization. We observed a significant decrease in proteasome activity in Jpk-treated cells. This was not a direct effect of Jpk, because its addition to the enzymatic reaction did not affect proteasome activity (not shown). This decrease could be caused by the tight association of proteasomes with the FAG. It is noteworthy that the decreased proteasome activity in the supernatant lasted longer (Fig. 8, +Jpk for 48 or 96 hours) than the presence of the FAG in the cytoplasm (Fig. 8G, 6 and 24 hours).

**Fig. 7.** Autophagy in cells containing a FAG. (A) Vero cells were transfected with LC3-GFP plasmid and cultured in medium without FBS, but containing peptatin A (Pep, 10 μM) plus E-64-d (10 μM). (B) LC3-GFP accumulated around the FAG in transfected cells cultured in complete medium without lysosomal inhibitors. (C) Representative experiment of an immunoblot using anti-LC3 antibody in cells treated with (to induce FAG formation) or without Jpk (+/– Jpk) and grown in medium with or without (4 hours) serum (+/–FBS) in the presence of lysosomal inhibitors. The positions of endogenous LC3-I and LC3-II are indicated. Fold increases in the ratios of LC3-II to tubulin (calculated as described in the Materials and Methods) from three independent experiments were 2.4±0.1 (Jpk–/FBS–), 3.8±0.3 (Jpk+/FBS–), 1.0 (control, Jpk–/FBS+) and 2.4±0.2 (Jpk+/FBS+). All differences were statistically significant at \( P < 0.005 \) using the Student’s t-test. (D) Ultrastructure of a FAG-containing cell (the FAG is limited by broken line), in which autophagic vacuoles appeared tightly associated with the FAG. (E) MDC-containing vesicles accumulated around the FAG. (F,G) During clearance of the FAG after Jpk removal (–Jpk for 16 or 24 hours), the number of MDC-containing vesicles and lysosomes (stained with MDC and/or anti-LAMP2 antibodies) increased and were associated with F-actin punctae and F-actin amorphous aggregates (arrowheads in G). (F,G) RpC (200 nM) added after Jpk removal accelerated the dissolution of the FAG (compare H with F and I with G). Inset in H shows the tight association and partial colocalization of F-actin punctae (red) with lysosomes and/or MDC-containing vesicles (green and blue, respectively). (J) The number of autophagic vacuoles increased during the dissolution of the FAG (–Jpk for 36 hours). (K-M) Some autophagic vacuoles contained fragments of microfilaments arranged in parallel (arrow in K; high magnification in L; M is an enlargement of the boxed area in L). Scale bars: 10 μm (A,B,E-I), 1 μm (J), 200 nm (D), 100 nm (K), 30 nm (L), 10 nm (M).
Cells can generate separate large inclusion bodies with segregated molecular identities.

Finally, we examined whether cells can generate only a single aggresome containing different altered proteins or whether aggresomes with a different content can be formed and maintained separately. We analyzed the structural dynamics (formation and disappearance) of the FAG in Vero cells expressing GFP-tagged exon 1 of mutant huntingtin (httm) containing a 103-trinucleotide CAG repeat expansion within its coding region that expresses a polyglutamine repeat (Canals et al., 2004). In Vero cells expressing GFP-tagged exon 1 httm and treated with Jpk, two large IBs were generated, one containing F-actin and the other the GFP-httm protein. Importantly, no IBs containing both molecular components were seen at any time (Fig. 9). Similar results were also obtained using striatal neuronal precursors M213 and HeLa cells (not shown). Therefore, cells can generate more than one IB, but the respective molecular inducers and components remain completely segregated.

Discussion

Here, using actin toxins, we report the formation of F-actin-enriched IBs. The IB produced by the F-actin stabilizer Jpk is particularly interesting because of its similarities with F-actin IBs of (neuro)diseases, as well as its presence in the totality of cells and its reversibility. The latter opens the possibility to examine in detail the proteolytic mechanisms involved in its clearance. The use of Jpk to induce a single, large FAG was previously reported in Dictyostelium discoideum amoebae (Lee et al., 1998). Here, we extend this study and report: (1) the formation and disappearance of this aggregate both in neuronal and non-neuronal mammalian cells, (2) the identification of some molecular components, (3) its ultrastructure and (4) the proteolytic processes involved in its clearance.

The single, large F-actin aggregate produced by jasplakinolide is an aggresome

In Jpk-treated cells, three different F-actin IBs were observed: F-actin punctae, F-actin amorphous aggregates and a FAG (single, large F-actin aggregate). This suggests that the FAG arises from the coalescence of F-actin punctae and F-actin amorphous aggregates as a result of active MT-dependent retrograde transport (Garcia-Mata et al., 2002; Johnston et al., 2002). Our results are

![Fig. 8. Proteasomes and proteasomal function in the FAG. (A,B) Vero cells containing a FAG (red) co-stained with anti-FK2 antibodies to show (poly)ubiquitylated proteins (A, green) or with anti-C9 antibodies to reveal the presence of proteasomes (B, green), indicating that the FAG contains proteasomes but not polyubiquitylated proteins. (C-F) FAG clearance was delayed in cells treated with lactacystin (Lac; compare E with C and F with D). (G) Proteasome activity measured in clarified lysates from untreated Vero cells (control, c), from cells treated with lactacystin (+Lac, 10 μM), or from cells treated with Jpk (+Jpk, 50 nM) for different times. Results are the mean ± s.d. from three independent experiments. Differences from control significant at P<0.01 (**) and P<0.001 (***) Scale bar: 10 μm.](image)

![Fig. 9. Cells can generate segregated aggresomes with different molecular compositions. Vero cells were transfected with a GFP-huntingtin mutant plasmid (GFP-httm). (A) At early expression times of GFP-httm (4 hours), a diffuse cytoplasmic staining was seen (green). (B,C) Subsequent treatment with Jpk (+Jpk, 50 nM) produced F-actin amorphous aggregates and a FAG (red) as long as the GFP-httm aggresome was also forming (green). (D,E) After longer Jpk treatment, the FAG is fragmented in F-actin amorphous aggregates and F-actin punctae (D, red) until its complete clearance (E), but the GFP-httm aggresome (E, green) remained in the cytoplasm. (F) Notice that the two aggresomes, one containing GFP-httm (green) and the other F-actin (red), constantly remain morphologically and molecularly segregated, even when cells were submitted to various Jpk pulses (also see supplementary material Fig. S1). Scale bar: 10 μm.](image)
consistent with this hypothesis, because they demonstrate that the formation of the FAG is MT-dependent. However, FAG structure became MT-independent once it was completely formed. Therefore – on the basis of its structure, MT-dependent formation and pericentriolar positioning, the rearrangement of vimentin intermediate filaments and the accumulation of mitochondria (Johnston et al., 2002; Bauer and Richter-Landsberg, 2006; Muquit et al., 2006; Podlubnaia and Nowak, 2006), the most representative of which being ADF/cofilin-actin rods (Nishida et al., 1987; Minamide et al., 2000) and Hirano bodies (Carrit et al., 1985; Hirano, 1994). In fact, because both structures are highly enriched in ADF/cofilin (Nishida et al., 1987; Maciver and Harrington, 1995), it has been postulated that actin rods present in neurites of hippocampal neurons are the precursors of Hirano bodies (Minamide et al., 2000). In this regard, the Jpk-induced F-actin aggresome also contains ADF/cofilin (see below), as well as cortactin and Arp2/3, which are involved in the nucleation and polymerization of actin (Weaver et al., 2003). Therefore, this aggresome might simply represent the aberrant accumulation of multiple fragments of F-actin trapped in the cytoplasm as a result of the reported actin-crosslinking stabilization and/or the aberrant actin-nucleation activity generated by Jpk (Bubb et al., 2000). The localization of cortactin, Arp2/3 and ADF/cofilin in F-actin aggresomes in astrocytes, neuroblastoma cells and/or hippocampal neurons, as well as in Hirano bodies (Galloway et al., 1987a; Galloway et al., 1987b; Goldman, 1983; Maciver and Harrington, 1995; Schmidt et al., 1989), suggests that the molecular machinery involved in actin dynamics and its subcellular organization is also perturbed in neural cells and in some IB-containing (neuro)diseases.

Importantly, actin rods and Hirano bodies are actin paracrystallike intracellular structures (Hirano et al., 1968; Izumiya et al., 1991; Hirano, 1994; Minamide et al., 2000). However, an ordered structure is usually not present in F-actin aggresomes, although it is occasionally seen in some areas, which by size and morphology most likely correspond to parallel arrays of microfilaments or actin bundles. The general absence of an ordered F-actin structure in the whole F-actin aggresome might be attributable to the mode of action of Jpk. When used at the concentrations reported here (50 or 500 nM), Jpk stabilizes F-actin and, at the same time, promotes its polymerization (Bubb et al., 2000). The final result is the formation of small F-actin masses (F-actin punctae and F-actin amorphous aggregates) throughout the cytoplasm, and their juxtanuclear coalescence gives rise to a FAG. Therefore, this aggresome simply represents the accumulation of multiple fragments of stable and aberrant F-actin trapped in the cytoplasm as a result of actin-crosslinking stabilization and/or aberrant actin-nucleation activity generated by Jpk. In this respect, photobleaching results indicate that the actin of the F-actin aggresome is not dynamic. Moreover, the presence in the aggregate of proteins that can sever F-actin without capping (ADF/cofilin) could further facilitate the intrinsic Jpk-induced actin polymerization.

Another difference with Hirano bodies described in histological preparations from brain is the absence of Tau in the F-actin aggresome, but notice that this marker has been detected in only 20% of Hirano bodies (Galloway et al., 1987b). It is proposed that the formation of Hirano bodies is either a cellular response of the actin cytoskeleton or a consequence of its aberrant function (Fechheimer et al., 2002). This postulate arises from the Hirano-body-like formation observed in Dicystostelium and mammalian cell lines by the expression of the C-terminal fragment of the Dicystostelium 4-kDa actin cross-linking protein (Maselli et al., 2002; Maselli et al., 2003; Davis et al., 2008). Mammalian cells containing model Hirano bodies showed normal growth, morphology and motility (Davis et al., 2008).

Cell viability, actin dynamics and proteolysis in cells containing the F-actin aggresome

Despite the perturbed subcellular distribution of mitochondria in cells containing an F-actin aggresome, the assay of mitochondrial activity was normal. It is possible that the clustering of mitochondria around the F-actin aggresome provide the high requirements of ATP for the proteolytic systems involved in its clearance. Strikingly, while the F-actin aggresome remained in the cytoplasm, most cells were binucleate, which indicates that they were arrested in cytokinesis, probably because these cells lack enough functional (unperturbed) G-actin and/or F-actin to complete cell division. Similarly, the F-actin aggresome might also hinder the formation of the myosin contractile ring, which would prevent cell cleavage. However, cells progress normally as soon as the F-actin aggresome disappears and hence its presence is apparently not harmful to cells. Importantly, in neurons, the appearance of an F-actin aggresome requires greater Jpk concentration and a longer treatment than in non-neuronal cells. This suggests that the former are more resistant to the formation of F-actin-enriched IBs, but that, once generated, they trigger apoptosis. Therefore, the appearance of disease-associated IBs in neuronal cells could render them more sensitive than other cell types, compromising their survival and leading to apoptosis. By contrast, intrinsic differences in the actin-cytoskeleton dynamics and/or regulation between neuronal and non-neuronal cells might also explain the reported differences in response to Jpk treatment.

The actin contained in F-actin aggresomes is not dynamic according to the photobleaching results with YFP-actin and to its insensitivity to LiB treatment. The latter is consistent with previous results in which actin filaments made by the addition of equimolar Jpk were resistant to depolymerization (Bubb et al., 1994). The stimulation of actin polymerization through the reconstitution of normal cell shape and actin-stress-fiber organization occurring after LiB withdrawal and the induced formation of plasma membrane ruffling or actin stress fibers do not produce any alteration in the structure of the F-actin aggresome. Moreover, the presence of an F-actin aggresome does also not interfere in the induced formation of these actin-based structures.
The high amount of F-actin contained in the aggregate suggests that it cannot be immediately chaperoned or degraded. Consequently, the cell strongly activates proteolysis mediated by proteasomes and autophagy (Fortun et al., 2007). The involvement of autophagy is supported by the spatial association of GFP-labeled autophagosomes with the F-actin aggregate, and the visualization by light and electron microscopy of autophagic vacuoles in their vicinity. Moreover, the presence of an F-actin aggregate increases the levels of LC3 II, the lipidated form bound to the autophagosome membrane and MDC-containing vesicles, almost all of which are localized around the F-actin aggregate. Finally, the autophagy induced by Rpe as well as chaperones stimulated by 17-DMAG decreased the lifespan of the F-actin aggregate. The use of pharmacological agents that impair the normal activity of proteasomes or lysosomes also increased the lifespan of the F-actin aggregate, suggesting their involvement in its clearance. The disruption of MTs also caused the aggregate to remain for longer in the cytoplasm. This might be because (1) lysosomes and autophagic vacuoles depend on MTs for their redistribution to aggresomes (García-Mata et al., 2002; Murray and Wolkoff, 2003; Iwata et al., 2005), and (2) autophagosome-lysosome fusion also depends on MTs and dynein motor activity (Rubinsztein et al., 2005; Webb et al., 2004). By contrast, pro tease activity might also depend on the integrity of cytoskeleton networks, because ubiquitylation enzymes and/or proteasomes could be transported along cytoskeleton towards aggresomes (Wojcik and De Martino, 2003). Therefore, taken together, these results indicate that the lysosomal and/or autophagic system is strongly activated in the dissolution of the F-actin aggregate.

Finally, the F-actin-aggresome model reported here offers numerous advantages: (1) it works in neuronal and non-neuronal mammalian cell lines; (2) it is simple, highly reproducible and reversible; and (3) it provides an opportunity to test pharmacological agents that could interfere with the formation, maintenance and/or disappearance of pathologic F-actin-enriched IBs.

MATERIALS AND METHODS

Antibodies, plasmids and reagents

Monoclonal antibodies against β- and γ-tubulin, ADIF/cofilin, myosin IIa, and vimentin were purchased from Sigma (St Louis, MO, USA). Monoclonal antibodies against cortactin, p21-Arc, RhoA, Rac and Cdc42 were obtained from BD Biosciences (Erembodegem, Belgium). Monoclonal antibody against LC3 (clone 5F10) was from Nanotools (Teningen, Germany). Monospecific rabbit polyclonal antibodies against β- and γ-actin, and KDELr were kindly provided by Christine Chapounnier (University of Geneva, Switzerland) and Hans-Dieter Soling (University of Göttingen, Germany). Monoclonal antibodies against giantin and MAP4 were supplied by Hans-Peter Hauri (Biozentrum, Basel University, Switzerland) and Jesus Avila (CSIC-UAM, Madrid, Spain), respectively. Polyclonal antibodies against N-WASP, spectrin and LAMP2 were a gift from Michael Way (MRC, London, UK), Jay S. Morrow (Yale University, CT) and Minoru Fukuda (La Jolla, CA), respectively. Mouse monoclonal antibodies that recognize ubiquitylated proteins (clone FK2) were purchased from Affinity (Exeter, Devon, UK), whereas those that recognize the α-subunit C9 of the 20S proteasome have been described previously (Fuertes et al., 2003). Secondary Alexa-Fluor-546 (Fab)2; fragments of goat anti-rabbit IgG, FITC-TRITC-phycoerythrin, DAPI and phalarginolide were obtained from Invitrogen (Carlsbad, CA). Goat anti-mouse-Cy2 IgG (Fab2); fragments were purchased from Jackson Immunoresearch (West Baltimore, PA, USA). pEGFP- LC3-encoding plasmid was a gift from Tatsuo Yoshimori and Noboru Mizushima (National Institute of Genetics, Japan); GFP-biotin-encoding plasmid was used as previously reported (Canals et al., 2004). TRITC- and FITC-phalloidin, monodansylcadaverine, cytochalasin D, taxol, brefeldin A and nocodazole, lactacystin, bafilomycin A1, pepstatin A, leupeptin, E-64-d, rapamycin and 17-DMAG were from Calbiochem (EMD Biosciences, Darmstadt, Germany). The EMBed-121 embedding media kit and the reagents used in the electron microscopy experiments were provided by Electron Microscopy Sciences (Hatfield, PA).

Cell culture and actin-toxin treatments

N2a, HeLa, Vero and M213 cells were grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and MEM sodium pyruvate (1 mM). Cell cultures were maintained at 37°C in a humidified CO2 (5%) atmosphere.

Primary cultures of mouse hippocampal neurons (E16 brains) were obtained and cultured as previously reported (Urena et al., 2005). Mouse neural stem cell 17.2 cells were grown in DMEM supplemented with 10% FBS, 5% horse serum, 2 mM glutamine and 20 μg/ml gentamicin as described previously (Snyder, 1992). Primary cultures of mouse hippocampal neurons (E16 brains) were obtained and cultured as previously reported (Urena et al., 2005).

Fluorescence microscopy and indirect immunofluorescence

Indirect immunofluorescence was performed as previously described (Valderrama et al., 2001) with the following antibody dilutions: anti-giantin, 1:500; coflin 1:1000; anti-vimentin, 1:100; anti-β-tubulin, 1:50; anti-γ-tubulin, 1:5000; anti-RhoA, -Rac and -Cdc42, 1:100; anti-p21Arc (ARP2/3), 1:300; anti-cortactin, 1:500; anti-MAP4, 1:200; anti-β- and γ-actin, 1:100; and anti-myosin IIA and B, 1:100. Secondary antibodies were used at the following concentrations: Alexa-Fluor-546-conjugated goat anti-rabbit, 1:500 and goat anti-mouse-Cy2, 1:50. TRITC- and FITC-phalloidin were used at 1:500 and DAPI at 1:100. For MDC-containing vesicles or mitochondria staining, cells were respectively incubated with MDC 200 nM or MitoTracker 100 nM for 30 minutes. Microscopy was performed with a BX60 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with an Orca ER cooled CCD camera (Hamamatsu Photonics, Japan) or with a TCS SL confocal microscope with argon and helium-neon lasers attached to a Leica DMIRE2 inverted microscope (Leica Microsystems, Heerbrugg, Switzerland). For 3D reconstruction of FAG and the Golgi complex, the entire 3D stack of images was obtained using the z stack present in the Leica TCS-SL microscope and the size of the confocal image between sections was 0.1 μm. The images were processed using Adobe Photoshop CS (Adobe Systems, San Jose, CA). ImageJ 1.33 (National Institutes of Health, Bethesda, MD) and IMOD 3.5.5 (Colorado, USA) 3D modeling software.

Transmission electron microscopy

Control and treated Vero cells were processed as previously described (Lazarro-Deiguez et al., 2006). Ultrathin sections were stained with 2% uranyl acetate for 30 minutes, then with lead citrate for 10 minutes and observed with a JEOL 1010 transmission electron microscope operating at 80 kV and provided with a Gatan BioScan model 920 module for acquisition of digital images with Digital Micrograph 3.4.3 acquisition software (Gatan, Pleasanton, CA).

Cell viability assay

Cell viability was measured either using the methylthiazol tetrazolium MTT reduction assay (which is indicative of mitochondrial function) or the Trypan Blue exclusion method. The reduction assay was performed using the Cell Proliferation Kit (Boehringer Mannheim, Germany) and MTT protein levels were measured spectrophotometrically at 550 nm. In the second method, cells were removed from Petri dishes and aliquots (50 μl) of the cells were mixed with PBS (40 μl) and 0.04% Trypan Blue (10 μl). Live (Trypan-Blue-excluding) cells were counted. Results are expressed as the percentage of surviving cells with respect to controls (100%). Statistical significance was evaluated by the Student’s t-test.

Subcellular fractionation and immunoblotting

Vero cells containing a FAG were washed in PBS, scraped and centrifuged at 300 g at 4°C for 5 minutes. Cells were washed twice in PBS and cell pellets were homogenized with a potter (ten strokes) in cold buffer A (50 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 100 mM sodium orthovanadate, 10 mM sodium fluoride and 10 μM leupeptin, 1 μg/ml pepstatin, and 2 mM phenylmethylsulfonyl fluoride). After further centrifugation of the suspension, the supernatant was removed. The pellet was resuspended in buffer A containing 2.5 M sucrose, which was overlaid with 630 μl of buffer A containing different sucrose concentrations (35, 30, 25, 20, 15, 10 and 5%). The discontinuous gradient was centrifuged at 130,000 g for 16 hours at 4°C in the SW55-Ti rotor. Subsequently, ten fractions were collected from the top (#1) to the bottom (#10). The capture of PAGs in the different fractions was checked by fluorescent microscopy using TRITC-phalloidin. The cytoskeleton components present in each fraction were analyzed by SDS-PAGE and immunoblotting.

Sucrose fractions (#1 to #10) were prepared for SDS-PAGE by boiling in sample buffer. Proteins were electrophoretically transferred onto nitrocellulose membranes. Blots were blocked in 5% non-fatty milk in Tris-buffered saline (TBS) with Tween-20 (v/v) (TBST) for 2 hours at room temperature. Primary-antibody incubations were performed in TBS containing 1.5% BSA for 2 hours at room temperature and used at 1:10,000 for anti-coflin, 1:500 for anti-Arp3, and 1:1000 for anti-myosin II and anti-actin. Secondary-antibody incubations were performed in TBST containing 5% non-fat dry milk at 1:10,000 for 1 hour at room temperature. Protein bands were visualized using a Supersignal West Pico Chemiluminescence Substrate Kit from Pierce (Rockford, IL). Immunoblotting procedure for endogenous LC3 forms was carried out as previously described (Esteban et al., 2007) using the
following antibody dilutions: anti-LC3, 1:500 and secondary antibody anti-mouse IgG-HRP, 1:10,000. To calculate the LC3-II fold-increase, densitometric measurements of the LC3-II bands were divided by those of the corresponding tubulin bands, and the resulting ratios were in turn divided by the LC3-II:tubulin ratio obtained in control cells (Jpk–, FBS+).

**Gene transfections**

Cells grown on 10-mm glass coverslips at 2 × 10⁵ cell/ml were transfected with GFP-LC3, GFP-hrGFP or YFP-actin using the FuGene6 (Roche) and expressed for 4–48 hours.

**Fluorescence recovery after photobleaching**

For FRAP experiments, vero cells transfected with YFP-actin were viewed using the Leica confocal microscope described above and equipped with an incubation system with temperature and CO₂ control. Cells (4 × 10⁵) were seeded on 22-mm glass coverslips (Micro cover glass; Electron Microscopy Sciences, Fort Washington, PA). After 24 hours, cells were transfected with 1 µg of GFP-actin construct. 24 hours after transfection, the glass coverslip was mounted in the video confocal chamber, keeping the cells at 37°C in a 5% CO₂ atmosphere. For visualization of YFP, images were acquired using a 63 × oil immersion objective lens (numerical aperture, 1.32), 488-nm laser line, excitation beam splitter RFP 500, 500- to 600-nm emission range detection and the confocal pinhole set at 2-3 Airy units to minimize changes in fluorescence efficiency due to the movement of YFP proteins away from the plane of focus. The same FAG or cytoplasmic area was photobleached using 40 scans with the 488-nm laser line at full power. To detect the fast component of the recovery, the first 30 images were taken every 0.5 seconds and the rest every 5 seconds for 5 minutes.

**Proteasomal activity**

We determined the chymotrypsin-like activity of proteasomes in the supernatants from crude cell extracts centrifuged at 10,000g with or without 10µM geldanamycin to the fluorogenic peptide substrate was determined by continuously monitoring the fluorescence (excitation, 355 nm; emission, 460 nm) of the released coumaryl-7-amide using a VICTOR plate reader (WALLAC VICTOR 2 V1420 1424 7739). The association of actin with Hirano bodies.

**References**


