Functional Interaction of the Auxilin J Domain with the Nucleotide- and Substrate-binding Modules of Hsc70*

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The uncoating of clathrin-coated vesicles requires the DnaJ homologue auxilin for targeting Hsc70 to clathrin coats. This function involves a transient interaction of the auxilin J domain with Hsc70. We have now identified the structural elements of Hsc70 that are responsible for the uncoating activity, and we show that the hitherto accepted view, which implicates the 10-kDa carboxyl-terminal variable domain of Hsc70, is incorrect. A 60-kDa chymotryptic or analogous recombinant fragment of Hsc70, which contains the ATPase- and substrate-binding domains, is sufficient to liberate clathrin from coated vesicles. Consistent with this was the observation that Hsp70 uncoats coated vesicles with the same efficacy as Hsc70 and that DnaK possesses vestigial uncoating activity. Direct binding studies demonstrated that the auxilin J domain undergoes an ATP-dependent reaction only with fragments of Hsc70 that contain both the ATPase- and substrate-binding domains. The individual domains by themselves did not bind to the J domain nor did a recombinant protein that contained the substrate-binding domain attached to the 10-kDa variable domain.

Proteins of the 70-kDa heat shock protein (Hsp70) family participate in numerous cellular functions such as protein folding, protein translocation across membranes, and reorganization of macromolecular complexes (1, 2). The underlying basis of these apparently diverse functions is the ability of Hsp70 proteins to interact reversibly with extended polypeptide segments of at least 7 residues generally containing large hydrophobic and basic side chains (3). Hsp70 proteins contain an amino-terminal 44-kDa ATPase domain, a 18-kDa substrate-binding domain, and a 10-kDa carboxyl-terminal domain (4). The carboxyl-terminal domain is the least conserved of the three Hsp70 domains and is therefore also referred to as the variable domain (5). The type of nucleotide present in the ATPase domain determines the accessibility of the substrate-binding site for substrates. Thus ATP promotes and ADP inhibits substrate release (6, 7).

The structure of a recombinant fragment of DnaK (bacterial Hsp70) that includes the substrate-binding domain and most of the 10-kDa variable domain reveals that the first half (Asp391-His606) has the form of a β-sandwich while the second (Glu509-His606) folds into five α-helical segments (8). Substrates bind in an extended conformation to a channel formed by loops of the β-sandwich. The second helix, designated aβ, covers the binding channel like a lid, thereby presumably preventing the escape of bound substrates (see Fig. 9). It has been suggested that the binding of ATP induces conformational changes that expose the substrate-binding channel by displacement of the αβ-helix (8). Hydrolysis of ATP would have the opposite effect.

The intrinsic ATPase activity of Hsp70 is very low but is synergistically stimulated by substrates and by members of the DnaJ protein family. Other factors, such as GrpE in bacteria (9) or Hip in eukaryotes, respectively, enhance or inhibit nucleotide exchange (10). DnaJ proteins consist of highly conserved elements, together with unique domains that distinguish the proteins from each other (11). The feature common to all DnaJ homologues is the J domain, a conserved module of about 70 residues, which is essential for the interaction with Hsp70 proteins (12–14). Nuclear magnetic resonance spectroscopy of the J domain of bacterial DnaJ and human Hdj-1 showed that it folds into four helices that contribute side chains to a hydrophobic core (15, 16). Two of the helices are engaged in coiled-coil interactions. The absolutely conserved and functionally essential tripeptide, “HPD,” forms part of a connecting loop between the two antiparallel helices. Less conserved charged residues on the helix exteriors may recognize particular Hsp70 isoforms (15, 16). Other unrelated domains in DnaJ proteins are believed to be involved in recognition of substrates for Hsp70s (11). Thus, the combination of a given J domain with a substrate recognition domain allows the targeting of a Hsp70 protein to a specific substrate or class of substrates. DnaJ and auxilin appear to be representatives of two types of DnaJ class proteins; the former displays affinity for a broad range of de-natured polypeptides (11) whereas auxilin acquired a high specificity for assembled clathrin (17–19).

Clathrin is the major structural component of the regular polygonal lattice that encases a certain type of intracellular transport vesicle (20). Binding of auxilin to assembled clathrin initiates Hsc70-dependent release of clathrin protomers (triskelia) from the vesicle membrane (18). Although the auxilin J domain differs considerably from that of DnaJ or Hdj-1, key residues, known to be engaged in the formation of the hydrophobic core, and the essential HPD segment are conserved (18). Moreover, the auxilin J domain binds to Hsc70 and stimulates its ATPase (19).

Nothing less than intact Hsc70 has previously been reported to uncoat clathrin-coated vesicles (4, 21). A chymotryptic 60-kDa fragment lacking the 10-kDa carboxyl-terminal domain would bind only to free clathrin triskelia and to unfolded peptide substrates. More recently a DnaK mutant with a deletion of 94 residues at the carboxyl terminus was reported to interact
poorly with immobilized DnaJ (22), and similarly, a human Hsp70 lacking the conserved carboxyl-terminal motif EEVD could no longer be stimulated by the DnaJ homologue Hdj-1 and failed to refold denatured luciferase (23). Taken together, these observations seemingly imply that the carboxyl-terminal domain of Hsp70 interacts with DnaJ homologues and is essential for a complete Hsp70-mediated reaction cycle.

The ATP-induced complex between the auxilin J domain and Hsc70 is relatively stable (19, 24). This made it possible to isolate complexes containing both proteins. It should therefore be in principal possible to identify the Hsc70 domains that participate in this interaction. Here we demonstrate that the variable 10-kDa carboxyl-terminal domain of Hsc70 is not required for the binding of auxilin or for the in vitro uncoating of clathrin-coated vesicles. Our results suggest instead that in the ATP-bound state of Hsc70 the ATPase domain interacts with the substrate-binding domain to create a binding site for the J domain.

MATERIALS AND METHODS

Plasmid Constructions—The construction of plasmids used for the expression of GST-auxilin-(547–910), GST-auxilin-(813–910), and GST-auxilin-(547–810) has been described in detail elsewhere (19). BovHsc70.pRSET which was used for the construction of recombinant Hsc70 fragments was a kind gift from Dr. McKay (Stanford). The construct no longer contained a mutation present in the original clone constructed no longer contained a mutation present in the original clone.

BovHsc70.pRSET was used for the construction of recombinant BovHsc70-(1–540) Klenow enzyme and inserted into the Smal site of the expression vector PGEX-4T-1. PGEXHsc70-(373–650) was obtained by digestion of BovHsc70.pRSET with NarI and HindIII. The resulting 876-bp fragment was blunted using Klenow enzyme and inserted into the Smal site of the expression vector PGEX-4T-1. PGEXHsc70-(373–540) was obtained by digestion of BovHsc70.pRSET with NarI and HindIII. The resulting 876-bp fragment was blunted using Klenow enzyme and inserted into the Smal site of the expression vector PGEX-4T-1. PGEXHsc70-(373–540) was obtained by digestion of BovHsc70.pRSET with NarI and HindIII.

Functional Domains of Hsc70

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Converting Hsc70 into its ATP- or ADP-bound state, the protein was incubated for 10 min at 25 °C in buffer C supplemented with either 5 mM ATP or 3 mM ADP. Digsitions with chymotrypsin were carried out as detailed in the legend to Fig. 3. The reaction was terminated with phenylmethylsulfonyl fluoride at a final concentration of 1 mM.

Enzymatic Dissociation of Coated Vesicles—Coated vesicles (0.4 μm) from bovine brain in buffer C were supplemented with GST-auxilin-(547–910) (0.2 μM) before they were used as substrate in the uncoating assay. This was done to make sure that the endogenous auxilin was not a limiting factor in the uncoating reaction. Dissociation reactions were performed in volumes of 75–100 μl in buffer C supplemented with 2 mM ATP, 5 mM phosphocreatine (Sigma), and 5 units/ml creatine phosphokinase (Sigma) (34). The incubation time was 15 min and the temperature 25 °C. Protein concentrations are stated in the legends to the figures. Dissociated clathrin was separated from baskets by ultracentrifugation (20 min at 109,000 × g) using a Beckman TL-A-45 rotor in a TL-100 benchtop centrifuge. The amount of clathrin present in the supernatant and pellet fractions was determined by densitometry after SDS-PAGE using a Molecular Dynamics instrument (35). For preparative uncoating experiments with digested Hsc70, brain coated vesicles (0.52 mg of protein) were incubated with 19 μg of GST-auxilin-(547–910) and 0.28 mg of chymotrypsin-digested hsc70 (composed mainly of 60- and 45-kDa fragments) for 20 min at 25 °C in the presence of 4 mM ATP. The reaction was terminated by adding hexokinase to a final concentration of 10 units/ml and 5 mM glucose. Released clathrin was separated from intact baskets by ultracentrifugation. The supernatant was applied directly to a Superose 6 gel filtration column equilibrated with buffer C containing 0.1 mM ADP. The column was eluted at room temperature with a flow rate of 0.5 ml/min. Fractions were analyzed by SDS-PAGE.

Interaction between Hsc70 Fragments and the Auxilin J Domain—A chymotryptic digest of Hsc70, prepared in ADP or ATP, respectively, or recombinant Hsc70 fragments without their GST-moieties, were incubated in buffer C with GST-auxilin-(813–910) for 10 min at 25 °C. As indicated in the figures, the buffer contained either 3 mM ATP or 3 mM ADP, respectively. Incubations with GST served as controls. At the end of the incubation period, 20–40 μl of glutathione-Sepharose 4B (Pharmacia) was added, and the incubation was continued for 15 min on ice to allow the fusion proteins to attach to the beads. The beads were pelleted by a 30-s spin at maximum speed in an Eppendorf benchtop centrifuge at 4 °C. The beads were extensively washed in buffer C containing either 0.1 mM ATP or 0.1 mM ADP, as detailed elsewhere (19), and then resuspended in 100 μl of SDS sample buffer to extract the bound protein which was subsequently analyzed by SDS-PAGE.

ATPase Assays and NADPH Analysis—To determine ATPase activities, 1.3 μM Hsc70 or 1.8 μM GST-Hsc70-(1–540), respectively, were incubated alone or with 0.5–2.1 μM GST-auxilin-(547–910) at 25 °C or 0.6–2.6 μM GST-auxilin-(547–814) in 30 μl of buffer C with 14 μM ATP, containing 0.3 MBq [32P]ATP (Amersham Corp.). The reaction was stopped by spotting a 1-μl aliquot onto a polyethyleneimine-cellulose TLC plate, and the ratio of ATP to ADP was determined by densitometry. Controls consisted of incubations of ATP without added proteins and with 1 μM GST-auxilin-(547–910), respectively. To follow the hydrolysis of Hsc70-bound ATP, 1 nmol of Hsc70 or GST-Hsc70-(1–540) was incubated for 6 min at 25 °C with 2 nmol of [32P]ATP (0.07 MBq). Unbound nucleotides were removed on a NAP-5 column (Amersham) and with 2.1 M perchloric acid. The ATPase reaction products were separated on a 15% polyacrylamide/7.5 M urea gel and visualized by autoradiography of a dried gel. The ratio of ATP to ADP was determined as described in the legend. Ratios of ATP to ADP were determined by densitometry.

Miscellaneous Techniques—SDS-PAGE and immunoblotting procedures were performed as described previously (35).
to isolate DnaK-clathrin complexes by gel filtration in the presence of ADP proved unsuccessful, indicating that the complexes that would have formed in the course of the uncoating reaction were unstable (data not shown). In contrast, complexes of Hsc70 with clathrin are readily demonstrated by this procedure (4, 19, 36).

In sum, when compared with bovine Hsc70, both human Hsp70 and bacterial DnaK, showed uncoating activity, strong in one case and weak in the other, despite extensive sequence differences between the three variable 10-kDa carboxyl-terminal domains and lesser differences between the other domains. While these results fall short of establishing definitively whether or not the variable domain participates, as had been supposed, in an interaction with DnaJ class proteins, they nevertheless place the issue in doubt. To eliminate any possibility that the in vitro uncoating assay may not have been sensitive enough to discriminate between an interaction of the 10-kDa domain with a “cognate” as against a “non-cognate” J domain, we extended our study to fragments of Hsc70.

Uncoating Activity of Hsc70 Fragments—Limited proteolysis of Hsc70 with chymotrypsin progresses through a 66-kDa fragment, which gives rise to two closely spaced bands of about 60 kDa and finally a stable 45-kDa fragment (Fig. 3). On immunoblots of the digest the Hsc70-specific monoclonal antibody 1B5 (37) stained only intact Hsc70 and the fusion protein GST-auxilin-(547–910) was included to make sure that the amount of endogenous auxilin present in brain coated vesicles is not a limiting component in the uncoating reaction. The amount of released clathrin was determined after ultracentrifugation by SDS-PAGE and densitometry. Data points are averages of three determinations.

Uncoating activity of human Hsp70 and DnaK. A constant amount of clathrin-coated vesicles (0.4 μg clathrin heavy chain), supplemented with 0.2 μg GST-auxilin-(547–910), was mixed with the indicated amount of Hsp70 protein or bovine Hsc70 for 15 min at 25 °C in the presence of an ATP-regenerating mixture. GST-auxilin-(547–910) was included to make sure that the amount of endogenous auxilin present in brain coated vesicles is not a limiting component in the uncoating reaction. The amount of released clathrin was determined after ultracentrifugation by SDS-PAGE and densitometry. Data points are averages of three determinations.
were found to be active (Fig. 4). When these fragments were tested in the uncoating assay they could be detected.

The supernatant containing the released clathrin was immediately applied to a S6 gel filtration column. Analysis of the clathrin-containing fractions by SDS-PAGE demonstrated the presence of the 60-kDa fragment and some 66-kDa fragment (Fig. 5). This result was in agreement with published data which showed that the 60-kDa fragment and contributed to the uncoating function of Hsc70-(1–540) and Hsc70-(1–540), respectively. The data for Hsc70 were taken from Fig. 2.

To confirm the auxilin dependence of the Hsc70-(1–540)-mediated uncoating reaction, we used coats prepared from adrenal gland coated vesicles, because these possess no endogenously active auxilin (40). Disintegration of these baskets was strictly dependent on the presence of Hsc70-(1–540) and GST-auxilin-(547–910) (Fig. 6).

Our results taken together suggest strongly that the Hsc70 segment 1–540 suffices for the auxilin-dependent dissociation of clathrin from coated vesicles.
Interaction between GST-Auxilin-(813–910) with Domains of Hsc70—Having excluded any direct role of the carboxyl-terminal 10-kDa domain of Hsc70 in the uncoating reaction, we wished to determine which part of the 60-kDa fragment binds to the J domain. To this end a mixture of intact Hsc70, 66-, 60-, and 45-kDa chymotryptic Hsc70 fragments was prepared and incubated with either GST or with GST-auxilin-(813–910) in the presence of ATP or ADP. Binding was assayed by retrieving GST-containing proteins and protein complexes on GSH-Sepharose. SDS-PAGE analysis of the adsorbed proteins demonstrated that intact Hsc70, as well as the 66- and the 60-kDa fragment, interacted with GST-auxilin-(813–910), whereas the 45-kDa ATPase domain did not (Fig. 7A). Intact Hsc70 appeared to be the marginally preferred substrate. Binding was stimulated by ATP and required the J domain (Fig. 7A). Fragments prepared in the presence of ATP did not behave any differently (Fig. 7A, track 4). These findings were corroborated with recombinant Hsc70 fragments. Like the 60-kDa fragment, Hsc70-(1–540) entered into a complex with the auxilin J domain, whereas the substrate-binding domain by itself (Hsc70-(373–540)) or in association with the carboxyl-terminal domain (Hsc70-(373–650)) did not bind (Fig. 7B). Generally the interaction of the auxilin J domain with Hsc70-(1–540) appeared to be not quite as strong as with intact bovine brain Hsc70 or with the 60-kDa fragment. B, recombinant fragments of Hsc70 incubated in ATP in the absence or presence of either GST-auxilin-(813–910) or GST-auxilin-(813–910) or GST. Track 1, incubation of 1.5 μM Hsc70-(1–540) with 8 μM GST-auxilin-(813–910); track 2, incubation of 1.5 μM Hsc70-(1–540) with 8 μM GST; track 3, incubation of 3.2 μM Hsc70-(373–540) with 5 μM GST-auxilin-(813–910); track 5, incubation of 3.2 μM Hsc70-(373–650) with 5 μM GST-auxilin-(813–910); track 6, incubation of 3.2 μM Hsc70-(373–650) with 5 μM GST-auxilin-(813–910); supernatant (S) and pellet (P). Note that only fragments containing both the nucleotide- and substrate-binding domain of Hsc70 bind to the auxilin J domain. The 27-kDa polypeptide in lane 4P corresponds to residual GST.

DISCUSSION

We have presented evidence that in vitro the variable 10-kDa carboxyl-terminal domain of Hsc70 is not essential for the interaction of Hsc70 with the auxilin J domain or for the uncoating of clathrin-coated vesicles. These results were unexpected, because several previous reports had suggested otherwise (4, 21, 23). However, when the experimental details and
Hsc70-(1–540) were incubated for 20 min at 25 °C with GST-Hsc70, which lacks the J domain. Data points are averages of three determinations. Note that GST-Hsc70-(1–540) immediately after the removal of unbound nucleotide. The purified recombinant 60-kDa fragment from rat Hsc70 in bacteria (21) was almost identical to our Hsc70-(1–540), except for four additional carboxyl-terminal residues. The failure of Hsp70 mutants with defective EEVD motifs to refold luciferase efficiently also could equally result from downstream effects unrelated to the interaction with Hsj1.

The effects of EEVD mutations and truncations of Hsc70 on substrate binding suggest that the segments between residue Asn540 and the EEVD motif may be involved in inter-domain interactions. EEVD mutants were shown to have low affinity for substrates, but the additional elimination of the ATPase domain restores the high affinity. Similarly, the removal of the carboxyl-terminal 10-kDa domain, which includes the EEVD motif, by chymotrypsin digestion or recombinant techniques, does not inhibit substrate binding (4, 21, 23, 30). Thus we may conjecture that the segment between Asn540 and the EEVD motif interacts with the nucleotide-binding domain and that this interaction leads to inhibition of substrate binding. One function of the EEVD motif may be to alleviate this inhibition. This scheme affords an explanation for the increase of high substrate-binding affinity when either the nucleotide-binding domain or the 10-kDa domain which includes the EEVD motif is lost. The relatively high sequence divergence between the carboxyl-terminal domains of Hsp70 proteins suggests that their inter-domain communication might be differentially regulated by specific cellular factors.

Exclusion of any requirement of the carboxyl-terminal 10-kDa domain for the in vitro interaction of auxilin’s J domain with Hsc70 and for dissociation of clathrin coats then raises the question of which of the other Hsc70 domains does interact with the J domain. Direct binding experiments demonstrated that only a fusion protein encompassing both the nucleotide-binding domain and the substrate-binding domains was capable of associating with the auxilin J domain. The failure of either the substrate-binding domain (Hsc70-(373–540)) or the nucleotide-binding domain by themselves to bind to the auxilin J domain suggests that segments from both domains may contribute to the J-domain-binding site. Alternatively, interactions between the two domains could result in the exposure of an otherwise cryptic site for the J domain on one of them. The initial interaction of the auxilin J domain with Hsc70 requires the Hsp70 protein to be in the ATP-bound state. The transition of Hsp70 proteins from their ADP-bound state to the ATP-bound state is known to be accompanied by large conformational rearrangements and monomerization of Hsp70 proteins, which can be detected by limited proteolysis, by fluorescence spectroscopy, and by small angle x-ray scattering (23, 38, 39, 41–43). The scattering data suggest a significant reduction in the radius of gyration of both the intact Hsc70 and the 60-kDa fragment. Although dissociation of the Hsp70 dimers could in principle account for the shape change, an ATP-induced internal rearrangement of domains was considered the more likely interpretation, because the 60-kDa fragment, unlike the intact protein, did not readily dimerize in the presence of ADP (43).
The models do not include the nucleotide-binding domains, which would appear to increase the accessibility of the substrate-binding channel. Of DnaK is occupied by a peptide. Note that the truncation of Hsc70 DnaK and Hsc70-(1–540), respectively. The substrate-binding channel of DnaK appeared also to interact poorly with clathrin, since no isoform would be expected to preclude an efficient interaction. Vitro advantage of a poorly matching Hsp70 can clearly be partly offset by this means. Although the auxilin J domain can associate of Hsc70-(1–540) indicates that helix aB is now too short to cover the entire substrate-binding channel. While one might suppose that this truncation of the helix might weaken the association of Hsc70-(1–540) with its substrates, it nevertheless appeared to be without any severe consequences for the uncoating reaction.

Our assay was also able to detect a vestigial uncoating activity in bacterial DnaK. Although the auxilin J domain can certainly not be the natural partner for DnaK, the disadvantage of a poorly matching Hsp70 can clearly be partly offset in vitro by simply raising the concentration of the chaperone. On the other hand under the competitive conditions that prevail in the cell a less than perfect match between J domain and Hsp70 isoform would be expected to preclude an efficient interaction. DnaK appeared also to interact poorly with clathrin, since no complex could be demonstrated by gel filtration. Differences in the substrate specificity between Hsc70 and DnaK have been noted previously (3).

The work described here and our previous work have defined the domains of auxilin and Hsc70, respectively, that are essential for the uncoating of clathrin-coated vesicles. It is hoped that this information will be helpful for elucidating the regulatory mechanism that controls the uncoating reaction in the cell.

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