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Role of Auxilin and Heat Shock Protein 70kDa in Clathrin Uncoating

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ABSTRACT

An interaction between auxilin and heat shock protein 70kDa (Hsc70) was initially discovered in 1995. There exists a large amount of data supporting the basis for their interaction in vitro and their function in clathrin uncoating in vivo. This review examines the key experiments in elucidating this interaction and introduces a third protein that may connect constriction or fission with hsc70/auxilin mediated clathrin uncoating.

INTRODUCTION

The formation of the clathrin coat in receptor mediated endocytosis (RME) requires a number of players. At the membrane, adaptor proteins (AP-1 and 2 recognize the cargo receptors and initiate the assembly of the clathrin triskelion. Then the triskelion legs interact with each other through their heavy chains and form the polyhedral lattice. The formation of this coat, which causes the membrane to deform and induces invagination, is thought to drive budding (Schmid, 1997). Once the vesicle is internalized, another set of protein players must come into action. They are required for the removal of the clathrin coat to allow two major events: (1) fusion of the vesicle to the target membrane and (2) recycling of coat components (Goldstein et al., 1979). This is the key transition step that connects two protein families, emphasizing the importance of protein localization.

The heat shock protein 70kDa (Hsp70) and heat shock protein 90kDa (Hsp90), both part of the heat shock family of proteins, as well as chaperonins are well known molecular chaperones that assist in protein folding and prevent aggregation of unfolded proteins (Frydman, 2001). Hsp70 and heat shock cognate 70kDa (Hsc70), which is constitutively expressed in the cytosol, use adenosine triphosphate (ATP) binding and hydrolysis to power their interactions with a wide range of unfolded substrates (Greene et al., 1995). One way to harness the chaperones' ability to modulate protein conformation is through binding co-chaperone domains in "adaptor proteins." These co-chaperones also regulate the intrinsic ATPase activity of Hsc70 (Frydman, 2001).

There are three classes of co-chaperone domains: (1) DNA J homology or J domain, (2) Bag-1 homology or Bag domain, and (3) Tetratricopeptide repeat (TRP) clamp domain. The J domain transfers a bound protein substrate to Hsc70-ATP and stimulates ATP hydrolysis, which enhances binding of Hsc70-ADP to target substrates (Suh et al., 1998). The Bag domain works in an opposite manner, where its activation of the Hsc70 ATPase domain stimulates ADP to ATP exchange and causes the release of substrates (Sodermann et al., 2001). The TRP clamp domains have been shown to bind both Hsp90 and Hsc70, but only inhibit Hsp90 ATPase cycle (Scheufler et al., 2000). There are around 15 characterized co-chaperone proteins, expressed from fungi to yeast and mammals. Each has their preference for Hsc70 or Hsp90, and each utilize an additional substrate interaction domain (i.e., clathrin, PLCgamma, or myosin binding sites) to help localize heat shock protein for specific functions (Young et al., 2003).

Auxilin is a specific family of J domain containing protein that is expressed in a wide range of organisms. Mammalian auxilin has a characteristic C-terminal J domain (a conserved HDP tripeptide) along with a clathrin-binding domain (Lemmon, 2001). Auxilin-1 is expressed mainly in the brain, whereas auxilin-2 is more ubiquitously expressed and contains an additional cyclin-G Associated protein Kinase (GAK) domain. Understanding the significance of Hsc70 interaction with auxilin may explain their functional role in uncoating of clathrin coated vesicles.

DEFINING INTERACTIONS

Hsc70 was first discovered in bovine brain cytosol as a participant of in vitro clathrin uncoating assays (Schlossman et al., 1984). Originally this chaperone was called 'the uncoating ATPase' until antibodies and sequence analysis using 2-dimensional gels and proteolysis mapping confirmed its identity as the 70kD stress protein (Ungewickell, 1985; Chappell et al., 1986). Additional studies suggested that a cofactor was involved in Hsc70 mediated clathrin uncoating. Adding purified bovine Hsc70 and ATP to purified clathrin with AP-2, forming preassembled baskets, was insufficient to stimulate uncoating unless a 100 kDA protein cofactor isolated from coated vesicles was added into the reaction (Prasad et al., 1993). This cofactor was identi-

fied as auxilin-1. Immunodepletion of this protein from brain cytosol inhibited coat dissociation, and uncoating was only restored when recombinant auxilin-1, Hsc70, and ATP was added back (Ungewickell et al., 1995). Structural analysis showed that auxilin-1 had a high affinity for certain domains of the clathrin baskets, along with an ability to recruit Hsc70 to the basket in an ATP dependent manner. When auxilin-1 lost its J domain, it was still able to bind to clathrin but was defective in Hsc70 mediated uncoating (Ungewickell et al., 1995).

Direct binding substrates for the J domain (Hsc70) and its adjacent clathrin binding domain were confirmed using glutathione-S-transferase (GST)-constructs. Both domains retained their respective binding properties when incubated separately with preassembled baskets and Hsc70/ATP, and only induced dissociation of clathrin baskets when expressed together in the presence of ATP (Holstein et al., 1996). Thus, rapid hydrolysis of ATP was observed when the J domain and Hsc70 interacted, supporting a model where (1) auxilin-1 interacts with the clathrin heavy chain via its clathrin binding domain and (2) the J domain then directs Hsc70 to this complex, which initiates basket dissociation in an ATP dependent manner.

FUNCTIONAL STUDIES

Understanding the functional significance of these interactions came with the design of highly specific antibodies and utilization of multiple microscopic techniques. Hsc70 antibodies that blocked in vitro clathrin uncoating were microinjected into cultured cells, reducing the amount of functional Hsc70 and disrupting the endocytic pathways of labeled ligand for low density lipoprotein (LDL) receptors and Fc (FcRII) receptors (Honing et al., 1994). Honing et al. used immunofluorescence, confocal laser scanning microscopy, scanning electron microscopy (EM), and thin section microscopy to show that blocking Hsc70 caused accumulation of ligand at the clathrin pits. Yet still, this was not direct evidence that uncoating was affected. A group in Germany discovered that GAK was the non-neuronal form of auxilin that was expressed ubiquitously in eukaryotic cells, called auxilin-2 (Umeda et al., 2000). They determined its binding specificities for alpha and gamma appendages (of AP2 and AP1, respectively) using GST-auxilin-2, and confirmed that ATP-dependent uncoating of clathrin vesicles required the J-domains of both Hsc-70 and auxilin-2. More importantly, when they overexpressed green fluorescent protein (GFP)-auxilin-2 in HeLa cells, not only was the endocytosis of transferrin (Tfn) inhibited, but clathrin was sequestered in cytosolic aggregates that co-localized with GFP-auxilin-2 while AP-1 and AP-2 remained membrane associated (Umeda et al., 2000). These results supported an in vivo interaction between auxilin and clathrin, but did not provide evidence for Hsc70-auxilin mediated clathrin uncoating. This required ablation of endogenous auxilin, via antisense or inactivating antibodies, or using dominant negative Hsc70 and looking into model systems to link genetic defects to physiological effects.

Studies by two groups separately isolated the yeast homolog of auxilin (Swa2p/Aux1) and showed for the first time auxilin's essential role in clathrin function in vivo (Gali et al., 2000; Pishavee et al., 2000). The first group, Gali et al., used Swa2p as bait in GST pull downs, confirming in vivo interactions with clathrin and mapping the activation of Hsc70 ATPase activity to the J and TRP domains. Generating SWA2 mutant yeast cells revealed hallmark phenotypes of clathrin deficient yeast strains: (1) secretion of unprocessed alpha factor, (2) mislocalization of a late golgi protein, and (3) endocytic defects in the uptake of 'a-factor plasma' membrane receptor during pulse chase experiments. Differential centrifugation was used to separate pellets containing clathrin coated vesicles (CCV) from free clathrin in the supernatant. SWA2 mutants showed a marked increase in clathrin association with vesicles at the expense of the free clathrin pool. Since this equilibrium of assembled and unassembled clathrin was observed in wild-type cells, it implied that Swa2p was needed to uncoat CCVs to allow recycling and continuous rounds of endocytosis to occur. Thin EM sections confirmed that Swa2 deletion mutant yeast accumulated abnormal amounts of clathrin at the membrane and not in the vesicles (Gali et al., 2000).

The second group, Pishvaee et al., called their auxilin yeast homolog Aux1 (Pishavee et al., 2000) and showed, using similar assays as described above, that targeted gene replacement of Aux1 impaired cargo delivery and caused an accumulation of CCVs. EM of the clathrin fraction and column chromatography results confirmed clathrin accumulation in the coated pits. In situ analysis and immunofluorescence, using antibodies against clathrin light chain, confirmed clathrin redistribution in vivo in the mutant cell. Immunogold labeling of clathrin light chain in ultra thin cryosections allowed quantification of a 5.5 fold increase in coated vesicles for the mutant cells. In addition, they created an Aux1 mutant lacking its J domain, which still resulted in CCV accumulation, implying that the J domain, which interacts with Hsc70, is important for uncoating in vivo. Uncoating is also required for the efficient delivery of cargo, which is perturbed in these mutant cells (Pishavee et al., 2000). These results confirm auxilin's role in Hsc70 mediated uncoating and exemplify how a system can be used to investigate the function of potential uncoating factors.

Additional work on clathrin uncoating has been performed in other organisms. Auxilin homologue in Caenorhabditis elegans (C. elegans) contains 18%

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amino acid identity and its J domain is 33% identical (Greener et al., 2001). Greener et al. took advantage of the fact that C. elegans oocytes yolk protein (YP170) uptake employs a similar mechanism to LDL endocytosis in mammals (Grant and Hirsch, 1999), and used ribonucleic acid interference (RNAi) to inhibit auxilin expression. They wanted to monitor clathrin uncoating/recycling by labeling the heavy chains or cargo with GFP, fixing the samples at each corresponding step, and using laser scanning confocal microscopy to monitor the vesicles and cargo. They first showed that endocytosis of GFP-YP170, but not its secretion, was reduced in RNAi treated worms. Auxilin RNAi treated larvae expressing a transgene for GFP-clathrin heavy chain (GFP-CHC) showed a brighter, punctate fluorescence in somatic cells, implying an accumulation of clathrin coated vesicles. This conclusion would be stronger if they used a clathrin antibody to check endogenous levels of clathrin and confirm that the accumulation was not an effect of overexpression. One striking result was that survival rate for RNAi treated worms increased from 40% to almost 100% when expressing GFP-CHC. Although auxilin is knocked down, thus preventing recycling of clathrin, GFP-CHC worms overexpressing clathrin may partially compensate for recycling defects. Additional evidence for recycling defects used fluorescence recovery after photobleaching (FRAP). After photobleaching a portion of the larvae, recovery of fluorescence can reveal whether clathrin exchange occurs, with unbleached fluorescence proteins replacing the photobleached proteins in a time dependant manner. As expected, GFP-CHC worms showed complete recovery of fluorescence whereas RNAi auxilin worms did not (Greener, Grant et al, 2001).

Drosophila melanogaster (*D. melanogster*) is a third model organism used to screen proteins for their function in RME. Endosomal internalization of the Boss receptor, stimulated by the Sevenless binding in *D. melanogster* eyes, uses a dynamin dependent pathway (Caganm et al., 1992). Screens of mutant flies with deficiencies in Boss internalization identified a point mutation in the fly homolog of Hsc70 (Chang et al., 2002). Mutant Hsc70-4 flies were generated by FLP/FRP recombination and were screened for horseradish peroxidase positive (HRP+) vesicles. There was a block in internalization seen by a lack of uptake of endocytic tracers and Texas red labeled avidin in flies with a transgene for endosomal markers. Immunostaining and EM analysis in these mutant cells confirmed that avidin endocytosis was blocked, whereas HRP+ vesicles, clathrin coated vesicles, and labyrinthine channels characteristic of shibere mutants (Kosaka and Ikeda, 1983), on or near the plasma membrane, were absent. GFP-clathrin light chain was used to confirm that clathrin was centrally located in Hsc70-4 mutant cells. HRP-Boss and 2-mercaptoethanesulfonic acid (MESNA) treated enzyme-linked immunoassay (ELISA) assays confirmed that mutant Hsc70-4 cells had proper Boss delivery to the plasma membrane but could not be internalized. In vitro binding analysis using Hsc70-4 mutants (R447H) did not affect clathrin or auxilin binding, yet clathrin uncoating was still compromised when measuring clathrin release from CCV (Chang et al., 2002). Only a 33% reduction was observed, probably since a null mutation in Hsc70-4 is lethal to cells. Nonetheless this modest reduction of in vitro uncoating activity was still sufficient to cause a dramatic reduction in endocytosis in vivo. One should note that these results only support a correlative observation between Hsc70-auxilin interaction and uncoating, and any cause and effect evidence requires clathrin uncoating to be measured in vivo.

BIOCHEMICAL CHARACTERIZATION

The in vitro and in vivo experiments described above support an interaction for Hsc70 and auxilin in clathrin uncoating. Another major focus is to understand the biochemistry of this interaction. It is believed that Hsc70 sequentially forms 2 types of complexes with the clathrin triskelions. In the first step, binding of auxilin to one clathrin triskelion causes further binding of three Hsc70-ATP molecules (Green and Eisenberg, 1990). Next, the rapid catalysis of Hsc70-ATP hydrolysis by auxilin triggers the dissociation of the clathrin triskelion from the basket. This leaves an uncoated triskelion with Hsc70-ADP bound to it, called the pre-steady state complex, and free auxilin continues to act catalytically by directly attaching to another triskelion in the basket and initiating another round of uncoating (Barouch et al., 1997).

The released Hsc70-ADP/clathrin complex transforms from the pre-steady state to a steady state complex after ATP exchange, and this complex requires the presence of AP-180 and Hsc70 to keep it intact (Jiang, et al., 2001). The pre-steady state/adenosine diphosphate (ADP) bound form cannot be immunoprecipitated easily and can dissociate rapidly, so the exchange of ADP for ATP alters and stabilizes the structure of the AP-clathrin-Hsc70 complex. This supports the model that Hsc70 acts stoichiometrically, where formation of the energetically stable steady state Hsc70-ATP/clathrin complex acts as the driving force for uncoating. This steady state complex also ties up the cytosolic triskelion, thus preventing it from inappropriately binding to proteins actively involved in endocytosis. This has been supported by in vivo studies showing redistribution of clathrin away from the coated pits/membranes when auxilin or AP-180 is overexpressed (Zhoa et al. 2001). The steady state complex is also proposed to "prime clathrin to interact with membranes" (Jiang et al., 2000) and allow rapid return of AP and clathrin to form a new coated pit. The steady state complex can now be used to characterize additional proteins and regulatory factors that are required for clathrin and AP recycling.

Much effort has also been placed in trying to determine the auxilin structure required to transfer clathrin to Hsc70-ADP. Structures for *Escherichia coli* (*E. coli*) J domain presenting iron-sulfur cluster protein to Hsc66 (Hsc70 homolog) (Cupp-Vickery and Vickery, 2000), and SV40 large T-antigen J domain binding retinoblastoma (Rb) tumor suppressor (Kim et al., 2001) have been determined. For auxilin, Ma et al. has mapped 2 separate domains: (1) a C-terminal sub domain, within the clathrin binding domain, that is required for auxilin catalytic activity and (2) an N-terminal domain that enhances the binding to clathrin (Ma et al., 2002). Scheele et al. has also characterized the C-terminal domain, Aux-C20, and showed it contains the key interactions with clathrin and AP-2 to support clathrin uncoating (Scheele, et al., 2001). Since full length 100kD auxilin and the 60kDa recombinant fragment which has full uncoating activity, were too large to crystallize, one group used the Aux-C20 fragment to determine the 3-dimensional structure of auxilin binding domain via nuclear magnetic resonance (NMR) (Gruschus and Hanetal, 2004). Their conclusion showed marked differences in the binding domains and the orientation in Aux-C20 compared to E. coli and large T antigen J proteins, implying that J domains determine which specific substrate is to be transferred to Hsc-70. This reflects the importance of understanding how J domain proteins interactions specify its function.

A NEW PROTEIN PLAYER

The dynamin superfamily of guanosine triphosphatase (GTPase) has been implicated in the scission of a wide range of vesicles and organelles. In clathrin mediated endocytosis, dynamin self assembles into coiled structures at the neck of the invaginated pits and stimulates its GTPase, which drives dynamin disassembly (Praefcke and McMahon, 2004). There exist 2 competing models for dynamin function in RME: (1) dynamin functions as a mechanochemical enzyme to physically drive membrane vesiculation by acting as a 'pinchase' to constrict and sever invaginated pits at their neck or (2) dynamin functions as a regulatory molecule in endocytic CCV formation to recruit and/or activate downstream effectors in its GTP-bound form (Yang and Cerione, 1999). Recent data from coated pit dynamics supports the latter model.

Uptake of fluorescently labeled transferrin at short time intervals was observed using a GFP-clathrin light chain fusion in COS-1 cells (Gaidarov et al., 1999). Automated tracking programs taking sequential time lapse images showed that clathrin coated pits had limited mobility and repeatedly formed coated vesicles at discrete sites. Addition of latrunculin B, an inhibitor to actin assembly, showed partial inhibition of RME. This implied that pit formation was initiated on the cytoskeleton and may be regulated by protein factors recruited by the cytoskeleton, one potentially being dynamin (Estes et al., 1996). Advances in microscopy now allow real-time imaging of cargo sorting and endocytosis in living cells (Ehrlich et al., 2004), which confirmed the dynamics of clathrin assembly and correlated it to the dynamics of dynamin in these coated pits. When a *D. melanogaster* Hsc70 mutant was crossed with a fly line containing a dominant negative allele of dynamin, shi, there was an enhancement of the rough eye phenotype (Chang et al., 2002), suggesting a connection between the two pathways. Characterization of dominant interfering Hsc70 mutants expressed in HeLa cells showed additional defects in endocytosis that could not be accounted for by uncoating alone (Newmeyer and Schmid, 2001). These mutants inhibited clathrin release during in vitro uncoating assays and caused retainment of all internalized transferrin, supporting a defect in uncoating and recycling. In addition, the mutant Tfn trafficking assays also showed defects in the initial uptake of biotinlayted Tfn, during avidin quenching ELISA and in the presence of MESNA treatment, suggesting that coated vesicle formation was also inhibited by the dominant interfering Hsc70.

The best direct link comes from a paper describing auxilin and Hsc70 interactions with dynamin in the early stage of coated vesicle formation (Newmyer et al., 2003). Dynamin has an intramolecular domain called the GTPase effector domain (GED) that controls its GTPase activity by acting as a GTPase activating protein (GAP). Effectors from rat brain cytosol that interacted with GTP bound dynamin were isolated from in vitro GST-dynamin GTPase columns, supplemented with its GED/GAP domain and GTP-gamma-S. These substrates, Hsc70 and auxilin, were eluted using GDP and were not pulled down in the GDP column, proving that their interaction with dynamin was GTP dependent. Direct binding was confirmed using recombinant auxilin and purified Hsc70 with Gst-dynamin: GTPgammaS. When GTP hydrolysis was measured using dynamin-GTP in the presence of its GAP, addition of auxilin, but not Hsc70, inhibited hydrolysis in a cooperative manner. Increasing concentrations of the GAP overcame the inhibition, suggesting that auxilin and GAP competed for the same binding site. Mapping domains in auxilin revealed 2 dynamin binding sites: an upstream site (405-491) and a downstream site (591-814) [previously shown to interact with clathrin an AP-2 (Scheele, Kalthoff et al., 2001]. The upstream binding site also did not inhibit clathrin uncoating in vitro, but was proved to be essential in coat formation. When HeLa cells were transfected with the 405-491 fragment, it acted as a dominant negative by blocking single round biotinylated Tfn sequestering into coated vesicles. This suggested that auxilin contains tandem binding sites which regulate CCV formation and uncoating in a sequential manner. A new model was proposed where: (1) auxilin and hsc70 are recruited by clathrin to be

involved in the initial stages of invagination, (2) auxilin may counteract dynamin's GAP activity promoting stabilization of the coat at the membrane which in turns promotes invagination, and (3) Hsc70-auxilin activity drives conformational changes in clathrin coat which drives constriction. Here dynamin acts as a regulatory protein that couples several rounds of clathrin uncoating to drive constriction and fission.

CONCLUSION

In vitro assays have proven that auxilin and Hsc70 both interact with clathrin, adaptor proteins, and now dynamin. The latter interaction supports a new role for both auxilin and Hsc70 in RME. In the current model auxilin is thought to bind to assembled clathrin vesicles and recruit Hsc70-ATP, where stimulation of the ATPase activity would disrupt clathrin-clathrin interactions and cause release of the coat. This model can now be modified to include additional steps for auxilin and Hsc70, where 2 separate binding domains in auxilin help regulate chaperone mediated activity spatially and temporally, which includes regulation of vesicle formation.

The role of Hsc70 in early stages of RME has been observed in vivo using dominant negative proteins, and overexpression of auxilin has been shown to inhibit clathrin formation. Future in vivo experiments should use knock down experiments of dynamin or Hsc70, with a focus on the early steps in endocytosis. They can take advantage of well-developed model organisms described above and advances in microscopy to help define the new roles for dynamin and auxilin/Hsc70 chaperone machinery in vesicle formation and fission.

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