

Mechanism of Clathrin Basket Dissociation: Separate Functions of Protein Domains of the DnaJ Homologue Auxilin

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Abstract. Auxilin was recently identified as cofactor for hsc70 in the uncoating of clathrin-coated vesicles (Ungewickell, E., H. Ungewickell, S.E. Holstein, R. Lindner, K. Prasad, W. Barouch, B. Martin, L.E. Greene, and E. Eisenberg. 1995. *Nature (Lond.)*. 378: 632–635). By constructing different glutathione-S-transferase (GST)–auxilin fragments, we show here that cooperation of auxilin's J domain (segment 813–910) with an adjoining clathrin binding domain (segment 547–814) suffices to dissociate clathrin baskets in the presence of hsc70 and ATP. When the two domains are expressed as separate GST fusion proteins, the cofactor activity is lost, even though both retain their respective functions. The clathrin binding domain binds to triskelion like intact auxilin with a maximum stoichiometry of

3 and concomitantly promotes their assembly into regular baskets. A fragment containing auxilin's J domain associates in an ATP-dependent reaction with hsc70 to form a complex with a half-life of 8 min at 25°C. When the clathrin binding domain and the J domain are recombined via dimerization of their GST moieties, cofactor activity is partially recovered. The interaction between auxilin's J domain and hsc70 causes rapid hydrolysis of bound ATP. Release of inorganic phosphate appears to be correlated with the disintegration of the complex between auxilin's J domain and hsc70. We infer that the metastable complex composed of auxilin, hsc70, ADP, and P_i contains an activated form of hsc70, primed to engage clathrin that is brought into apposition with it by the DnaJ homologue auxilin.

CLATHRIN-COATED vesicles are agents for the vectorial transport of selected membrane proteins from the plasma membrane and the *trans*-Golgi network to the endosomal system (4, 17). After budding and pinching off from the donor membrane, coated vesicles rapidly shed their coats. An enzyme catalyzing the uncoating of purified bovine brain coated vesicles was first purified from brain cytosol (34) and shown to be a member of the 70-kD heat shock protein (hsp)¹ 70 family (7, 40). These proteins participate in numerous functions including the folding of newly synthesized proteins, translocation of proteins across membranes, disassembly of oligomeric complexes, and protection of proteins against irreversible denaturation under conditions of stress (16, 19). Hsc70 is supported in its many activities by cofactors, notably members of the DnaJ protein family, which target hsc70 to its various substrates and stimulate its ATPase (9, 38). Other factors such as GrpE in bacteria (46) and Hip in eukaryotes (20) regulate nucleotide exchange.

The dissociation of the clathrin coat requires hsc70 and,

as cofactor, the eukaryotic DnaJ homologue auxilin (41). Auxilin was originally identified as a clathrin assembly protein (2). This loose term refers to proteins that bind to clathrin and promote its polymerization into baskets. Auxilin binds to the region in the clathrin heavy chain that forms the hub and the proximal part of the triskelion leg. Light chains are not required for the interaction of auxilin with clathrin (25). Auxilin has in common with members of the DnaJ protein family a so-called J domain. This element of ~70 residues occupies the carboxyl-terminal end of the auxilin chain. When compared with the known J domains of bacterial DnaJ. However, segments such as tripeptide H₃₂P₃₃D₃₄ in the J domain of DnaJ, which are known to be required for interaction with DnaK (bacterial hsp70), are conserved (11, 42). Outside the J region, auxilin bears little resemblance to DnaJ. It is more than twice the size of DnaJ (99 kD vs 41 kD), and it lacks the zinc finger domain as well as the G/F-rich domain of the latter. This evolutionary divergence is consistent with the differences in function: whereas auxilin acquired a clathrin binding domain, DnaJ possesses a zinc finger region, which is thought to be the site of attachment of unfolded polypeptides (39). It is also conceivable that the limited divergence within the J region may have occurred to tune the interaction of auxilin with hsc70.

Here we show that the clathrin binding domain of auxi-

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1. *Abbreviations used in this paper:* GST, glutathione-S-transferase; hsp, heat shock protein; AMP-PNP, adenylyl-imidodiphosphate.

lin adjoins the J region in the sequence. A recombinant fragment of auxilin bearing both domains possesses cofactor activity, but when the domains are expressed separately, this activity is lost. The region that contains the clathrin binding domain behaves like an assembly protein, while a fragment with auxilin's J domain interacts with hsc70 in an ATP-dependent reaction. Hsc70 associated with cofactor contains bound ADP+P_i and may be inferred to represent an activated state of hsc70 ready to engage substrate molecules.

Materials and Methods

Purification of Clathrin, Hsc70, and DnaK

Clathrin and hsc70 were obtained from frozen bovine brains (Pel-Freez, Rogers, AR). Coated vesicles and clathrin were purified as detailed elsewhere (6). Bovine brain hsc70 was purified as described previously (18). Hsc70 used for ATPase determinations was chromatographed on Superose 6 (Pharmacia Biotech, Piscataway, NJ) in buffer C (20 mM Hepes, 25 mM KCl, 2 mM MgCl₂, 10 mM NH₄ SO₄, pH 7.0) containing 0.1 mM ATP. Unbound nucleotide was removed from hsc70 by desalting using a PD-10 gel filtration column (Pharmacia Biotech, Piscataway, NJ). If required, the protein was concentrated using a microconcentrator (Centricon 30; Amicon Inc., Beverly, MA). DnaK was purified from *Escherichia coli* strain TG1 that was transformed with plasmid pJMII (27). The bacteria (200 ml) were grown for 3 h at 37°C before the expression of recombinant DnaK was induced with 1 mM isopropylthio-β-D-galactoside for 3 h at room temperature. Cells were lysed by sonification (6 bursts for 30 s each at a setting of 200 W) in buffer C containing 0.5% Triton X-100, 0.1 mM PMSF, 0.01 mM leupeptin, 1 mM benzamide (all from Sigma Chemical Co., St. Louis, MO), and 1 μg/ml E64 (Boehringer Mannheim Biochemicals, Indianapolis, IN). DnaK was purified from the clarified lysate by affinity chromatography on ATP-agarose (Sigma Chemical Co.) as described for hsc70 (18). To remove a contaminating ATPase activity from DnaK, the protein was further purified by gel filtration on Superose 12 (Pharmacia Biotech) in buffer C. Fractions containing the main peak of DnaK were pooled, and then directly loaded onto a 1 ml hydroxypatite column connected to an FPLC system (Pharmacia Biotech). A 20 ml gradient from 0–500 mM phosphate was applied at a flow rate of 0.5 ml/min. DnaK was desorbed with 150 mM phosphate and eluted in a very narrow peak. The purified protein was desalted on a PD-10 column that was equilibrated in buffer C. 0.1-ml aliquots of both hsc70 and DnaK were rapidly frozen in dry ice/methanol and stored frozen at –80°C.

Construction and Expression of Recombinant Auxilin Fragments

To generate the GST-auxilin(1–814) DNA, full-length auxilin DNA in pQE-30 (36) was digested with BamHI, and the resulting 2,472-bp fragment was subcloned into the BamHI-site of pGEX4T-1 (Pharmacia Biotech). GST-auxilin DNA was constructed by digestion of full-length auxilin DNA with HindIII, followed by treatment with Klenow enzyme and digestion with ClaI. This generated a 2,259-bp fragment. GST-auxilin(1–814) DNA was digested with ClaI and SmaI to remove a 1,947-bp fragment. The 2,259-bp fragment was put in its place. All restriction enzymes and the Klenow enzyme were from Boehringer Mannheim Biochemicals; all subsequent constructs were generated from this material. The DNA coding for GST-auxilin(1–546), corresponding to residues 1–546 of auxilin, was obtained by digestion with AvaI, which removed a 1,138-bp fragment from the GST-auxilin DNA. The vector was religated. The excised 1,138 bp was subcloned into the AvaI-site of pGEX4T-1 vector to generate the DNA for GST-auxilin(547–910). Digestion with BamHI and subcloning the 812-bp fragment into the BamHI-site of pGEX4T-1 resulted in the DNA coding for GST-auxilin(547–814). The J domain sequence contained in the GST-auxilin DNA was amplified by PCR with Taq DNA polymerase (Promega, Madison, WI) using ATCTATCTGAATTC-GATGGATCCTGAGAAGTTAAAGATTCTAGAA as forward primer and CATGCTAGAATTCAGTCAGTCACGATCGGCCGCTCGA-GTCGACCCAGCT as reverse primer. The 396-bp PCR product was subcloned into the pGEM-T vector (Promega) and digested with BamHI, blunted with Klenow, and digested with XhoI. Subcloning of the 340-bp

fragment into the SmaI/XhoI sites of pGEX4T-2 resulted in a construct coding for GST-auxilin(813–910). Fusion proteins were expressed in *E. coli* strain BL21. Bacteria were grown for 3 h at 37°C, and then induced with 1 mM isopropylthio-β-D-galactoside for 3 h at room temperature. Pellets from 1 liter of bacteria culture were resuspended in 30 ml of lysis buffer (PBS/1% Triton/1 mM PMSF/1 mM leupeptin) and lysed by sonication as described above. Glutathione-S-transferase (GST) fusion proteins were purified from lysates by affinity chromatography on glutathione-Sepharose 4B (Pharmacia Biotech) according to the manufacturer's instructions. In most cases, the fusion proteins were further purified by gel filtration on Superose 12 (Pharmacia Biotech) equilibrated in buffer C. Protein concentrations were determined spectroscopically. Extinction coefficients were calculated from the amino acid composition. In some instances, the GST moiety of GST-auxilin(547–910) and GST-auxilin(547–814) was cleaved by digesting 1 mg fusion protein with 1 U thrombin (No. 154163; ICN, Costa Mesa, CA) for 16 h at 20°C in 20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM CaCl₂. The reaction was terminated with 1 mM PMSF. The GST moiety was removed by adsorption to glutathione-Sepharose and thrombin by gel filtration on Superose 12 in buffer C. The purified fusion proteins were concentrated in Centricon microconcentrators and stored frozen at –80°C in small aliquots.

Enzymatic Dissociation of Clathrin Baskets

Baskets composed of pure clathrin and recombinant H6-AP180 were prepared as described previously (29). For some experiments, the baskets were stabilized by H6-17-AP180, a recombinant fragment of AP180 covering the segment from residues 746–896. Reassembled baskets were pelleted at 109,000 g for 20 min in a rotor (TLA-45; Beckman Instruments, Inc., Palo Alto, CA) and resuspended in buffer C. Dissociation experiments were performed in 75–100 μl buffer C supplemented with 2 mM ATP, 5 mM phosphocreatine (Sigma Chemical Co.), and 5 U/ml creatine phosphokinase (Sigma Chemical Co.) (3). The incubation time was usually 15 min and the temperature was 25°C. Protein concentrations are stated in the figure legends. Dissociated clathrin was separated from baskets by ultracentrifugation as described above. The amount of clathrin present in the supernatant and pellet fractions was determined by densitometry after SDS-PAGE using a Molecular Dynamics (Sunnyvale, CA) instrument (26).

Clathrin Binding and Assembly Experiments

Binding of fusion proteins to clathrin baskets was tested by incubating them in buffer A (0.1 M MES, 0.5 mM MgCl₂, 1 mM EGTA, pH 6.5) or C at concentrations indicated in the legends with clathrin baskets (0.45 μM triskelia) for 15 min on ice. The baskets were pelleted by ultracentrifugation as described above, and the protein composition of the supernatants and pellets was analyzed by SDS-PAGE and quantitated by densitometry. The amount of fusion protein sedimenting in the absence of baskets was subtracted as background. Auxilin(547–814) comigrates with the smaller clathrin light chain (LCB). Since the ratio of light to heavy chain is constant, the contribution of LCB to the intensity of the auxilin(547–814) band is readily inferred from the intensity of the heavy chain band and corrected for accordingly.

Dissociation of Auxilin-containing Clathrin Baskets on a Preparative Scale

Clathrin triskelia (1.3 nmol) polymerized into baskets were incubated with 6 nmol GST-auxilin(547–910) for 30 min on ice in buffer A. The baskets were separated from unbound ligand by ultracentrifugation and resuspended in 0.4 ml buffer C. For dissociation, the baskets were incubated for 20 min at 25°C with 4 nmol hsc70 in the presence of 4 mM ATP. Released clathrin was separated from intact baskets by ultracentrifugation. The supernatant was divided into two 0.25-ml aliquots. One was applied directly to a Superose 6 gel filtration column equilibrated with buffer C containing 0.1 mM ATP. The column was eluted at room temperature with a flow rate of 0.5 ml/min. Fractions were analyzed by SDS-PAGE. The second aliquot was incubated for 2 h with 10 U hexokinase and 5 mM glucose to eliminate residual ATP, and then applied to a Superose 6 column, equilibrated in buffer C + 0.1 mM ADP, and eluted at room temperature.

Hsc70 Binding Assays

Hsc70 was preincubated with respective nucleotides (see figure legends) in buffer C for 10 min at 25°C. ATP was usually supplemented with regen-

erating mixture, and ADP incubations with 10 U hexokinase (Boehringer Mannheim Biochemicals) and 5 mM glucose. Typically, 8 μM hsc70 were incubated in a volume of 100 μl with GST-auxilin fusion proteins for 15 min at 25°C. 20–40 μl glutathione–Sephadex beads were added, and the incubation was continued for 20 min on ice to allow binding of the fusion proteins while slowing the dissociation of the auxilin–hsc70 complex. The beads were then pelleted by a 30-s spin at 4°C in an Eppendorf benchtop centrifuge at maximum speed and resuspended in 0.4 ml of buffer C containing 0.1 mM of the original nucleotide. The solution was then underlaid with an equal volume of 10% sucrose in buffer C and the respective nucleotide. The beads were pelleted by centrifugation for 2 min at maximum speed and washed once more in 1 ml buffer C with the respective nucleotide. The final pellet was resuspended in 100 μl of sample buffer, and bound protein was analyzed by SDS-PAGE.

ATPase Assays and Nucleotide Analysis

To determine ATPase activity, 2 μM hsc70 was incubated alone or with 3.4 μM auxilin(547–910) at 25°C in 30 μl buffer C with 20 μM ATP, containing 0.3 MBq α -[^{32}P]ATP (Amersham Corp., Arlington Heights, IL). The reaction was stopped by spotting a 1- μl aliquot onto a PEI-cellulose TLC plate (Aldrich Chemical Co., Milwaukee, WI), and the ratio of ATP to ADP was determined by densitometry. Controls consisted of incubations of ATP without added proteins and with 3.4 μM auxilin(547–910), respectively. Gel-filtered fusion proteins were used because they lacked endogenous ATPase activity. To follow the products of the hydrolysis reaction, 1.2 nmol hsc70 was incubated for 4 min at 25°C with 4 nmol ATP containing equal amounts of α - and γ -[^{32}P]ATP (0.07 MBq). Unbound nucleotide was removed on a NAP-5 column as described before. Hsc70_{ATP} was then incubated in the presence and absence of GST-auxilin(547–910) as indicated in the figure legends. Before TLC analysis, hsc70 was desalted on a NAP-5 column. In one instance, the complex composed of GST-auxilin(547–910), hsc70, and labeled nucleotide was first isolated by incubation with glutathione–Sephadex for 15 min at 4°C, and then desalted and processed for nucleotide analysis as described.

Nucleotide Composition of Clathrin-associated Hsc70

Clathrin baskets (2.6 μM triskelia) were incubated with 5.5 μM GST-auxilin(547–910) for 30 min on ice in a vol of 88 μl . Unbound GST-auxilin(547–910) was removed by ultracentrifugation. The pelleted baskets were resuspended in 250 μl of buffer C. Hsc70 (13.7 μM) in buffer C was preincubated for 10 min at 25°C with 20 μM ATP containing a mixture of γ - and α -labeled [^{32}P]ATP (ratio 2:1). Unbound nucleotides were removed by desalting on a NAP-5 column. The baskets were incubated with a twofold molar excess of hsc70 (per clathrin heavy chain) for 30 min at 5°C. Dissociated clathrin (>80%) was separated from residual baskets by ultracentrifugation. The supernatant was applied to gel filtration on Superose 6. The column was equilibrated in buffer C in the absence of nucleotides and eluted at 5°C. 0.75-ml fractions were collected, and their protein composition was determined by SDS-PAGE, followed by densitometry of the stained protein zones. Hsc70-bound nucleotides and P_i were extracted by heating 20- μl aliquots of each fraction for 1 min to 95°C in the presence of 0.1% SDS. The nucleotide composition was determined by spotting 2- μl aliquots onto polyethyleneimine-cellulose TLC plates. Nucleotides were separated by ascending chromatography in 0.4 M NH_4HCO_3 and visualized by autoradiography. The ratio of P_i to ADP was determined by densitometry.

One-Round Hydrolysis of Hsc70- and DnaK-bound Nucleotide

Hsc70 (9 μM) was preincubated with 20 μM ATP containing 0.37 MBq α -labeled ATP in a vol of 200 μl for 5 min at 25°C. Thereafter, unbound nucleotide was removed by gel filtration on a precooled NAP-5 column. Similarly 3.7 μM DnaK were loaded with α -labeled ATP. Both proteins were incubated for 5 min at 25°C in the presence and absence of cofactors as detailed in the figure legends. Incubations were stopped by addition of 0.1 vol of 1% SDS and heating the samples for 1 min at 95°C as described above. Extracted nucleotides were analyzed by TLC as described.

Basket Dissociation with GST Fusion Protein Heterodimers as Cofactors

Heterodimers of GST fusion proteins consisting of GST-auxilin(547–814)

and GST-auxilin(813–910) were generated by mixing 5.5 μM GST-auxilin(547–814) with 75 μM GST-auxilin(813–910) in the presence of 1.25 M guanidinium hydrochloride, 2 mM DTT, 2 mM EDTA, 42 mM sodium phosphate, pH 7.0 (10). After 15 min at 22°C, the sample was desalted on a NAP-5 column that was equilibrated in buffer C and concentrated in a Centricon microconcentrator. To determine the extent of heterodimerization, the renatured mixture was subjected to gel filtration on Superose 12 equilibrated in buffer C. Fractions containing GST-auxilin(547–814) were pooled and concentrated in a Centricon 30 microconcentrator. 0.76 μM light chain–free triskelia were incubated for 30 min on ice either with 1.6 μM of the column-purified putative heterodimeric fusion proteins or with a mixture of 3.2 μM homodimeric GST-auxilin(547–814) and GST-auxilin(813–910). The baskets were subsequently pelleted by ultracentrifugation, and the protein composition in the pellets and supernatants was analyzed by SDS-PAGE as described above. Incubation of fusion proteins in the absence of clathrin baskets served as controls. Light chain–free clathrin (35) was preferred for this experiment, to avoid the interference of LCB with the densitometric evaluation of the GST-auxilin(547–814) band. For uncoating experiments, baskets (0.46 μM triskelia) were incubated with hsc70 (2.2 μM) and GST-auxilin(813–910) and GST-auxilin(547–814) as homodimeric mixture and heterodimeric mixtures, respectively, in amounts that are specified in Fig. 14.

Miscellaneous Techniques

SDS-PAGE and immunoblotting were performed as previously described (26). Clathrin baskets were negatively stained with 1% uranyl acetate (wt/vol) as described (1). Electron micrographs were taken on an electron microscope (JEOL USA, Peabody, MA) at 80 kV.

Results

Localization of a Clathrin Binding Domain in Auxilin

We have previously shown that truncation of auxilin at residue 814, which eliminates its J domain, abrogates all cofactor activity in the hsc70-dependent dissociation of clathrin baskets, but does not suppress clathrin binding (41). To identify the regions of auxilin involved in clathrin binding, we generated four GST fusion proteins that spanned the auxilin segments 1–546, 547–910, 547–814, and 813–910, by expression in bacteria (Fig. 1 a). Of these only GST-auxilin(1–546) proved difficult to express and to purify. Immunoblotting and staining with an anti-GST antibody indicated that the recombinant protein was unstable and largely degraded into smaller fragments, the most prominent of which migrated in gel electrophoresis as a 35-kD polypeptide (Fig. 1 b). In contrast, GST-auxilin(547–910), GST-auxilin(547–814), and GST-auxilin(813–910) were obtained in high yield from bacterial lysates by affinity chromatography on glutathione–Sephadex. For some purposes the GST moiety was cleaved from GST-auxilin(547–910) with thrombin and removed by adsorption to glutathione–Sephadex and gel filtration.

The ability of the recombinant products to assist hsc70 in the dissociation of clathrin baskets was assayed. To this end, clathrin baskets were incubated with increasing amounts of fusion proteins in the presence of hsc70 and ATP. Liberated clathrin was separated by ultracentrifugation from residual baskets and quantified by SDS-PAGE as described previously (41). Only GST-auxilin(547–910) and its GST-free form, auxilin(547–910), supported hsc70-catalyzed dissociation of clathrin baskets (Fig. 2). Removal of GST moiety from GST-auxilin(547–910) increased its activity probably because GST fusion proteins are dimers, and this may allow only one of the paired auxilin fragments at a time to engage clathrin productively. The failure of

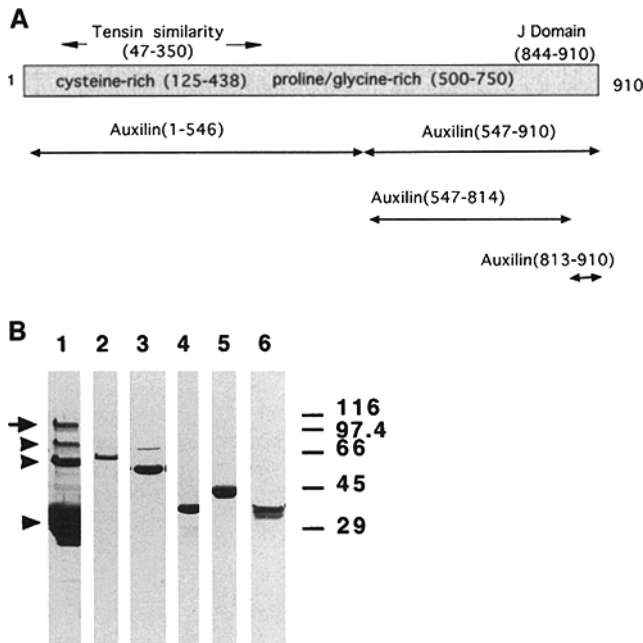


Figure 1. Recombinant auxilin fragments used in this study. (a) Block diagram showing the position of the recombinant fragments in auxilin. (b) SDS-PAGE of purified recombinant auxilin fragments. All proteins were affinity purified on glutathione-Sepharose and, except for GST-auxilin(1-546), gel filtered on Superose 12. GST-auxilin(1-546) (lane 1); GST-auxilin(547-910) (lane 2); GST-auxilin(547-814) (lane 3); GST-auxilin(813-910) (lane 4); auxilin(547-910) after removal of the GST moiety (lane 5); and auxilin(547-814) after removal of GST moiety (lane 6). The arrowheads denote fragments of GST-auxilin(1-546) that reacted with an anti-GST serum. The arrow denotes the position of undegraded GST-auxilin(1-546). The molecular masses (kD) of marker proteins are indicated on the right.

GST-auxilin(1-546) and GST-auxilin(547-814) was not unexpected because both proteins lack the J domain. GST-auxilin(813-910), which contains only auxilin's J region, was also inactive, implying that the J domain is necessary but not sufficient for cofactor activity.

We previously proposed that the cofactor function of auxilin rests on its ability to recruit hsc70 via the J domain and to deliver it via its clathrin binding domain to clathrin baskets (41). GST-auxilin(547-910) would therefore be expected to contain a clathrin binding domain, which, however, might be missing from GST-auxilin(813-910). This prediction was tested in a cosedimentation assay using preassembled clathrin baskets as substrate for the different fragments. We found that only those fusion proteins that contained the segment 547-814 (Fig. 1) were able to bind to clathrin baskets (Fig. 3). No specific association of GST-auxilin(813-910) or of GST-auxilin(1-546) with the baskets could be detected. The small amount of GST-auxilin(1-546) that was pelleted in the presence and in the absence of clathrin baskets probably reflects aggregation or stickiness of the fusion protein, rather than binding.

To determine the stoichiometry of the interaction of GST-auxilin(547-910) with clathrin, the GST moiety was eliminated by digestion with thrombin, leaving auxilin (547-910) in its monomeric state. The purified fragment

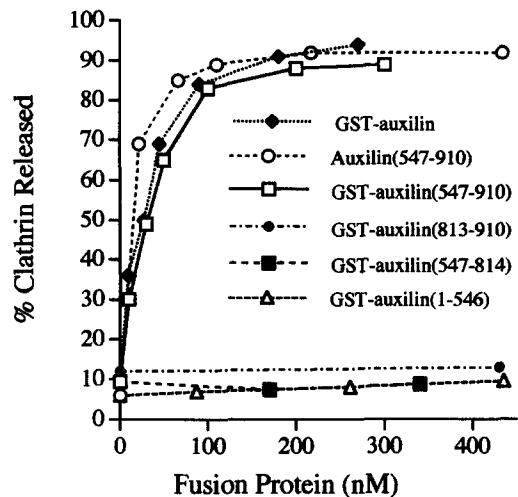


Figure 2. Cofactor activity of auxilin fusion proteins. A constant amount of clathrin baskets ($0.46 \mu\text{M}$ triskelia) was mixed with the indicated fusion proteins and incubated with hsc70 ($3.1 \mu\text{M}$) for 15 min at 25°C . The amount of released clathrin was determined after ultracentrifugation by SDS-PAGE and densitometry. Data points are averages of two determinations.

bound to clathrin in a ratio of three per triskelion, like intact auxilin (Fig. 4). We also tested auxilin(547-814) that lacks the J domain. Its binding properties were indistinguishable from those of auxilin(547-910), thereby ruling out any involvement of the J domain in clathrin binding. While the existence of further clathrin binding sites in the segments 1-546 and 813-910 cannot yet be ruled out entirely, because they may be too weak to be detected in our assay or, alternatively, the recombinant protein may not be correctly folded, it remains clear that the strong binding site that we have identified between residues 547 and 814 suffices in conjunction with the J domain to create an active cofactor. This conclusion is also supported by our observation that an eightfold molar excess of auxilin(547-814) over GST-auxilin reduces the binding of the latter by $\sim 85\%$ (data not shown).

Auxilin was previously characterized as a clathrin assembly protein (2). To determine whether the segment 547-814 retains this function, we performed assembly experiments with auxilin(547-814). The GST moiety from the fusion protein was removed to ensure that GST-induced dimerization of the clathrin binding domain could not fortuitously cause clathrin assembly. Clathrin triskelia were mixed with auxilin(547-814), and then dialyzed against assembly buffer lacking calcium. In this condition, efficient assembly of intact clathrin is dependent on the presence of factors such as adaptors, AP180, and auxilin (21, 26). Polymerized clathrin was separated from triskelia by ultracentrifugation. The amount of clathrin in the pellet clearly increased with increasing amounts auxilin(547-814) added (Fig. 5a). EM of the resuspended pellet confirmed that the clathrin had polymerized into regular baskets (Fig. 5b). Densitometry of the Coomassie-stained gel lanes (Fig. 5a) indicated that the assembled baskets bound an average of 2.1 auxilin(547-812) molecules per triskelion. If clathrin binding is essential for the delivery of hsc70

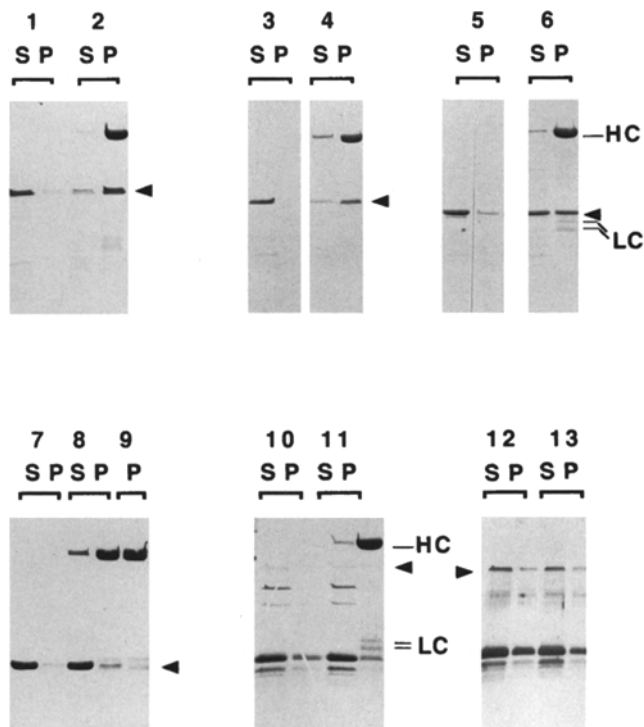


Figure 3. Clathrin binding activity of recombinant auxilin fragments. The respective fragments were incubated in buffer A either alone (lanes 1, 3, 5, 7, 10, and 12) or with the indicated amount of preassembled clathrin baskets (lanes 2, 4, 6, 8, 11, and 13). As a control to the experiment in lane 8, clathrin baskets ($0.45 \mu\text{M}$ triskelia) were incubated also alone (lane 9). To separate bound and aggregated fragments from unbound and soluble ones, the incubation mixtures were centrifuged for 20 min at $88,000 g$ in a Beckman TL-100 ultracentrifuge. The protein content of pellets (P) and supernatants (S) was analyzed by SDS-PAGE. The arrows point to the respective fusion proteins. (Lanes 1 and 2) GST-auxilin(547-910) ($2.3 \mu\text{M}$), clathrin baskets ($0.55 \mu\text{M}$ triskelia). (Lanes 3 and 4) GST-auxilin(547-814) ($2 \mu\text{M}$), clathrin baskets ($0.23 \mu\text{M}$ triskelia). (Lanes 5 and 6) Auxilin(547-910) ($0.73 \mu\text{M}$), clathrin baskets ($0.38 \mu\text{M}$). (Lanes 7 and 8) GST-auxilin(813-910) ($5.2 \mu\text{M}$), clathrin baskets ($0.45 \mu\text{M}$). (Lanes 10 and 11) GST-auxilin(1-546) (6.3 nM), clathrin baskets ($0.38 \mu\text{M}$). (Lanes 12 and 13) Immunoblot to lanes 10 and 11 stained with anti-GST antibody. The clathrin control in lane 9 was included to demonstrate comigration of GST-auxilin(813-910) with the smaller of the clathrin light chains.

to clathrin baskets, one might expect that auxilin(547-814) would also inhibit basket dissociation by preventing binding of auxilin(547-910). This was indeed observed in a competition experiment in which auxilin(547-814) reduced the extent of clathrin release by about half when added at fivefold molar excess over auxilin(547-910) (data not shown).

In sum, our results demonstrated that the structural elements for auxilin's cofactor activity are located between residues 547 and 910. Since the deletion of the J domain (segment 815-910) did not interfere with auxilin's ability to bind to clathrin, it can be concluded that a high affinity clathrin binding domain is located between residues 547 and 814.

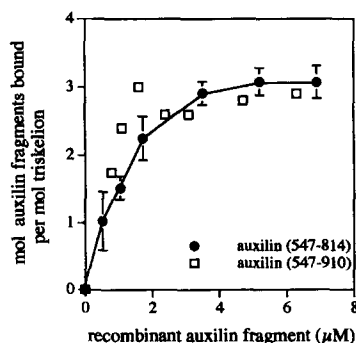


Figure 4. Titration of clathrin baskets ($0.38 \mu\text{M}$ triskelia) with auxilin(547-910) and auxilin(547-814). Binding data were obtained by densitometry of SDS-PAGE lanes, such as those shown in Fig. 3. Data points are averages of two (auxilin[547-910]) or three (auxilin[547-814]) independent determinations.

Dissociation of GST-Auxilin(547-910) from Clathrin

To follow the movement of the cofactor during clathrin basket dissociation, preformed baskets were combined with stoichiometric amounts of GST-auxilin(547-910). Free GST-auxilin(547-910) was removed by ultracentrifugation. The resuspended baskets were then incubated with hsc70 in the presence of ATP to allow basket dissociation. Remaining clathrin baskets were removed by ultracentrifugation. The supernatant with the released clathrin was divided into two aliquots. The first was treated with hexokinase and glucose to eliminate residual ATP, and the second was left undisturbed. Both aliquots were analyzed by gel filtration on Superose 6 in the presence of ADP or ATP, respectively. As expected in the presence of ADP, hsc70 coeluted with clathrin between fractions 4 and 9, but GST-auxilin(547-910) had separated. Instead, it eluted from fractions 10-20 with a peak in fraction 18 (Fig. 6). In ATP, clathrin eluted essentially free of other components between fractions 4 and 9 followed by GST-auxilin(547-910), which now gave rise to two peaks: the first between fractions 11-14 and a second between fractions 17 and 20. The first peak also contained hsc70, which, when chromatographed alone in ATP, eluted exclusively as a monomer between fractions 16 and 21 (data not shown). GST-auxilin(547-910) chromatographed by itself eluted between fractions 17 and 20 (data not shown). Taken together, our gel filtration experiments showed that GST-auxilin(547-910) dissociates completely from clathrin liberated by the basket dissociation reaction, but in the presence of ATP, it apparently enters into a complex with hsc70.

Interaction of GST-Auxilin(547-910) with Hsc70

To confirm the inferences drawn from gel filtration on the interaction between hsc70 and GST-auxilin(547-910), we incubated hsc70 with ATP in the presence and absence of GST-auxilin(547-910). Glutathione-Sepharose resin (Fig. 7, lane 1) was then added to adsorb the GST fusion protein. In the absence of GST-auxilin(547-910), only very little hsc70 was nonspecifically retained by the Sepharose resin, but in the presence of GST-auxilin(547-910), near stoichiometric quantities of hsc70 (relative to auxilin) were recovered with the resin (Fig. 7, lane 2). The interaction be-

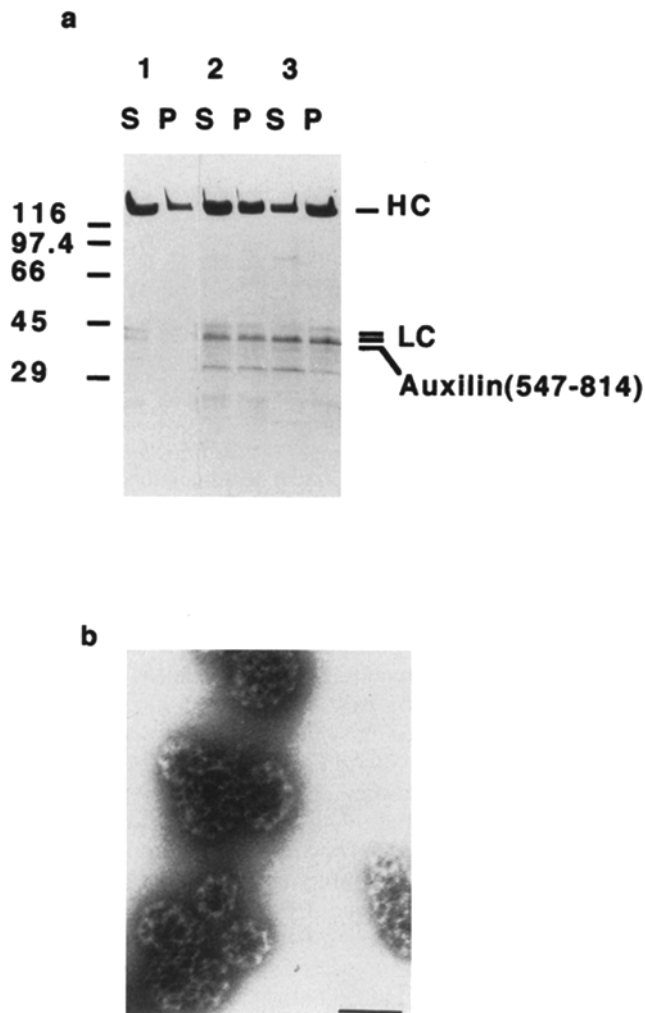


Figure 5. Auxilin(547-814) promotes assembly of clathrin baskets. 0.6 μM clathrin triskelia were dialyzed into buffer A in the absence or presence of auxilin(547-814). Assembly was evaluated after ultracentrifugation. (a) SDS-PAGE analysis of assembly in the absence (lane 1) and presence of 0.83 μM (lane 2) and 1.6 μM (lane 3) auxilin(547-814). The molecular masses (kD) of marker proteins are indicated on the left. S, supernatants; P, pellets. (b) EM of reassembled baskets after negative staining with uranyl acetate. Bar, 100 nm.

tween GST-auxilin(547-910) was strictly dependent on ATP, which could not be replaced by ATP- γ -S, adenylylimidodiphosphate (AMP-PNP), ADP, or ADP+ P_i (Fig. 7, lanes 3-10). When GST-auxilin(547-814), which lacks the J domain, was used as a ligand, hsc70 could not be recovered with the resin, suggesting that the J domain is essential for complex formation (Fig. 7, lane 12). Given that the J domain is necessary for the interaction of auxilin(547-910) with hsc70, we next asked whether it is also sufficient. To address this question, we tested GST-auxilin(813-910) in our assay and found that it readily associated with hsc70 in the presence of ATP (Fig. 7, lanes 14 and 15). GST by itself, which was used as a control, did not interact with hsc70 (Fig. 7, lane 16). Thus, the J domain-containing segment of auxilin is both necessary and sufficient for binding to hsc70. Hsc70 is known to bind unfolded polypeptides at its peptide binding domain, which is located on the

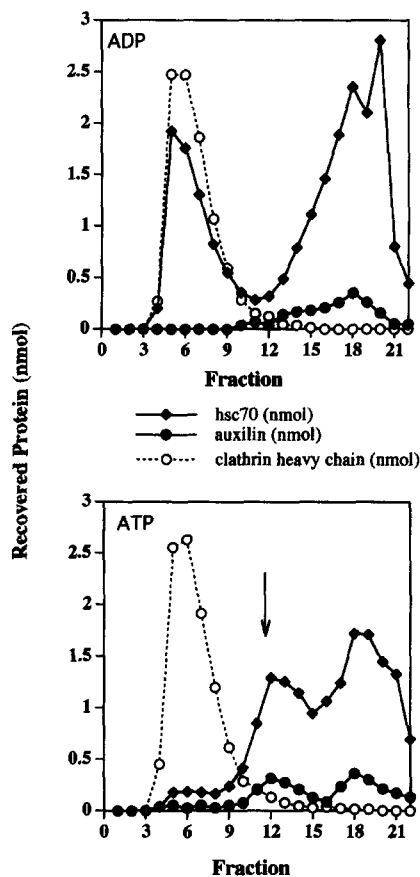


Figure 6. Dissociation of GST-auxilin(547-910) from clathrin. Clathrin baskets containing prebound GST-auxilin(547-910) were incubated with hsc70 in the presence of 4 mM ATP for 20 min at 25°C. Remaining baskets were removed by ultracentrifugation. The supernatant was divided into two aliquots. One aliquot was depleted of ATP (see Materials and Methods), and then applied to a Superose 6 gel filtration column and eluted in the presence of ADP (upper panel). The other was directly applied to gel filtration in the presence of ATP (lower panel). Eluted proteins were identified by SDS-PAGE, and the intensities of the bands in each fraction were quantified by densitometry. The arrow denotes the position of the putative hsc70 GST-auxilin(547-910) complex. Note that dissociated clathrin elutes free of previously bound GST-auxilin(547-910).

COOH-terminal side of the nucleotide binding domain (43). The COOH-terminal motif EEVD of hsc70 has been implicated in the interaction with the DnaJ homologue Hdj-1 (13). Bacterial DnaJ is believed to stabilize via its J domain the interaction of DnaK with substrates by stimulating its ATPase and thereby locking DnaK in its "ADP conformation" onto the substrate. To examine whether the substrate binding domain of hsc70 is involved in complex formation with GST-auxilin(547-910), 6 μM GST-auxilin(547-814) was incubated with 4 μM hsc70 in the presence of ATP and 2 μM auxilin(547-910). If the substrate binding site of hsc70 engages in complex formation, it might do so upon activation by the J domain carrying auxilin(547-910) (which remains silent in our assay because it lacks the GST moiety). GST-auxilin(547-814) would constitute an abundant substrate for the activated hsc70. Thus, cosedimentation of hsc70 with the glutathione-

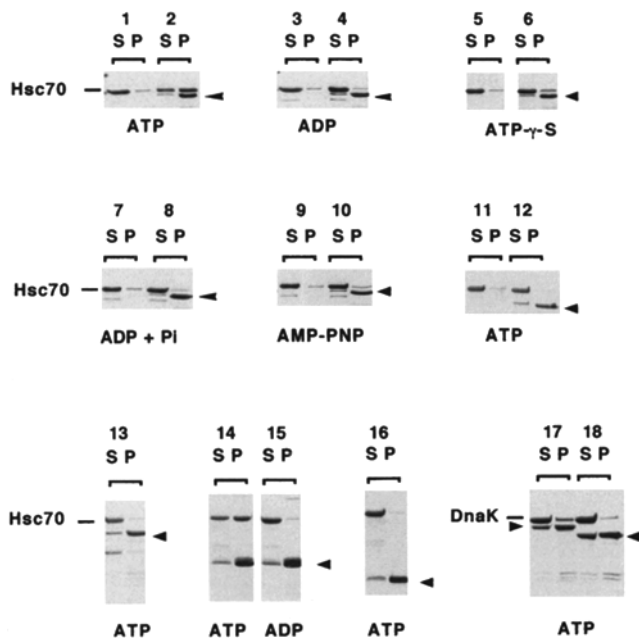


Figure 7. Interaction of recombinant auxilin fragments with hsc70 and DnaK. Hsc70 (8 μ M) was incubated for 15 min at 25°C with 9 μ M GST-auxilin(547–910) (lanes 2, 4, 6, 8 and 10) and with 6 μ M GST-auxilin(547–814) (lane 12); hsc70 (4 μ M) was incubated simultaneously with 6 μ M GST-auxilin(547–814) and 2 μ M auxilin(547–910) (lane 13); with 17 μ M GST-auxilin(813–910) (lanes 14 and 15); and with 45 μ M GST (lane 16). DnaK (4.3 μ M) was incubated with 5 μ M GST-auxilin(547–910) (lane 17) or with 9 μ M GST-auxilin(547–814) (lane 18) for 15 min at 25°C. The type of nucleotide is indicated underneath the gel lanes; nucleotide concentrations were 5 mM for ATP, ADP+P_i, ATP- γ -S, and 10 mM for AMP-PNP. The GST fusion proteins were recovered by adsorption on glutathione–Sephadex beads. Protein was eluted from the washed resin with SDS sample buffer and analyzed by SDS-PAGE together with the corresponding supernatant. Supernatants (S) and pellets (P) are directly comparable. The arrowheads denote the respective GST fusion proteins.

Sephadex in these circumstances would indicate binding of GST-auxilin(547–814) to the substrate binding domain of hsc70. In fact, no association was detected, and we therefore conclude that the interaction between GST-auxilin(547–910) involves a site on hsc70 that interacts specifically with the J domain (Fig. 7, lane 13). That not any J domain is as suitable as auxilin's for this interaction is shown by the reduced binding of DnaK to GST-auxilin(547–910) (Fig. 7, lane 17). The absence of a contaminating ATPase activity from DnaK preparations proved essential for the detection of this interaction (see Materials and Methods for the purification of DnaK).

The stability of the auxilin(547–910) hsc70 complex was investigated next. To this end, the complex was preformed in ATP and adsorbed to glutathione–Sephadex resin. Unbound protein was removed and free ATP was exchanged for 5 mM ADP. The complex was then incubated on ice or at 25°C for up to 2 h. At 25°C, ~50% of the hsc70 was released within 8 min (Fig. 8). This again indicated that hsc70 did not bind GST-auxilin(547–910) to its peptide binding site, since this interaction should have been main-

tained in ADP (30, 32). On ice, however, no significant dissociation occurred over a period of 2 h (Fig. 8).

Relations between ATP Hydrolysis and Complex Stability

Bacterial DnaJ interacts transiently with DnaK and stimulates its ATPase activity about twofold (23). Similarly, GST-auxilin(547–910) stimulated the ATPase of hsc70 by about fivefold (Fig. 9). The extent of this stimulation varied between 2.5- and fivefold when different preparations of either hsc70 and GST-auxilin(547–910) were examined. This stimulation was strictly dependent on the presence of auxilin's J domain, as demonstrated by experiments in which the hydrolysis of hsc70-bound ATP was followed: when preformed hsc70- α -[³²P]ATP complexes were incubated at 25°C for 5 min, not >25% of the bound ATP was converted to ADP (Fig. 10). In contrast, when GST-auxilin(547–910) was included, almost all of the bound ATP had been hydrolyzed (Fig. 10). GST-auxilin(547–814), which lacks the J domain, and GST by itself did not enhance the ATPase activity of hsc70. However, the fusion protein GST-auxilin(813–910), which contains only auxilin's J domain, was also fully active in stimulating the ATPase of hsc70 (Fig. 10).

Since we had observed that DnaK has some affinity for GST-auxilin(547–910) (see Fig. 7), we also tested its ability to stimulate the ATPase of DnaK. To this end, DnaK preloaded with α -labeled ATP was incubated either alone or in the presence of GST-auxilin(547–910) or GST-auxilin(547–814), respectively. Only GST-auxilin(547–910) stimulated the ATPase of DnaK, which suggests that despite

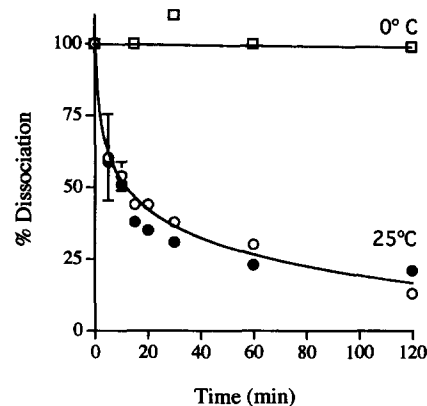


Figure 8. Stability of GST-auxilin(547–910)–hsc70 complex in ADP. GST-auxilin(547–910)–hsc70 complexes bound to 40 μ l glutathione–Sephadex were diluted into 1 ml of buffer C containing 5 mM ADP, incubated for the indicated times at 0° and 25°C, respectively, and then recovered by centrifugation in a benchtop centrifuge. Bound protein was eluted with SDS sample buffer and analyzed by SDS-PAGE. The ratio of bound hsc70 to GST-auxilin(547–910) was determined by densitometry. The results of two independent experiments at 25°C are shown. One represents the averages of duplicate determinations, and the other (with error bars) represents that of triplicate determinations. Nonspecific adherence or trapping of hsc70 by unwashed beads accounts for a background of ~10% that was not subtracted. Both experiments, performed at 25°C, indicate a half-life of ~8 min for the GST-auxilin(547–910)–hsc70 complex.

the evolutionary divergence between the J domains of auxilin and DnaJ, DnaK is still capable of recognizing both. Thus, auxilin's J domain behaves like DnaJ in that it causes almost instant hydrolysis of hsc70- and DnaK-bound ATP. The rapid hydrolysis of bound ATP suggested that the GST-auxilin(547-910)-hsc70 complex should contain ADP rather than ATP. This appeared paradoxical because the ADP complex of hsc70 did not bind auxilin (Fig. 7). To determine the type of nucleotide associated with hsc70 in the presence of GST-auxilin(547-910), hsc70 was preincubated with an equimolar mixture of α - ^{32}P ATP and γ - ^{32}P ATP, and then incubated with and without GST-auxilin(547-910) for 4 min at 4°C and 25°C, respectively. After the removal of free nucleotide by gel filtration, bound nucleotide was extracted from hsc70 after rapid denaturation of the protein in hot 0.1% SDS. Analysis by TLC revealed that in the absence of GST-auxilin(547-910), the predominant nucleotide present was ATP, irrespective of the incubation temperature. GST-auxilin(547-910) had only a small effect on the hydrolysis of hsc70-bound ATP at 4°C, but at 25°C, the predominant nucleotide species associated with hsc70 was ADP and, in addition, P_i (Fig. 11 a). To determine the nucleotide state of hsc70 that is associated with GST-auxilin(547-910), the complex was (after the desalting step) purified by adsorption to glutathione-Sepharose at 4°C. TLC analysis of the bound radioactivity showed it to contain equimolar amounts of ADP and P_i (Fig. 11 b). However, when the adsorbed complex was incubated for 30 min at 25°C to release hsc70, the analysis of the bound nucleotide showed it to be only ADP (Fig. 11 b). We infer that GST-auxilin(547-910) can interact with hsc70_{ADP+P_i} but not with hsc70_{ADP}.

To determine the nucleotide composition of clathrin-bound hsc70, we used hsc70 that contained α - and γ -labeled- ^{32}P ATP in the absence of free nucleotide to dissociate baskets that contained GST-auxilin(547-910). In a deviation from the standard coat dissociation assay, the incubation with the baskets was performed at 5°C for 30 min to reduce the rate of nucleotide exchange. In the course of this work, we had observed that dissociation of baskets proceeds at low temperatures as long as at least a twofold

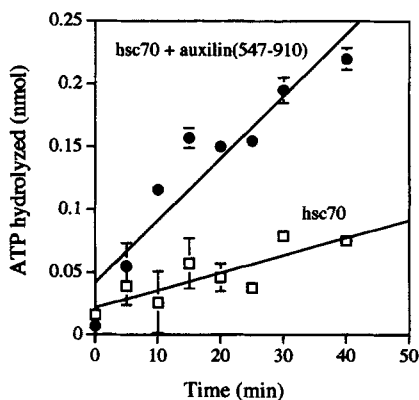


Figure 9. Stimulation of the hsc70 ATPase by auxilin(547-910). Hsc70 (2 μM) was incubated in the absence or presence of 3.4 μM auxilin(547-910) in buffer C at 25°C. Nucleotides were separated by TLC. The total amount of hydrolyzed ATP was calculated from the ratio of ATP to ADP. Data points represent the average of three determinations.

GST	-	-	-	-	6.0	-	-	-
GST-auxilin 813-910	-	-	-	6.5	-	-	-	-
GST-auxilin 547-814	-	-	6.7	-	-	-	-	3.4
GST-auxilin 547-910	-	7.0	-	-	-	-	3.6	-
DnaK	-	-	-	-	-	1.69	1.69	1.69
Hsc70	1.7	1.7	1.7	1.7	1.7	-	-	-

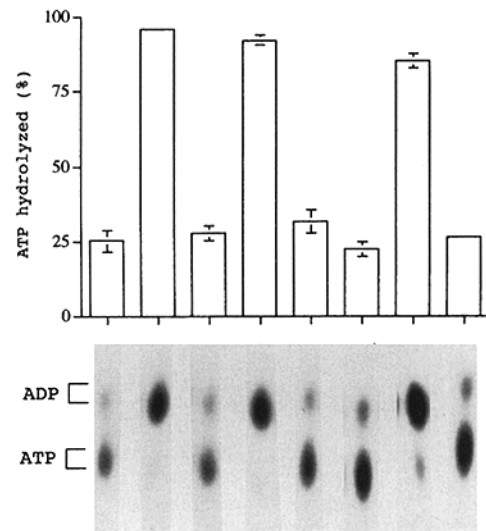


Figure 10. Stimulation of hsc70 and DnaK ATPase depends on auxilin's J domain. Hsc70 or DnaK containing bound α -labeled ATP were incubated for 5 min at 25°C in the absence or presence of the indicated GST fusion proteins. Numbers in the grid refer to the respective fusion protein concentrations in μM . The extent of the hydrolysis of bound ATP (as percentage) is shown by the bars. Each bar represents the average of four independent determinations. Representative autoradiographs of the nucleotide fractionation by TLC are shown in the lower panel.

molar excess of hsc70-containing bound ATP was provided (not shown). Released protein was fractionated by gel filtration on Superose 6 at 5°C in the absence of free nucleotides. Fractions were analyzed for their protein and nucleotide composition by SDS-PAGE and TLC, respectively (Fig. 12). Clathrin-bound hsc70 was associated with ADP, but surprisingly also with P_i , accounting for $79 \pm 7\%$ of the hydrolyzed γ -phosphate. In contrast, unbound hsc70 contained in addition to ADP only $26 \pm 5\%$ of the liberated P_i . This result suggests that binding of hsc70 to clathrin inhibits the release of P_i .

Activity of GST-Auxilin(813-910)·GST-Auxilin(547-814) Heterodimers

Since neither GST-auxilin(547-814) nor GST-auxilin(813-910) supported hsc70 in basket dissociation (Fig. 2), we tested if coat dissociation would occur when both fusion proteins were added together. Again, no significant dissociation of clathrin baskets was observed (see Fig. 14). This suggested that the mere presence of a suitable J domain does not suffice to complement GST-auxilin(547-814). GST exists under physiological conditions as a dimer (24); GST fusion proteins are therefore also at least dimers. GST dimers dissociate in 1-2 M guanidinium hydrochloride and reform upon removal of the denaturant (10). Using this property of GST, we generated GST-auxilin(813-910)·GST-auxilin(547-814) heterodimers by pass-

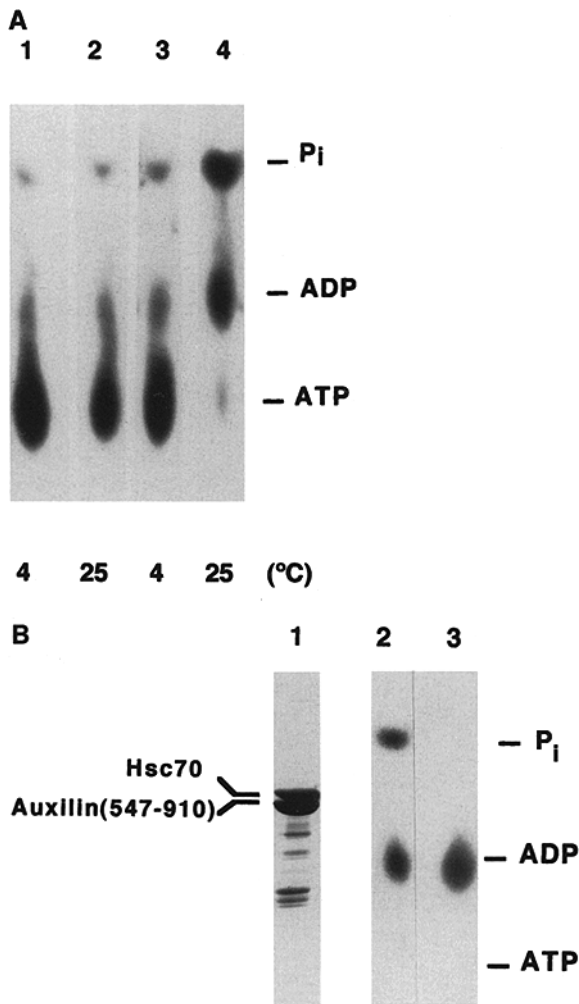


Figure 11. Association of P_i with hsc70. (a) Hsc70 containing equal amounts of α - and γ -[³²P]ATP was incubated for 4 min at 4° and 25°C, respectively, in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of GST-auxilin(547-910). Hsc70 was gel filtered, and bound nucleotide was analyzed on TLC plates. Note that after ATP hydrolysis, P_i remains quantitatively associated with hsc70. (b) State of bound nucleotides in hsc70, which is complexed with GST-auxilin(547-910). The hsc70-GST-auxilin(547-910) complex was isolated by adsorption to glutathione-Sepharose, and an aliquot was analyzed by SDS-PAGE (lane 1). Complexed hsc70 contains bound ADP and P_i (lane 2). Hsc70 released from GST-auxilin(547-910) during a 30-min incubation at 25°C is associated only with ADP (lane 3).

ing a mixture of the respective homodimers through a denaturation/renaturation cycle. To test for heterodimer formation, renatured putative heterodimers were assayed in a basket binding experiment. Cosedimentation of GST-auxilin(813-910), which does not possess a clathrin binding site, with clathrin basket would indicate its association with the clathrin binding protein GST-auxilin(547-814). To this end, heterodimers were first purified by gel filtration to remove the excess of GST-auxilin(813-910), which was used to drive the majority of the GST-auxilin(547-814) into an association with GST-auxilin(813-910) (see Materials and Methods for details). The purified putative heterodimers and a mixture of homodimers, respectively, were incubated with preformed clathrin baskets. Specific

cosedimentation of GST-auxilin(813-910) with clathrin baskets was observed only with the putative heterodimer preparation (Fig. 13). In contrast, from an untreated mixture of GST-auxilin(813-910) and GST-auxilin(547-814), only the latter cosedimented with clathrin baskets. Next, the heterodimerized fusion proteins were tested in the basket dissociation reaction. Significant recovery of cofactor activity was indeed observed after heterodimerization, but not with the untreated mixture (Fig. 14). In contrast, the untreated mixture even reduced spontaneous dissociation of the baskets during the incubation with hsc70 when added at high concentration. This effect is probably due to the assembly-promoting property of GST-auxilin(547-814). Our results suggest that the clathrin binding domain must be physically connected to the J domain if it is to support hsc70-driven basket dissociation, but the position of the J domain relative to the clathrin binding domain need not be fixed in space.

Discussion

The clathrin assembly protein auxilin, which was recently

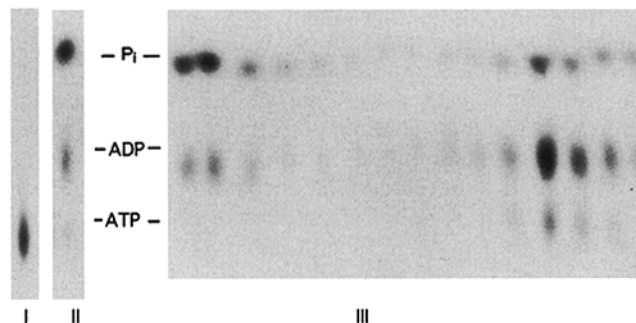
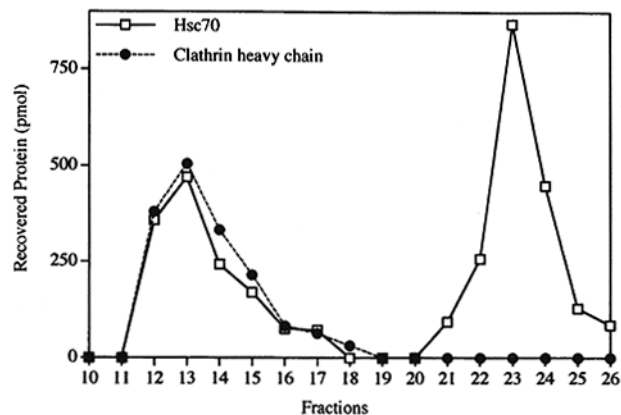


Figure 12. Inhibition of P_i release from hsc70 by clathrin. Clathrin baskets containing GST-auxilin(547-910) were dissociated by hsc70 containing a mixture of α - and γ -labeled ATP. Released protein was fractionated by gel filtration on Superose 6 (upper panel). Protein-associated nucleotide was analyzed by TLC and visualized by autoradiography (lower panels I-III). Hsc70-associated nucleotide before incubation with clathrin baskets (I); total nucleotide after incubation of hsc70 with clathrin (II); hsc70-bound nucleotide after incubation with clathrin and fractionation by gel filtration (III). Note that clathrin-associated hsc70 retained 79 ± 7% of the P_i (fractions 12-14), while free hsc70 (fractions 22-24) retained only 26.5 ± 5% of the P_i.

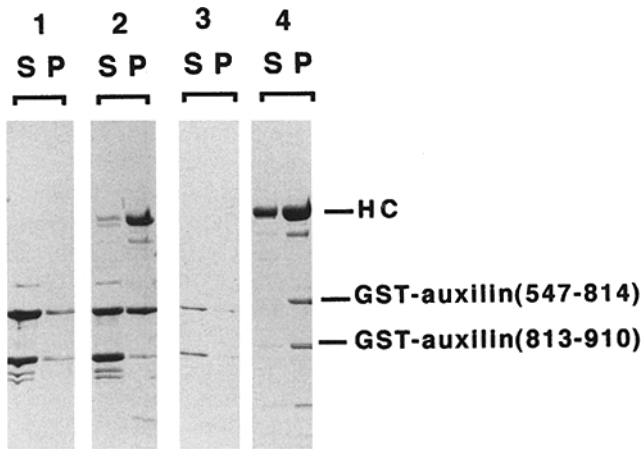


Figure 13. Binding of GST-auxilin(813-910)-GST-auxilin(547-814) heterodimers to clathrin baskets. 0.76- μ M light chain-free triskelia were incubated either with 1.6 μ M of the column-purified putative heterodimeric fusion proteins or with a mixture of 3.2 μ M homodimeric GST-auxilin(547-814) and GST-auxilin(813-910). Baskets were pelleted by ultracentrifugation, and the protein composition in the pellets (P) and supernatants (S) was analyzed by SDS-PAGE. (Lane 1) Mixture of untreated fusion proteins; (lane 2) mixture of untreated fusion proteins with clathrin baskets; (lane 3) GST-auxilin(813-910)-GST-auxilin(547-814) heterodimers; (lane 4) GST-auxilin(813-910)-GST-auxilin(547-814) heterodimers with clathrin baskets. Note that GST-auxilin(813-910) cosediments specifically with clathrin baskets only upon forming heterodimers with GST-auxilin(547-814).

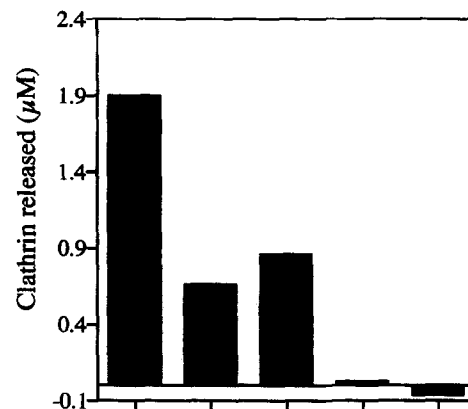
identified as a member of the DnaJ protein family, cooperates with hsc70 in dissociating clathrin baskets (41). This function requires a domain that recognizes preferentially assembled clathrin, in addition to the J domain that interacts with hsc70. Both are located within a 38-kD fragment extending from Pro-547 to the carboxyl terminus of the protein. The two domains are autonomous in their respective functions. Auxilin(547-814), which lacks the J domain, interact with clathrin on its own. This function does not require ATP and is in this respect analogous to the ATP-independent interaction of DnaJ with unfolded and partially folded substrates (44). Like intact auxilin (2), this fragment binds with a maximal stoichiometry of 1 per clathrin heavy chain and supports the polymerization of triskelia into regular baskets *in vitro*.

Why should a cofactor for an uncoating enzyme (hsc70) support clathrin assembly? Promotion of assembly is probably not the function of auxilin in the cell, but this may merely reflect its preference for binding to clathrin baskets as against triskelia. By binding to a site on the clathrin heavy chain that is exposed in assembled baskets, auxilin would stabilize the assembled state, behaving like an assembly-promoting protein. It is also possible that under assembly conditions, auxilin(547-910) binds as a dimer with higher affinity for assembled clathrin than for triskelia. At present we cannot distinguish between these possibilities.

Other clathrin binding proteins are the adaptor complexes, AP1 and AP2. Their β -type subunits are known to contain a clathrin binding site (1) that was more recently narrowed down to a 50-residue region located in the hinge domain of the β chains (37). The auxilin sequence between residues 547 and 814, which contains the clathrin binding

site, has no homology to the clathrin binding segment of the β chain, suggesting that auxilin and the β subunits bind to different sites on the clathrin heavy chain. This is to be expected, because auxilin is and indeed must be able to uncoat clathrin-coated vesicles that contain adaptor molecules. We know, in addition, that auxilin does not compete with the adaptors for binding to clathrin baskets (Lindner, R., S. Holstein, and E. Ungewickell, manuscript in preparation).

In the course of the basket dissociation reaction, GST-auxilin(547-910) is released from the clathrin heavy chain. At present, it is not clear whether this dissociation is a direct consequence of an interaction between hsc70 and auxilin or results from the disintegration of the baskets. There are two reasons why the latter explanation seems more plausible: first, we have shown that the J domain and the clathrin binding domain must be physically joined to each other to form an active cofactor. This suggests very strongly that auxilin has to bring hsc70 into close proximity with its binding site on the clathrin heavy chain. If the attachment of hsc70 to auxilin were to disrupt the auxilin-clathrin interaction, both proteins could diffuse away before the complex between hsc70 and the heavy chain of clathrin has had time to form. Second, auxilin has a much higher affinity for assembled clathrin than for triskelia,



GST-auxilin(547-910)	0.36 μ M	-	-	-	-
[GST-auxilin(547-814) GST-auxilin(813-910)]	-	0.34 μ M	-	-	-
[GST-auxilin(547-814) GST-auxilin(813-910)]	-	-	0.85 μ M	-	-
GST-auxilin(547-814) +GST-auxilin(813-910)	-	-	-	0.34 μ M	-
GST-auxilin(547-814) +GST-auxilin(813-910)	-	-	-	-	0.85 μ M

Figure 14. Cofactor activity of GST-auxilin(813-910)-GST-auxilin(547-814) heterodimers. Clathrin baskets (0.46 μ M triskelia) were incubated with hsc70 (2.2 μ M) and with the indicated fusion proteins for 15 min at 25°C in buffer C. Concentrations of the fusion proteins in the assay are indicated by the numbers in the grid. Heterodimeric fusion proteins are marked by brackets. Data points, which are averages of two independent measurements, are corrected for spontaneous dissociation of clathrin baskets during the incubation in the absence of cofactor. Note that 0.85 μ M of the homodimeric fusion protein mixture causes slight stabilization of the baskets rather than their destabilization.

and therefore dissociation of the baskets under the influence of hsc70 is likely to effect the release of auxilin. After its release, auxilin(547–910) readily reenters into a complex with hsc70 in the presence of ATP. This suggests that auxilin can make repeated deliveries of hsc70 molecules to baskets, and thus explains why substoichiometric amounts of auxilin (relative to clathrin and hsc70) suffice for basket dissociation (31).

In the presence of ADP at 25°C the preformed hsc70–auxilin complex has a half-life of ~8 min. In contrast, the analogous DnaJ·DnaK complex has long eluded detection, and its existence was only recently demonstrated directly, both by gel filtration and a solid phase binding assay, using glutaraldehyde as a stabilizing agent (44). The requirements for complex formation are similar for both systems. Thus, ATP supports complex formation, while ATP- γ -S and AMP-PNP do not. While the GST–auxilin(547–910)–hsc70 complex did not form in ADP or ADP+P_i, a slight stimulation by ADP was noted in the solid phase binding assay (44). Like bacterial DnaJ and other DnaJ homologues, auxilin modestly stimulates the ATPase of hsc70, but in the absence of other factors, nucleotide exchange appears to be relatively slow compared with the rapid cleavage of the γ -phosphate of the hsc70-bound ATP (Fig. 10). Unexpectedly, however, dissociation of the hsc70–auxilin complex did not accompany ATP hydrolysis, but rather the release of P_i. Thus, it is the hsc70_{ADP+P_i} state of hsc70 that interacts stably with auxilin's J domain. A causal relationship between release of P_i and dissociation of auxilin is likely, but this was not proven by our experiments. Hsc70 that is associated with clathrin triskelia after the uncoating process retained, in addition to ADP, ~80% of the P_i, which was generated by hydrolysis of hsc70-bound ATP. This was contrasted by unbound hsc70, which after the reaction retained only 27% of the original P_i (Fig. 12). This result suggests strongly that binding of hsc70 to clathrin inhibits the release of P_i. Moreover, clathrin can apparently also dislodge auxilin from hsc70 without prior release of P_i (Fig. 6). The clathrin-associated hsc70 did most likely bind in the course of the uncoating reaction. First, for the basket dissociation reaction, we used conditions in which the sole ATP source was the hsc70-associated ATP. Moreover, the reaction and subsequent gel filtration step was performed at 5°C, conditions that do not favor rapid nucleotide exchange (14). Since ADP/ATP exchange appears to be a prerequisite for the rapid dissociation of hsc70 from clathrin (32), our experimental conditions were not favorable for the dissociation of hsc70 from clathrin. Moreover, when triskelia rather than baskets were incubated in buffer C with hsc70 in the presence of ADP, under otherwise the same experimental conditions, no binding of hsc70 to clathrin was observed (data not shown). The existence of a stable hsc70_{ADP+P_i} complex had been previously inferred from the analysis of the crystal structure of the nucleotide binding domain of hsc70 (12). More recently, Buxbaum and Woodman also had presented evidence for the longevity of the hsc70_{ADP+P_i} complex by demonstrating that the release of P_i from intact hsc70 is slow but enhanced by the presence of coated vesicles, especially by those purified from brain tissue (5). The authors had speculated that clathrin or a DnaJ homologue might be responsible for the liberation of the phos-

phate. Our results do not support the notion that clathrin is responsible for this effect, and it remains to be seen if auxilin has, in addition to the stimulatory effect on the ATPase of hsc70, an effect on the rate of phosphate release. Alternatively, it is possible that coated vesicle preparation contains exchange factors that stimulate the release of P_i and ADP. For the interaction between DnaK and DnaJ, it has been suggested that only DnaK_{ADP} generated directly by ATP hydrolysis (rather than from incubation of DnaK with ADP) can bind to DnaJ (44). This observation, taken together with the features of the interaction between auxilin and hsc70, suggests that the fleeting complex between DnaJ and DnaK might be in principle analogous to the auxilin hsc70_{ADP+P_i} complex, and that both represent a physiologically important activated form of hsc70/DnaK poised to engage substrate molecules. The rate of the spontaneous dissociation of auxilin or DnaJ, respectively, might govern the lifetime of the activated state of hsc70/DnaK. This feature may serve as a safeguard against “nonspecific action” of an activated hsc70/DnaK. It may be surmised that activated hsc70/DnaK can engage substrates only within a narrow time frame. The probability of such an event will be low unless the hsc70/DnaK is brought into the proximity of the substrate by a DnaJ-like protein.

Very recently, Eisenberg and co-workers (22) reported that the yeast DnaJ homologue Ydj1p induces polymerization of hsc70 in the presence of ATP. However, this interaction appears to be quite different from that of auxilin with hsc70, since the Ydj1p-induced hsc70 polymer contained only negligible amounts of Ydj1p and it sedimented with 100,000 g. By contrast, auxilin(547–910) and hsc70 form an equimolar complex, as we have shown, that does not sediment in the same conditions (data not shown).

Different members of the hsp70 protein family are known to differ in their specificity for DnaJ homologues (33, 38), in their affinity for nucleotides (45), and possibly also in their affinity for substrates. DnaJ-like proteins are likely to be even more diverse in structure because they not only bind to selected members of the hsp70 protein family, but also need to target them to many different substrates. Divergence in the J domain is likely to determine the type of hsp70 with which the DnaJ-homologue interacts. For example, we have observed that while auxilin's J domain can still stimulate the ATPase of DnaK (Fig. 10), it appears nevertheless to bind with lower affinity to DnaK (Fig. 7). Consistent with this finding are our preliminary observations that indicate that DnaK only functions poorly in the dissociation of clathrin baskets (data not shown). While a low affinity between “noncognate” chaperone pairs might be overcome in biochemical assays by simply increasing the protein concentrations, they might, however, be insufficient to drive reactions in the cell. It was recently shown that Kar2p, a yeast hsp70 found in the lumen of the ER, recognizes not only Sec63p but also the closely related J domain of the luminal ER protein Scj1p (38). However, Kar2p failed to interact productively with the J domains of the two cytosolic DnaJ homologues, Sis1p and Mdj1p. On the other hand, the cytosolic hsp70 homologues SSA1 and SSA2 function well in the uncoating of clathrin-coated vesicles from bovine brain, while the closely related yeast isoforms SSB1 and SSB2 do not (15).

This implies that bovine brain auxilin interacts with SSA1/2 but not with the other isoforms. SSA1 has in common with a number of cytosolic hsp70 family members, including bovine brain hsc70, the carboxyl-terminal motif, EEVD. It was recently shown that the deletion or alteration of this motif impairs binding of the cytosolic DnaJ homologue Hdj1 (13). The absence of the carboxyl-terminal motif from SSB1 probably explains why it does not uncoat clathrin-coated vesicles. The identity between the J domain of Ydj1p, a natural partner of SSA1 (8), and the J domain of auxilin is only 32% (with 55% homology), but despite the considerable sequence differences, both J domains evidently interact with the same hsp70 isoform, namely SSA1.

Why is there a need for so many different isoforms of hsp70? The probable and perhaps trivial reason is that some isoforms are better adapted to perform certain cellular functions than others. The controlled dissociation of a macromolecular complex may demand properties different from those of hsp70 isoforms that are active in protein translocation across membranes or in protecting proteins against irreversible denaturation. This conjecture could be experimentally addressed by exchanging auxilin's J domain for that of DnaJ or for that of Sec63p. The clathrin basket dissociation assay could then be used to quantitatively test the suitability of DnaK or Bip to function as an uncoating enzyme.

Another important question concerns the regulation of the uncoating of coated vesicles. Clearly the uncoating reaction must not interfere with the assembly of coated pits. It is conceivable that either the interaction of auxilin with clathrin or the binding of hsc70 to auxilin's J domain is subject to tight regulation. Posttranslational modifications such as phosphorylation could be involved, since auxilin is known to be heavily phosphorylated by coat-associated kinases *in vitro* (28). However, we do not yet know whether this modification inhibits cofactor function.

Thus far, auxilin has been identified and characterized only in neuronal tissue. Western and Northern blotting experiments failed to detect the protein or its RNA in non-neuronal tissue (36), but given the ubiquity of the uncoating reaction, it must be inferred that auxilin homologues are active in the uncoating of clathrin-coated vesicles in non-neuronal tissue also. It is therefore not surprising that the expressed sequence tag database yielded a number of cDNA clones from nonneuronal tissue (e.g., NCBI: 263616, human placenta; or NCBI: 128990, human liver), which at the amino acid level show ~70% identity with the carboxyl-terminal segment of auxilin. This score can be further improved if the expressed sequence tag sequences are corrected for frequent sequencing errors by comparing all available sequences from one tissue and species and by eliminating obvious frame shift errors.

The cofactor function of auxilin requires only ~40% of its sequence. We do not yet know the function of the amino-terminal 60% of the molecule. The most notable feature of this part of its sequence is a homology to the actin binding protein tensin (36), which is located in focal contacts. However, we still have no experimental evidence to suggest that auxilin interacts with actin. On the other hand, it is tempting to speculate that hsc70 is involved in the dynamics of focal contacts. Thus, the function of the

amino-terminal half of auxilin might be to target hsc70 to a substrate unrelated to clathrin.

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