Identification of Domain Required for Catalytic Activity of Auxilin in Supporting Clathrin Uncoating by Hsc70*

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During clathrin-mediated endocytosis Hsc70, supported by the J-domain protein auxilin, uncoats clathrin-coated vesicles. Auxilin contains both a clathrin-binding domain and a J-domain that binds Hsc70, and it has been suggested that these two domains are both necessary for auxilin activity. To test this hypothesis, we created a chimeric protein consisting of the J-domain of auxilin linked to the clathrin-binding domain of the assembly protein AP180. This chimera supported uncoating, but unlike auxilin it acted stochiometrically rather than catalytically because, like Hsc70, it remained associated with the uncoated clathrin. This observation supports our proposal that Hsc70 chaperones uncoated clathrin by inducing formation of a stable Hsc70-clathrin-AP complex. It also shows that Hsc70 acts by dissociating individual clathrin triskelions rather than cooperatively destabilizing clathrin-coated vesicles. Because the chimera lacks the C-terminal subdomain of the auxilin clathrin-binding domain, it seemed possible that this subdomain is required for auxilin to act catalytically, and indeed its deletion caused auxilin to act stochiometrically. In contrast, deletion of the N-terminal subdomain weakened auxilin-clathrin binding and prevented auxilin from polymerizing clathrin. Therefore the C-terminal subdomain of the clathrin-binding domain of auxilin is required for auxilin to act catalytically, whereas the N-terminal subdomain strengthens auxilin-clathrin binding.

Many of the functions carried out by the Hsc70 (the constitutive isoform of Hsp70) class of molecular chaperones both in vivo and in vitro require the presence of J-domain proteins (1, 2). For example, Hsc70 alone cannot uncoat clathrin-coated vesicles in vitro (3); the J-domain protein auxilin must also be present in catalytic amounts (4). Furthermore, knockout of auxilin in yeast or knockdown of auxilin in Caenorhabditis elegans prevents endocytosis from taking place (5–7), showing that auxilin is required for clathrin-coated vesicle uncoating (5, 16). Interestingly, yeast auxilin, which contains only the clathrin-binding domain and J-domain, supports uncoating (5, 18). Moreover, in vitro, a recombinant fragment of mammalian auxilin consisting of just the clathrin-binding domain and the J-domain supports uncoating and clathrin polymerization just like intact auxilin (16, 19), and therefore in this paper we will simply refer to this recombinant fragment as auxilin. Recently, Ungewickell and co-workers (20) found that the clathrin-binding domain of auxilin can be further divided into two subdomains. The C-terminal subdomain, which is conserved in both GAK and C. elegans auxilin (7, 16, 17), binds only to the distal domain of clathrin whereas the N-terminal subdomain binds to both the distal and terminal domains of clathrin (20). This has important implications in understanding how auxilin might interact with clathrin because we had previously shown that the terminal domain of clathrin is not necessary for the uncoating of clathrin baskets by Hsc70 (4).

Ungewickell and co-workers (19) also found that auxilin activity is partially restored when its clathrin-binding domain and J-domain of auxilin are linked head to head via glutathione S-transferase dimerization. On this basis they suggested that the simple combination of a clathrin-binding domain and a J-domain, with the two domains bringing clathrin baskets and Hsc70 in proximity to each other, may be sufficient for auxilin to support uncoating by Hsc70. If this is indeed the case it seemed possible that the source of the clathrin-binding domain would not affect the activity of auxilin. To test this hypothesis, we produced a chimeric protein derived from the 58-kDa C-terminal clathrin-binding domain of neuronal-specific, monomeric assembly protein AP180 (21, 22), and the J-domain of auxilin (4). We then determined whether this chimera supports uncoating by Hsc70 in vitro.

Our results showed that the chimera supports uncoating by Hsc70, but surprisingly it acts stochiometrically rather than...
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catalytically. Rather than dissociating from uncoated clathrin and recycling to support further uncoating, like both Hsc70 and APs, it is stably incorporated into the uncoated Hsc70-clathrin complex. This observation supports our earlier proposal that a stable Hsc70-clathrin-AP complex forms following uncoating (23). These results also show that, despite the intertwined nature of clathrin baskets, Hsc70 acts by dissociating individual clathrin triskelions rather than destabilizing the entire clathrin baskets.

Because the chimera lacks the C-terminal subdomain of the auxilin clathrin-binding domain that specifically binds to the distal domain of clathrin, it seemed possible that it was this domain needed for auxilin to act catalytically. To test this point we linked the auxilin J-domain directly to the N-terminal subdomain of auxilin and found that this deletion mutant protein also acts stoichiometrically rather than catalytically. On the other hand, we found that the 20-kDa C-terminal portion of auxilin consisting only of the C-terminal portion of the clathrin-binding domain and the J-domain acts catalytically but does not support polymerization of clathrin into baskets and binds to clathrin baskets much more weakly than intact auxilin.

Therefore, it appears that the C-terminal portion of the clathrin-binding domain of auxilin is required for auxilin to act catalytically, whereas the N-terminal portion of the clathrin-binding domain is required for auxilin to induce clathrin polymerization and strengthens the binding of auxilin to clathrin baskets.

EXPERIMENTAL PROCEDURES

Materials—ATP, creatine phosphokinase, and phosphocreatine were from Sigma. SDS-polyacrylamide gels were from Invitrogen. PQE30 vector, host cell M15, and His6 affinity resin were from Qiagen (Valencia, CA). PCR kits were from Roche Molecular Biochemicals, and restriction enzymes were obtained from New England Biolabs (Beverly, MA). [14C]Formaldehyde and [32P]ATP were from New England Nuclear (Norwich, CT).

Construct of Plasmids—The constructs derived from auxilin and AP180 were shown in Fig. 1. To make the 58-kDa C-terminal domain of AP180 and the chimera composed of the 58-kDa C-terminal domain of auxilin and C58J, respectively, we used mouse AP180 cDNA (accession number M98385) as a template for a PCR. In making C58J, the primers were 5′-ACTATCGACGGATCCGGCCAGTG-3′ (sense primer) and 5′-ATGAACTTAAATGTGTTACAGAATCTCTT-3′ (antisense primer). A BamHI site was introduced into the sense primer, and a HindIII site was introduced into antisense primer. The PCR product that encodes AP180 and the chimera was ligated together at the HindIII site and a NarI site was introduced into antisense primer. This was subcloned into PQE30 at the NarI site to make the expression vector, host cell M15, and His6 affinity resin were from Qiagen (Valencia, CA). PCR kits were from Roche Molecular Biochemicals, and restriction enzymes were obtained from New England Biolabs (Beverly, MA). [14C]Formaldehyde and [32P]ATP were from New England Nuclear (Norwich, CT).

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Preparation of Clathrin Baskets—AP baskets were made by overnight dialysis against 0.1 M MES, 1 mM DTT (pH 6.5), and pure clathrin baskets were made by dialysis against 0.1 M MES, 1 mM DTT (pH 6.0). After centrifugation for 1 h at 150,000 × g, the pellet was resuspended in buffer A, 20 mM MES (pH 6.0) or 20 mM imidazole (pH 7.0), 2 mM magnesium acetate, 25 mM KCl, 10 mM (NH4)2SO4, 1 mM DTT. The concentration of clathrin in prepared baskets was determined after solubilizing the baskets with 0.5 M Tris, pH 8.0.

Pure clathrin baskets used in the uncoating experiments at pH 7.0 were made by polymerizing double-purified clathrin by overnight dialysis against 10 mM MES, 2 mM CaCl2, 1 mM DTT, pH 6.5. Polymeric AP-clathrin baskets were prepared as described previously (23). Both the AP-clathrin baskets and the pure clathrin baskets were stable for at least 2 days.

Uncoating Assay and Binding Assay—Uncoating and binding assays were done at 25 °C in buffer A at pH 7.0 and 6.0, respectively. Uncoating of clathrin by Hsc70 and binding of Hsc70 to baskets were routinely performed by incubating Hsc70 with baskets at 25 °C for 15 min in the following buffer: 1 mM ATP and 50 mM of creatine phosphate, 0.5-1 mg/ml of clathrin, and 1 mM MgSO4. The amount of clathrin released from baskets was determined by ultracentrifugation of 0.15-ml aliquots of reaction mixture in a TL-100 ultracentrifuge at 400,000 × g for 6 min. The clathrin released or the unbound Hsc70 in the supernatant was quantified by SDS-PAGE followed by gel scanning on a LKB Ultrascan XL densitometer.

The constructs used are shown in Fig. 1. We first compared the ability of C58 and the chimera C58J, composed of the 58-kDa C-terminal clathrin-binding domain of AP180 and the J-domain of auxilin, to bind to clathrin and induce its polymerization. Both recombinant proteins induced the polymerization of clathrin to clathrin baskets (Fig. 2A) and bound polymerized clathrin (Fig. 2B) at a 1:1 molar ratio of recombinant
protein to clathrin triskelions. Furthermore, when equal amounts of both recombinants were added in excess to the clathrin baskets, they bound in equal amounts, and together bound at a 1:1 molar ratio to the clathrin baskets. This shows that the two recombinant proteins bind with equal affinity to the same binding site on clathrin (Fig. 2, open circles and diamonds). Therefore, the addition of the auxilin J-domain to C58 had no effect on its ability to polymerize clathrin or to bind to the resulting clathrin baskets. Interestingly, although auxilin, like C58 and C58J, induced clathrin polymerization at a 1:1 molar ratio to clathrin (Fig. 2, solid triangles), it bound to clathrin baskets at a 3 to 1 molar ratio to clathrin (Fig. 2B, solid triangles). Therefore, there are clear differences in the interactions of auxilin and C58J with clathrin baskets, making it particularly interesting to determine whether C58J, like auxilin, supports uncoating of clathrin baskets by Hsc70.

C58J Supports Uncoating Stoichiometrically Rather than Catalytically—When catalytic amounts of auxilin were added to clathrin baskets prepared with the assembly protein C-58, there was stoichiometric uncoating by Hsc70 (Fig. 3, circles). In contrast, when catalytic amounts of C58J were added to these same clathrin baskets, we obtained almost no uncoating of the clathrin baskets (Fig. 3, squares). At first glance, this suggests that C58J does not support uncoating by Hsc70. However, when we prepared the clathrin baskets by using C58J rather than C58 as an assembly protein, the C58J-clathrin baskets were uncoated by Hsc70 with the same biphasic time course as occurs when auxilin supports uncoating (Fig. 3, diamonds). Furthermore, three Hsc70s were required to uncoat each clathrin triskelion during the rapid initial burst of uncoating, showing that Hsc70 uncoated the C58J-clathrin baskets stoichiometrically, just as occurs with auxilin (24). Because C58J was used as an AP in this experiment, it was present at a 1:1 molar ratio to clathrin. Therefore, these data suggest that C58J indeed supports uncoating but does so stoichiometrically rather than catalytically.

The inability of C58J to support catalytic uncoating does not occur because it is unable to move rapidly from site to site on the clathrin. Like auxilin (Fig. 4A, section 1), clathrin baskets with bound C58J also rapidly exchanged with free C58 and C58J (Fig. 4A, sections 2–4). Furthermore, the close agreement between the experimental data and the theoretical values given in sections 2–4 confirm that, as shown in Fig. 2, C58 and C58J bind with equal affinity to the same single binding site on clathrin. It also appears that one of the three sites where auxilin binds to clathrin baskets probably overlaps the site where C58 and C58J bind because the addition of a large excess of free auxilin displaced about two-thirds of the bound C58J (data not shown).

If auxilin and C58J bind to the same site on clathrin baskets and exchange rapidly with free recombinant protein, why does auxilin act catalytically whereas C58J does not? To investigate this question, we first confirmed that C58J acts stoichiometrically by measuring the uncoating of clathrin baskets polymerized with varying ratios of C58J and C58. Fig. 5A shows that the amount of uncoating was proportional to the amount of C58J present in the clathrin baskets. Therefore, our data strongly suggest that only clathrin molecules complexed with C58J are uncoated, and once this uncoating occurs, unlike auxilin, C58J is not able to dissociate from the uncoated clathrin and return to the baskets to support further uncoating. This conclusion is supported by the data in Fig. 4B where we show that when baskets polymerized with equal concentrations of

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**Fig. 1.** Schematic representation of the recombinant fragments used in this study. The nomenclature shown in the figure is the same as that used throughout the text, except auxilin-C54 is referred to as auxilin because its properties are the same as full-length auxilin (16, 19).

**Fig. 2.** Ability of C58, C58J, and auxilin to polymerize clathrin and bind to clathrin baskets. In A, varying concentrations of C58 (solid circles), C58J (solid diamonds), or auxilin (solid triangles) were added to column purified clathrin (0.5 μM), dialyzed against 0.1 M MES, pH 6.5, overnight, and then centrifuged. In B, the amount of APs bound to clathrin (0.5 μM) was determined using the same method as in A in the presence of the following APs: C58 (solid circles), C58J (solid diamonds), equal concentrations of C58 and C58J (open circles and diamonds), and auxilin (solid triangles). The clathrin or APs remaining in the supernatant were quantified by SDS-PAGE electrophoresis.

**Fig. 3.** Uncoating of baskets by Hsc70. Hsc70 (0.6 μM) was incubated for the indicated times with either clathrin baskets (0.5 μM) polymerized with C58J (diamonds) or clathrin baskets (0.5 μM) polymerized by C58 and uncoated in the presence of 0.04 μM auxilin (circles) or 0.04 μM C58J (squares).
C58 and C58J were uncoated by Hsc70, only C58J but not C58 was released from the baskets as the clathrin was uncoated. Therefore, the simplest explanation of our data is that, in contrast to auxilin but like Hsc70 (23), the released C58J forms a stable complex with the uncoated clathrin preventing C58J from supporting further uncoating.

These results suggest that, following uncoating, C58J is acting as a typical AP because we previously showed that the uncoated clathrin-Hsc70 complex is stable only if it also has an AP associated with it (23). In the presence of trace amounts of auxilin, Hsc70 acted catalytically rather than stoichiometrically in uncoating pure clathrin baskets that lacked APs presumably because in the absence of APs the Hsc70 could not form a stable complex with the released clathrin (23). In contrast, if C58J were acting as an AP, both Hsc70 and C58J should act stoichiometrically rather than catalytically during the uncoating of pure clathrin baskets because both Hsc70 and C58J should form a stable complex with the released clathrin, and thus the amount of uncoating should be equal to the amount of added C58J. Fig. 5B shows that this was the case.

Even with pure clathrin baskets the amount of uncoating was limited by the amount of C58J added to the baskets (circles), unlike the results obtained with trace amounts of auxilin (squares).

**Auxilin Domain Required for Catalytic Activity**—The N-terminal portion of the clathrin-binding domain of auxilin shows some similarity with the clathrin-binding domain of AP180 and has been found to bind to both the terminal and distal domains of clathrin (20). On the other hand, the C-terminal portion of the clathrin-binding domain binds only to the distal portion of clathrin (20) and shows no homology with the clathrin-binding domain of AP-180. Because one of the major differences between C58J and auxilin is that C58J lacks this C-terminal domain of AP-180, it is possible that this portion of the auxilin clathrin-binding domain is required for auxilin to act catalytically. To test this point we first investigated the properties using the truncated auxilin, auxilin-C20.

Auxilin-C20 was unable to induce polymerization of clathrin under our standard conditions for polymerization, although at high concentrations it did induce a small amount of clathrin to sediment. Examination of this sedimentable material by electron microscopy showed that it did not consist of clathrin baskets (data not shown). In contrast, Ungewickell and co-workers (20) carried out polymerization with a similar recombinant fragment in the presence of 2 mM Ca$^{2+}$ and found that it did promote polymerization. In agreement with the results of Ungewickell and co-workers (20), we find that the presence of auxilin-C20 inhibits polymerization (Fig. 2).
gewickell and co-workers (20), we found that auxilin-C20 did support clathrin uncoating by Hsc70, and furthermore, we found that it acted catalytically rather than stoichiometrically (Fig. 6A). Although approximately three times more auxilin-C20 than auxilin was necessary to support uncoating by Hsc70 (Fig. 6A, open circles versus open squares), we also found that at pH 7 auxilin-C20 bound to clathrin baskets polymerized by C58 more weakly than either C58J or auxilin, both of which bound stoichiometrically to clathrin baskets (data not shown). When this weak binding is taken into consideration, our data suggest that the bound auxilin-C20 is as effective as auxilin in supporting catalytic uncoating of clathrin baskets.

We next investigated whether the 10-kDa clathrin-binding region adjacent to the J-domain is required for auxilin to support catalytic uncoating of AP-clathrin baskets. First, we found that a auxilin-C16 was almost completely inactive in supporting uncoating by Hsc70 (Fig. 6A, solid squares). Therefore, the minimum portion of auxilin capable of supporting uncoating by Hsc70 is the J-domain in combination with the full 10-kDa clathrin-binding region. Next, we deleted this 10-kDa region clathrin-binding region from auxilin and examined the ability of this mutated auxilin to bind to clathrin, to polymerize it, and to catalytically support uncoating. Table I shows that Δ-auxilin induced polymerization of clathrin just like auxilin, and furthermore, like auxilin it bound to the resulting clathrin baskets at a 3:1 molar ratio. However, Δ-auxilin supported uncoating poorly. It did not support catalytic uncoating of clathrin baskets prepared with C58 as an assembly protein (Fig. 6A, open circles). Rather, as shown in Fig. 6B (squares), Hsc70 only uncoated clathrin baskets polymerized with Δ-auxilin, and even in this case, the time course of uncoating was considerably slower than with intact auxilin. Furthermore, when clathrin was polymerized with two Δ-auxilins with mutated J-domains and unmodified Δ-auxilin per clathrin (Fig. 6B, circles), the resulting clathrin baskets still could not be uncoated by Hsc70, showing that only clathrin baskets containing three Δ-auxilins/clathrin can be uncoated. Altogether these data show that the C58J was more effective in supporting uncoating than the Δ-auxilin. Therefore, the 10-kDa domain adjacent to the J-domain appears to be necessary for auxilin to catalytically support uncoating by Hsc70.

Induction of Hsc70 Binding to Clathrin Baskets at pH 6.—We previously showed that at pH 6 where uncoating does not occur, intact auxilin catalytically induces the binding of Hsc70-ATP to both pure clathrin baskets (8) and AP180-clathrin baskets (4), a reaction that is reversed when the ATP is hydrolyzed to ADP. Fig. 7 confirms this effect and also shows that somewhat more auxilin is required to induce Hsc70 binding to C58-clathrin baskets than to pure clathrin baskets, although in both cases the auxilin acts catalytically (open versus solid circles). We obtained a similar result with C58J (Fig. 7, open versus solid triangles). Therefore, although C58J acts stoichiometrically at pH 7, because it forms an irreversible complex with the uncoated clathrin, at pH 6, where uncoating does not occur, it acts catalytically to induce Hsc70 binding just like auxilin. In contrast, Δ-auxilin did not induce binding of Hsc70 even when added to pure clathrin baskets (Fig. 7, diamonds). It induced Hsc70 binding only when it was used to polymerize the clathrin baskets (data not shown), just as was required for it to induce uncoating by Hsc70. Therefore, the absence of the 10-kDa domain of auxilin adjacent to the J-domain seems to interfere more with the activity of auxilin itself than with the activity of the C58J chimera.

We previously showed that the K71E Hsp70 mutant, which does not hydrolyze ATP, cannot uncoat clathrin-coated vesicles at pH 7 and is not induced to bind to mixed AP clathrin baskets at pH 6 by catalytic amounts of auxilin (25). We now find that auxilin can induce binding of K71E Hsp70 with bound ATP to clathrin baskets at pH 6, but it does so stoichiometrically rather than catalytically (Fig. 8A). When one K71E Hsp70 was added per clathrin triskelion in the baskets, one auxilin was required for each of the Hsp70s induced to bind to the baskets (Fig. 8A), but as with the binding of WT Hsp70 to clathrin baskets, the binding of K71E Hsp70 was reversed in ADP (data not shown). As expected, in contrast to what we observed with WT Hsc70 (8), this binding was not accompanied by activation of the ATPase activity of K71E Hsp70 by auxilin baskets (data not shown), presumably because K71E Hsp70 does not have any ATPase activity. The auxilin-induced binding was not due

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**Table I**

Polymerization of clathrin by Δ-auxilin

| Δ-Auxilin added (µM) | Clathrin polymerized (µM) |
|---------------------|---------------------------|
| 0.3                 | 0.2                       |
| 0.5                 | 0.4                       |
| 1.0                 | 0.5                       |
| 1.5                 | 0.5                       |
| 2.0                 | 0.5                       |

* The resuspended pellet had three Δ-auxilins bound per clathrin triskelion as determined from the densitometry of the Coomassie Blue bands on SDS gels.
Domain Needed for Auxilin to Support Catalytic Uncoating

During clathrin-mediated endocytosis, the molecular chaperone Hsc70 carries out key functions for which the J-domain protein auxilin is required. It has been postulated (10, 28) that, in general, J-domain proteins act by inducing substrates to bind to the J-domain of Hsc70. This result leads to several important conclusions. First, it demonstrates unequivocally that, despite the intertwined nature of the clathrin triskelia in the clathrin basket, Hsc70 is able to uncoat individual clathrin triskelions from the basket, leaving the remainder of the basket intact. Even when pure clathrin baskets, which are relatively unstable at pH 7, were uncoated in the presence of small amounts of C58J, uncoating was proportional to the amount of C58J added. Therefore, uncoating is not due to a nonspecific destabilization of whole clathrin baskets after Hsc70 removes a few clathrin triskelions from the baskets. Rather, the three Hsc70s that bind to each clathrin triskelion before it is uncoated apparently uncoating stoichiometrically. Like C58 and auxilin bound to clathrin baskets, the uncoated clathrin triskelions does not return to the clathrin coated Hsc70-clathrin-AP complex because in the absence of APs, following uncoating a stable Hsc70-clathrin-AP complex forms. The observation that Hsc70 can remove individual clathrin molecules individually from a polymerized network of clathrin is consistent with our view that Hsc70 plays an important role in the ATP-dependent clathrin exchange that occurs during clathrin-mediated endocytosis in vitro (29).

The second important conclusion we can draw from our results is that, as we previously proposed in an earlier study (23), the catalytic induction of Hsc70 binding to clathrin by auxilin requires ATP hydrolysis by Hsc70, perhaps to release the auxilin from the uncoated Hsc70-clathrin complex so that it can induce multiple binding cycles of Hsc70 to the baskets, in addition to the 10-kDa clathrin-binding domain adjacent to the J-domain of Hsc70. However, the catalytic binding of K71E Hsp70 to auxilin requires ATP hydrolysis by Hsc70, perhaps to release the auxilin from the uncoated Hsc70-clathrin complex so that it can induce multiple binding cycles of Hsc70 to the baskets, in addition to the 10-kDa clathrin-binding domain adjacent to the J-domain of Hsc70.

**FIG. 7.** Induction of Hsc70 binding to clathrin baskets at pH 6. Hsc70 (0.6 µM) and varying concentrations of C58J (open circles), auxilin (open circles), or J-auxilin (diamonds) were added to pure clathrin baskets (0.6 µM). Hsc70 was also incubated with C58-clathrin baskets in the presence of varying concentrations of auxilin (solid circles) and to clathrin baskets polymerized using a varying ratios of C58J to C58 (solid triangles) in which the concentration given on the abscissa is that of just C58J.

**FIG. 8.** Induction of the binding of K71E Hsp70 to auxilin-clathrin baskets at pH 6. A, either 0.6 µM WT Hsp70 (squares) or K71E Hsp70 (triangles) was added to clathrin baskets (0.6 µM) polymerized with varying concentrations of auxilin. B, K71E Hsp70 was added to baskets (0.4 µM) polymerized with one auxilin/clathrin triskelion, and the amount bound was determined after centrifugation.
with Hsc70, it is stably attached to the released clathrin triskelions. In this regard, it differs from auxilin, which to act catalytically must detach from the released clathrin triskelions and return to the partially uncoated clathrin baskets to support further uncoating by Hsc70. Thus, auxilin supports uncoating catalytically, whereas C58J, like C58 in the presence of APs, acts stoichiometrically. The ability of Hsc70 to form a stable complex with uncoated clathrin and an associated AP is consistent with the view that Hsc70 not only dissociates clathrin from clathrin-coated pits during clathrin exchange in vivo but also chaperones this dissociated clathrin and may be involved in its rebinding to the clathrin-coated pits (29).

Although both auxilin and C58J act as clathrin APs, our data demonstrate that they interact with clathrin in different ways. First, we found that, although both auxilin and C58J induce polymerization of clathrin at a 1:1 molar ratio, C58J, like C58, binds to the polymerized clathrin at a 1:1 molar ratio, whereas auxilin binds at a 3:1 molar ratio. In addition, as we discussed above, C58J supports uncoating stoichiometrically, whereas auxilin supports uncoating catalytically. Presumably these differences are related to differences in the way that auxilin and C58J interact with clathrin. C58 binds to the terminal domain of clathrin (30), and because C58J binds to the same site, it must support uncoating by binding to the terminal domain of clathrin. However, auxilin is able to support uncoating when the terminal domains of clathrin are removed from clathrin baskets. These data would suggest that auxilin interacts with the distal domain of clathrin, but Ungewickell and co-workers (20) found that the N-terminal portion of the auxilin clathrin-binding domain not only interacts with the distal domain of clathrin but also with its terminal domain. In addition, they pointed out similarities between the N-terminal portion of the auxilin clathrin-binding domain and the clathrin-binding domain of AP180.

On the other hand, the C-terminal portion of the auxilin clathrin-binding domain adjacent to the J-domain interacts only with the distal domain of clathrin (20), and we find that auxilin-C20 consisting of only this domain linked to the J-domain supports uncoating catalytically, binds quite weakly to clathrin baskets, and, under our polymerization conditions, cannot induce clathrin to polymerize. In contrast, Δ-auxilin, with only the N-terminal portion of the auxilin clathrin-binding domain present, binds strongly to clathrin baskets, induces clathrin to polymerize, but does not catalytically support clathrin uncoating by Hsc70. Rather, it supports uncoating stoichiometrically and then only does so when it is used to polymerize the clathrin baskets. Similarly, at pH 6, in contrast to C58J, which is similar to auxilin in that it catalytically induces binding of Hsc70 to C58-clathrin baskets, Δ-auxilin only induces Hsc70 binding to clathrin baskets when it is used to polymerize the clathrin baskets. Therefore, paradoxically, removal of the C-terminal portion of the auxilin clathrin-binding protein has a greater effect on the activity of auxilin itself than on the activity of C58J. This may be because C58 only binds to the terminal domain of clathrin, whereas the N-terminal portion of the clathrin-binding domain of auxilin interacts not only with the terminal domain but also with the distal domain of clathrin. Therefore, removal of the C-terminal portion of the auxilin clathrin-binding domain may interfere not only with the binding of auxilin to the distal domain of clathrin but also with proper binding of auxilin to the terminal domain of clathrin. In summary, our data suggest that the N-terminal portion of the auxilin clathrin-binding domain, which interacts with the terminal domain as well as the distal domain of clathrin, strengthens the binding of auxilin to clathrin and enables it to act as a clathrin AP. In contrast, the C-terminal portion of the auxilin clathrin-binding domain, which interacts only with the distal domain of clathrin, is crucial to the ability of auxilin to act catalytically, i.e. to detach from the uncoated clathrin rather than remaining stably bound like a typical AP.

In addition to relating the domain structure of auxilin to its ability to support uncoating, we investigated the role of ATP hydrolysis by Hsc70. We had previously shown that the Hsp70 mutant, K71E Hsc70, which does not hydrolyze ATP, is not able to carry out uncoating and is not induced to bind to mixed AP-clathrin baskets at pH 6 by catalytic amounts of auxilin (25). However, we had not tested whether auxilin was able to induce stoichiometrically the binding of K71E Hsc70 to clathrin baskets at pH 6. Based on our studies on the ability of J-domain proteins to induce polymerization of Hsc70, we previously suggested that the ability of J-domain proteins to induce the binding of substrates to Hsc70-ATP was independent of ATP hydrolysis by Hsc70 (9). In support of this view, in our present study we found that the induction by auxilin of the binding of K71E Hsc70 to clathrin baskets at pH 6 is ATP-dependent even though K71E Hsc70 does not hydrolyze ATP. However, just as with Δ-auxilin, this induction of binding was stoichiometric rather than catalytic. These data show that, in addition to the presence of the C-terminal subdomain of the auxilin clathrin-binding domain, ATP hydrolysis by Hsc70 is involved in the ability of auxilin to induce catalytically the binding of Hsc70 to clathrin baskets at pH 6. Perhaps ATP hydrolysis is required to release the auxilin from the clathrin baskets after it induces Hsc70 binding so that it can rapidly move from triskelion to triskelion to induce Hsc70 binding catalytically. Alternatively, ATP hydrolysis may be required to prevent rapid dissociation of the Hsc70-ATP from the clathrin baskets which, in turn, would increase the amount of auxilin required for steady-state Hsc70 binding. In any event, our data show that both the distal portion of the clathrin-binding domain of auxilin and ATP hydrolysis by Hsc70 are required for catalytic property of auxilin in inducing binding of Hsc70 to clathrin baskets at pH 6 and catalytically support uncoating of clathrin baskets at pH 7.

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