The removal of the clathrin coat is essential for vesicle fusion with acceptor membranes. Disassembly of the coat involves hsc70, which is specifically recruited by members of the auxilin protein family to clathrin lattices. In vitro, this function of auxilin does not require the globular amino-terminal domain of the clathrin heavy chain, which is known to play a prominent role in the interaction of clathrin with adaptors and numerous endocytic accessory proteins. Here we report the unexpected finding that the neuron-specific form of auxilin (auxilin 1) can also associate with the clathrin amino-terminal domain. This interaction is mediated through tandemly arranged sites within the auxilin 1 carboxyl-terminal segment 547–910. The overlapping auxilin 1 fragments 547–714 and 619–738 bind the clathrin terminal domain with high affinity, whereas auxilin 1-(715–901) interacts only poorly with it. All three fragments also associate with the clathrin distal domain and the α-appendage domain of AP-2. Moreover, they support efficient assembly of clathrin triskelia into regular cages. A novel uncoating assay was developed to demonstrate that auxilin 1-(715–901) functions efficiently as a cofactor for hsc70 in the uncoating of clathrin-coated vesicles. The multiple protein-protein interactions of auxilin 1 suggest that its function in endocytic trafficking may be more complex than previously anticipated.

Vesicular membrane carriers facilitate the transport of lipids and proteins between membrane-bound organelles (1). They form upon recruitment of soluble cytosolic coat proteins onto the surface of donor membranes where they collect cargo, the components for identifying acceptor membranes and those of the fusion apparatus (1). Moreover, they provide mechanical support for vesicle budding and their release into the cytosol. Clathrin-coated vesicles are the most extensively studied class of transport vesicles. They are involved in receptor-mediated endocytosis and sorting of lysosomal proteins. The dissociation of the clathrin coat is not only a prerequisite for fusion with acceptor membranes, but may also be required for the interaction of the vesicle with microtubule motor proteins (2). Uncoating involves the chaperone hsc70 and, depending on the cell type, either the DnaJ cofactors auxilin 1 or auxilin 2/GAK (cyclin G-associated protein kinase) (3–6). Whereas auxilin 2 is expressed ubiquitously (7), auxilin 1 is found only in neuronal cells (8). Orthologs of auxilin have been described in yeast and nematodes (9–12). A carboxyl-terminal segment of 360 residues, present in both auxilins, binds to assembled clathrin, to the α-appendage domain of the coated vesicle adaptor AP-2, and to hsc70 (3, 4, 13). Hsc70 in its “ATP-bound conformation” interacts with the auxilin J domain, a highly conserved motif common to all members of the DNAJ protein family (14). Auxilin-stimulated hydrolysis of bound ATP converts hsc70 to the ADP conformation, which is characterized by a tight association with unfolded proteins (15). Thus the function of the auxilins is to target hsc70 with bound ATP to clathrin cages and to couple ATP hydrolysis with clathrin-hsc70 complex formation (3). Clathrin heavy chain trimers, also known as clathrin triskelia, are released from the coat structure together with three tightly bound hsc70 molecules (16–18). During this process auxilin 1 is also released from clathrin (3).

Auxilin 2 differs from auxilin 1 in two important aspects: first, auxilin 2 recognizes not only the plasma membrane adaptor AP-2 but also the Golgi-adaptor protein AP-1 (4), and second it contains an amino-terminal Ser/Thr-kinase domain (7). This domain shares sequence homology with members of the Ark kinase family (19). Ark2p is associated with Sla2p, an actin-binding protein, which is also present in clathrin-coated vesicles (20). The in vivo function of hsc70 in the uncoating process was first addressed by injecting a monoclonal antibody directed against this protein into living cells (21). The antibodies interfered with endocytosis and increased the number of membrane-associated clathrin structures. More recently this observation was extended by overexpressing dominant negative hsc70 mutants, which also inhibit receptor-mediated endocytosis and interfere with other aspects of clathrin dynamics (22). The importance of functional auxilin orthologs in uncoating was recently demonstrated for yeast and nematodes (9, 10, 12). A regulatory role for phosphoinositides in uncoating is suggested from the analysis of mice that lack the phosphoinositide phosphatase synaptojanin and from synaptojanin mutants in Caenorhabditis elegans (23, 24). In both instances defects in uncoating were observed. In this context it may be of significance that both auxilins contain a PTEN-like domain, which might attach these proteins to membranes enriched in phosphoinositides and regulate their activity (25). Thus, there is now a wealth of evidence implicating the auxilins and hsc70 in the uncoating of clathrin-coated vesicles in vivo. However, little is known about the molecular events that lead to auxilin-mediated placement of hsc70 on the clathrin heavy chain, which causes disruption of clathrin-clathrin interactions and clathrin-adaptor associations (26).

The clathrin heavy chain consists of a proximal leg segment, which is closest to the vertex where the three heavy chains join, and a distal leg segment linked by a flexible linker to a com-
pactly folded amino-terminal domain (TD). The clathrin TD is not required for the self-assembly of clathrin, but is essential for its interaction with peripheral and integral membrane proteins that control clathrin assembly on membrane surfaces. For *in vitro* uncoating the clathrin TD was shown to be dispensable as were the clathrin light chains that bind along the proximal leg segment (5).

Our unexpected finding that the clathrin TD associates with auxilin 1 prompted this study. Here we show that the auxilin 1 segment 547–910 contains at least two distinct binding sites for each, the TD and α-appendage. An AP180 fragment, which lacks the ENTH-domain (epsin N-terminal homology domain), competes efficiently with auxilin 1 for binding to the TD. A second auxilin 1 binding site on the clathrin heavy chain is located on the distal domain.

### MATERIALS AND METHODS

#### DNA Manipulations and Expression of Recombinant Protein Fragments

Full-length bovine auxilin 1 cDNA in pGEX 4T-1 (Amersham Pharmacia Biotech) was used for constructing recombinant auxilin 1 fragments. To generate the cDNA for auxilin 1 (amino acid residues 715–901) the NcoI and ApoI-cleaved fragment was ligated into pET32a between the NcoI and EcoRI restriction sites.

The auxilin 1 fragment (619–738) was first subcloned into the SphI site of pUC57 (Qiagen, Hilden, Germany). Next the insert was excised from pUC57 with Sall and HindIII and inserted into the expression vector pET32a (Novagen) that was linearized with the same enzymes. Polyhistidine-tagged auxilin 1 fragments were expressed in *Escherichia coli* BL21(DE3)pLysS bacteria (Novagen, Madison, WI). After 3 h of growth at 37 °C the culture was transferred to room temperature and 0.5 mM isopropyl thiolgalactopyranoside (Eurobio, Le Ulis, France) was added to induce expression of recombinant proteins for 3 h. Bacteria were harvested by centrifugation, resuspended, and lysed by sonication as described previously (3). 1% Triton X-100 (Sigma) and Complete Protease Inhibitor mixture (Roche Molecular Biochemicals) were included in the lysis buffer. Recombinant proteins were purified by affinity chromatography on Ni-NTA agarose (Qiagen) according to the manufacturer’s recommendations. Auxilin 1 fragments were further purified by size exclusion chromatography on a Superdex 200HR 10/30 FPLC-system (Amersham Pharmacia Biotech). The column was equilibrated in 25 mM HEPES, 12.5 mM potassium acetate and 5 mM magnesium acetate, pH 7.1 (buffer G).

The cDNA of human clathrin heavy chain in pBluescript (KIAA 0034) was a gift from T. Nagase, Kazusa DNA Research Institute, Kisarazu, Japan. Clathrin proximal domain (clathrin-PD); amino acid residues 1073–1675) was generated by cleavage with MspI while the distal domain of clathrin (clathrin-DD); amino acid residues 438–1073) was excised with StspI and BspMI. The clathrin fragment coding for the proximal domain and about half of the distal domain (clathrin-D*PD); amino acid residues 730–1675) was obtained using the restriction enzyme DvaI.

All clathrin constructs were ligated into the pET32a vector and expressed with an amino-terminal polyhistidine tag in BL21(DE3)pLysS as described above. Only the bacteria used for expressing the clathrin DD were grown for 2.5 h at 37 °C and induced with 0.5 mM isopropyl thio galactopyranoside at room temperature for 4.5 h. The protocol for the purification of recombinant clathrin fragments was identical to that for the recombinant auxilin 1 fragments. The AP180 fragment His6-AP180-(328–901) was a gift from T. Nagase, Kazusa DNA Research Institute, Kisarazu, Japan. Clathrin heavy chain in pBluescript (KIAA 0034) was a gift from T. Nagase, Kazusa DNA Research Institute, Kisarazu, Japan. Clathrin heavy chain in pBluescript (KIAA 0034) was a gift from T. Nagase, Kazusa DNA Research Institute, Kisarazu, Japan.

#### Protein Binding Studies

**Protein Binding Studies**—The molar concentration of clathrin used in the following experiments refer to the heavy chain and light chain of one triskelia leg.

**Cage Binding**—1.3 × 10^17 mol clathrin assembled into cages by dialysis against buffer A (100 mM 2-(N-morpholino)ethanesulfonic acid, 1 mM EDTA, 0.5 mM MgCl₂ and 2 mM CaCl₂). Clathrin was incubated on ice for 1 h with 1.9 × 10^15 mol of either clathrin 1-(547–714), auxilin 1-(715–901) or GST-auxilin 1(547–910) in a total volume of 100 μl and then centrifuged at 90,000 × g for 20 min in a TLA-10 rotor (Beckman Optima TL-100). Resulting supernatant and pellet fractions were analyzed by SDS-PAGE. In this and all subsequent experiments the pellets were resuspended in a final volume that was identical to that of the supernatants.

**Limited Digestion of Clathrin Cages**—To remove the TD from about two-thirds of the clathrin heavy chains, cages (6.8 × 10^10 mol of clathrin in 300 μl) were digested with 0.21 mg/ml trypsin (Worthington, Freehold, NJ) for 10 min on ice in buffer A containing 1.6 mM CaCl₂. The reaction was terminated with 74 μg of soybean trypsin inhibitor (Worthington). To quantitatively remove the TD from all heavy chains, 1.4 × 10^10 mol assembled clathrin in 600 μl of buffer A plus 1.6 mM CaCl₂ were preincubated with 0.2 mg/ml trypsin for 10 min on ice and then further digested for 50 min at 10 °C with a total amount of 0.4 mg/ml trypsin in a volume of 760 μl. The reaction was stopped with 378 μg of soybean inhibitor. Trypsin-treated cages were pelleted by ultracentrifugation at 90,000 × g for 30 min. The clathrin pellets were washed in 1 ml of buffer A containing 25 or 126 μg of soybean inhibitor depending on the stringency of the digestion. After a second centrifugation the pellets were resuspended in 80 μl of buffer G with 20 μg of soybean inhibitor (40 μg of inhibitor when high concentrations of trypsin were used).

**Binding of Auxilin 1 Fragments to Trypsin-clipped Clathrin Cages**—Trypsin-digested cages containing 9 × 10^10 mol of clathrin were incubated in 40 μl of buffer G for 30 min on ice with either 1.7 × 10^10 mol of auxilin 1-(547–714), 1.8 × 10^10 mol auxilin 1-(619–738) or 1.9 × 10^10 mol auxilin 1-(715–910). 3.5 × 10^10 mol of GST-auxilin 1-(547–910) were incubated together with equimolar amounts of trypsin-clipped cages and His6-AP180 (50) in a reaction volume of 100 μl. The cages were separated from unbound protein ligands by ultracentrifugation at 90,000 × g for 30 min. Supernatants and resuspended pellets were analyzed by SDS-PAGE.

**Clathrin Assembly Experiments**—Purified clathrin was preincubated in buffer G for 10 min on ice and centrifuged at 90,000 × g for 30 min to remove aggregates. The supernatant containing 1.4 × 10^10 mol of clathrin was incubated on ice with 2.8 × 10^10 mol of either auxilin 1-(547–714), auxilin 1-(715–910), auxilin 1-(619–738), or GST-auxilin 1(547–910) for 1 h in a volume of 100 μl. The reaction mixture was divided into two aliquots and centrifuged at 90,000 × g for 30 min. One aliquot was analyzed by SDS-PAGE while the other one was diluted to 100 μg/ml, negatively stained with uranylacetate, and used for electron microscopy (3).

**Selective Proteolysis of Clathrin Coat-associated Auxilin 1**—500 μg of clathrin-coated vesicles from pig brain in 250 μl of buffer A were digested with 0.3–1.5 μg of trypsin as detailed in Fig. 8. Addition of 0.36–3.6 μg of soybean trypsin-inhibitor terminated the reaction. The cages were pelleted by ultracentrifugation and resuspended in 20 μl of buffer G containing 0.1 μg of the inhibitor.

**Coat Dissociation Experiments**—To test the cofactor function of recombinant auxilin 1 fragments 250 μg of trypsin-treated pig brain-coated vesicles were incubated with 6.9 × 10^11 μl of hec70 (5) and 6.4 × 10^11 μl of either GST-auxilin 1(547–910) or the His6-tagged auxilin 1 fragments for 20 min at 25 °C in uncoating buffer C (20 mM HEPES, 25 mM KCl, 10 mM ammonium sulfate, 2 mM MgCl₂, pH 7.0). hec70 was preincubated on ice for 15 min with regeneration mixture containing 2 mM ATP, 5 mM creatine phosphate, 1 mM dithiothreitol and 5 units/ml creatine kinase as final concentrations. Assayed clathrin and free triskelia were separated by centrifugation at 90,000 × g for 20 min. Supernatants and pellets were analyzed by SDS-PAGE.

**Binding of Purified Auxilin 1 Fragments to GST-a-Appendage and GST-Clathrin-TD**—GST-a-Appendage and GST-TD fusion proteins immobilized on 10 μl of GSH-Sepharose beads (Amersham Pharmacia Biotech) with ~25 μg of bound fusion protein were diluted with 10 μl of Sepharose CL-4B (Amersham Pharmacia Biotech). Binding experiments were carried out in 200 μl of buffer C (5 × 10^14 mol of the auxilin 1 fragments (547–714, 619–738 and 715–910) or thrombin-digested GST-auxilin 1(547–910). 2.4 × 10^10 mol of thrombin-digested GST-auxilin 1(547–910) were incubated with 20 μl of GST-TD beads in a reaction volume of 100 μl. 0.1% Triton X-100 and 0.5 mg/ml of highly purified bovine serum albumin (Roche Molecular Biochemicals) were added to prevent nonspecific interactions. The
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Beets were pelleted by centrifugation for 1 min at 11,000 rpm in a A–8–11 swinging bucket rotor (Eppendorf, Hamburg, Germany) and washed twice with 500 μl of buffer G. For the first wash the resuspended beeds were underlayed with 60 μl of 10% sucrose in the same buffer. SDS-PAGE and immunoblotting were performed with the supernatant and bead fractions.

For immunoblot analysis 2.5 μg/ml mAb100/4 directed against auxilin 1 (8) and the anti-penta-His antibody (0.2 μg/ml, Qiagen) were used. When rat brain cytosol was used in pull-down experiments auxilin 1 was stained with the affinity-purified polyclonal rabbit antibody R5C3, which was developed in our laboratory. Antibody binding was detected with goat anti-rabbit IgG conjugated to horseradish peroxidase or goat anti-rabbit IgG (Cappel) using Renaissance Enhanced Luminol substrate (PerkinElmer Life Sciences, Zaventem, Belgium).

**Binding of Cytosolic Auxilin 1 to GST-TD and GST-α-Appendage—** Fresh porcine brains were obtained from a local abattoir. The tissue was washed in ice-cold phosphate buffered saline and then homogenized in 1 ml of buffer G containing 0.1% Triton X-100 using a PD-10 desalting column (Amersham Pharmacia Biotech) that was equilibrated in buffer G. The protein concentration in the cytosol varied between 10–15 mg/ml. Pull-down experiments with immobilized GST-TD were performed as described. Unfractionated bacterial lysate containing full-length recombinant His6 auxilin 1 (5) was transferred into phosphate buffered saline and 0.5 mg/ml bovine serum albumin. 1.5 × 10−10 mol clathrin-D*PD or 2 × 10−10 mol clathrin-PD were preincubated on ice for 10 min without any beads. After a short prespin in a benchtop centrifuge the supernatants of the recombinant proteins were added to the immobilized fusion proteins and incubated for 30 min on ice. Binding was analyzed as described above.

**RESULTS**

**Binding of Auxilin 1 to the TD of the Clathrin Heavy Chain—** A GST fusion protein containing the clathrin amino-terminal domain (GST-TD) attached to GSH-Sepharose beads was used to pull down TD-binding proteins from pig brain cytosol. Among the proteins that associated specifically with the clathrin TD was auxilin 1 (Fig. 1). This result was unexpected because the clathrin TD is not required for in vitro uncoating of clathrin coats (5). To rule out the possibility of an indirect interaction between auxilin 1 and the clathrin TD through other cytosolic proteins, we tested and confirmed the ability of the recombinant clathrin TD to bind recombinant auxilin 1 directly from bacterial lysates (Fig. 1). Next we expressed the carboxyl-terminal segment GST-auxilin 1-(547–910), which was previously shown to be fully functional in an in vitro uncoating assay (3), and removed its GST moiety with thrombin. The resulting auxilin 1-(547–910) associated in pull-down experiments with the immobilized TD. Among the proteins known to interact with the clathrin TD is the neuronal assembly protein AP180 (29). To determine whether AP180 and auxilin 1 bind to the same or overlapping sites on the TD, we expressed a polyhistidine-tagged recombinant fragment of AP180 (segment 328–896) that was previously shown to bind the clathrin TD (29). First we confirmed its interaction with the TD and then demonstrated that GST-auxilin 1-(547–910), when added in excess, blocked the interaction of the recombinant AP180 fragment with the clathrin TD almost completely (Fig. 2). This effect was not due to the displacement of GST-TD by GST-auxilin 1-(547–910) from the GSH-beads, because the amount of GST-TD in the pellets remained constant (data not shown). Thus we conclude that auxilin 1 and AP180 share overlapping binding sites on the clathrin TD. Recombinant auxilin 1-(547–813), which lacks the J-domain was also tested and shown to bind with similar efficiency to the TD as auxilin 1-(547–910) (data not shown here, but see also Fig. 8). Taken together we have demonstrated that auxilin 1 is capable of associating directly with the globular clathrin TD and that it competes with AP180 for binding.

**Interaction of Auxilin 1 Fragments with Trypsin-clipped Clathrin Coats—** Auxilin 1 was previously demonstrated to bind and to assist hsc70 in the uncoating of trypsin-clipped clathrin cages that lack the TD (5). This suggests that auxilin 1 recognizes at least two distinct sites on clathrin: one on the TD, as shown above, and another somewhere else on the heavy chain. This could clearly be shown by incubating AP180 together with auxilin 1-(547–813) with intact cages and with cages that had been treated with increasing trypsin to remove the TD either partially or completely. Auxilin 1-(547–813) could be used for this experiment, because we knew from previous work that it binds trypsin-clipped clathrin cages (3). In contrast AP180 is known to require an intact heavy chain for binding (30). The different binding characteristics of the two proteins are clearly demonstrated here: while auxilin 1-(547–813) binds to cages, irrespective of the presence of the TD, binding of AP180 declines dramatically upon its removal (compare lane P3 with the control lane P4 in Fig. 3).

**Tandemly Arrayed Clathrin and AP-2 Binding Sites on Auxilin 1—** To localize the clathrin binding elements within auxilin 1 more precisely, we expressed the segments 547–714, 619–738, and 715–901 with a polyhistidine tag in bacteria (Fig. 4). The recombinant proteins were purified on a Ni-NTA affinity resin and then subjected to size exclusion chromatography. All fragments interacted with GST-TD and GST-α-appendage in...
Role of Auxilin 1 Fragments in Clathrin Assembly and Disassembly—Auxilin was originally described as a clathrin assembly protein because it supports the polymerization of soluble triskelia into regular coat structures (8). To determine whether any of our new auxilin 1 fragments would also support assembly of soluble clathrin into regular cages, we incubated them with triskelia under conditions that ensure efficient cage formation is strictly dependent on assembly promoting factors. The extent of assembly was analyzed by ultracentrifugation followed by SDS-PAGE of pellet and supernatant fractions and by electron microscopy. We observed that all three fragments supported significant assembly of clathrin into cages, although in this respect auxilin 1-(619–738) appeared to be the least efficient fragment (Fig. 6). Cages that were assembled by auxilin 1-(619–738) and by auxilin 1-(547–714) tended to clump together, while auxilin 1-(715–901)-induced cages were smaller and less heterogeneous in size, reminiscent of those that are assembled by AP180 (Fig. 6).

Binding of Auxilin 1 Fragments to Trypsin-clipped Cages—To determine which of the auxilin 1 fragments might bind exclusively to the clathrin TD, we conducted binding experiments with trypsin-clipped cages that lack this domain. While the association of auxilin 1-(715–901) and auxilin 1-(619–738) was hardly affected by the removal of the TD, binding of auxilin 1-(547–714) was slightly reduced, suggesting that its association with the clathrin TD increases its overall affinity for cages (Fig. 7).

Auxilin 1 Binding Sites on Heavy Chains—Next we set out to identify the heavy chain segments that mediate auxilin 1 binding to cages that lack the TD. To this end we constructed and expressed three recombinant clathrin fragments in bacteria. The first corresponds to the proximal leg domain including the trimerization region (1072–1675), here referred to as clathrin-PD; the second slightly longer fragment extended into the distal domain and is referred to as PD* (730–1675). The third fragment corresponded to the distal leg (clathrin-DD; segment 438–1073). All three fragments contained a polyhistidine tag. Purified intact triskelia were readily pulled down by GST-auxilin 1-(547–910) beads (Fig. 8), whereas GST-beads, used here as a control, and GST-auxilin 1-(813–910) beads failed to do so. The latter result confirmed our previous findings that the J-domain-containing auxilin 1 segment 814–910 does not associate with clathrin (3). The clathrin proximal leg segment (clathrin-PD) interacted only poorly with the auxilin 1 fragment. However, significant amounts of the clathrin-DD were pulled down with the immobilized auxilin 1 fragment. Similarly, the fragment that contained both, the proximal and most of the distal leg segment (clathrin PD*JD), associated with the immobilized auxilin 1 (547–910) (Fig. 8).

Uncoating Activity of Auxilin1 Fragments—Finally we asked whether auxilin 1-(715–901) would be able to uncoat clathrin-coated vesicles. Auxilin 1 is a component of coated vesicle purified from bovine and pig brain (8). Therefore adding hsc70 and ATP to clathrin-coated vesicles is already sufficient to induce their uncoating. To assess the contribution of exogenously added auxilin to the uncoating reaction, the endogenous auxilin has to be removed or at least to be inactivated. To achieve this we took advantage of the high protease sensitivity of auxilin (8) and searched for digestion conditions that would inactivate the cofactor function of auxilin without degrading clathrin and adaptors. We observed that already very low amounts of trypsin proved sufficient to abolish auxilin immunoreactivity (Fig. 9). The disappearance of auxilin from immunoblots corresponded to reduced susceptibility of the coated vesicles toward uncoating by hsc70. Addition of recombinant GST-auxilin 1-(547–910) restored uncoating (Fig. 9). Under the same digestion conditions neither the clathrin heavy chain nor adaptors or clathrin light chains were significantly affected by the protease (Fig. 9).

Using such trypsin-treated coated vesicles as substrates, we confirmed the cofactor activity of auxilin 1-(547–714) and auxilin 1-(715–901) with that of auxilin 1-(547–910). As expected auxilin 1-(547–714), which lacks the J-domain, was unable to support uncoating, whereas auxilin 1-(715–901) was fully functional (Fig. 9). Taken together our data suggest that for in vitro uncoating of clathrin-coated vesicles the J-domain plus the clathrin binding sites that are present within segment 715–813 are sufficient.

**DISCUSSION**

This study shows that the interaction between auxilin 1 and clathrin is mediated on both proteins by multiple independent
contact sites. On the clathrin heavy chain they involve the distal leg segment and the globular TD. The latter finding was unexpected because we had previously shown that auxilin 1 binds with high affinity to clathrin cages from which the TD had been removed with trypsin. Moreover, these cages were efficiently dissociated by hsc70 in a reaction that strictly depended on the presence of auxilin 1. Thus these earlier results clearly suggested that the cofactor function of auxilin does not require the clathrin TD. Apart from auxilin 1 numerous ligands such as the β-subunit of adaptors, AP180, the epsins, amphiphysin, non-visual arrestins, the LDL receptor, and as shown most recently the tyrosine kinase ACK1 associate with clathrin through the TD (29, 31–35). In general these interactions are of low affinity and are often difficult to detect unless

**Fig. 4.** Auxilin 1 fragments characterized in this study. A, location of the segments within the auxilin 1 sequence. Dark shaded segments correspond to the J-domain. B, the purified fusion proteins used in this study shown after SDS-PAGE and staining with Coomassie Blue. Auxilin 1-(547–714), auxilin 1-(619–738), and auxilin 1-(715–901) were expressed with an amino-terminal polyhistidine tag, while auxilin 1-(547–910) and auxilin 1-(813–910) were fused to the carboxy-terminal end of GST (3).

**Fig. 5.** The auxilin 1 fragments encompassing the segments 547–714, 715–901, and 619–738, respectively, interact with the clathrin TD and the α-appendage domain of the AP-2 adaptor complex. GST-fusion proteins immobilized on GSH-Sepharose were incubated with various purified recombinant auxilin 1 fragments in buffer G for 30–60 min. The concentration of the recombinant fragments was 3.5 μM. The beads were washed and recovered by low speed centrifugation and then analyzed by SDS-PAGE and immunoblotting. For competition experiments with the α-appendage and auxilin 1-(619–738) a constant amount of the auxilin 1 fragment was added together with increasing concentrations of α-appendage to GST-TD-Sepharose beads (E). The His6-tagged proteins were labeled with a monoclonal antibody directed against the His tag (A, B, and D) and the GST-free auxilin 1-(547–910) (C) was detected with mAb100/4 (8). Note that auxilin 1-(715–901) appears to have the lowest affinity for the TD and for the α-appendage domain of AP-2.

**Fig. 6.** Auxilin 1 fragments with clathrin assembly promoting activity. 1.4 × 10^10 mol of clathrin was incubated on ice in buffer G for 1 h in a final volume of 100 μl with 2.8 × 10^10 mol of either His6-auxilin 1-(547–714), His6-auxilin 1-(715–901), His6-auxilin 1-(619–738), or GST-auxilin 1-(547–910) or without any additions (control). Assembled clathrin was separated from free triskelia by ultracentrifugation. Aliquots of supernatant and resuspended pellets were subjected to SDS-PAGE. Proteins were stained with Coomassie Blue. Negatively stained images of cages assembled by His6-auxilin 1-(547–714), by His6-auxilin 1-(715–901), and by His6-auxilin 1-(619–738), respectively, are shown in B. Note that the cages assembled by segment 715–901 appear smaller.
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FIG. 7. Binding of auxilin 1 fragments to clathrin cages lacking the TD. Cages were digested with increasing amounts of trypsin to remove the TD of the clathrin heavy chain. All tested auxilin 1 fragments are shown to interact with trypsin-clipped clathrin. A, His<sub><i>a</i></sub>-auxilin 1-(547–714). B, His<sub><i>a</i></sub>-auxilin 1-(715–901). C, His<sub><i>a</i></sub>-auxilin 1-(619–738). Note that only His<sub><i>a</i></sub>-auxilin 1-(547–714) appears to bind with reduced affinity to clathrin that lacks the TD. Protein bands were stained with Coomassie Blue.

The avidity of the interaction is increased. This is the case when the three TDs of a triskelion bind to ligands that are immobilized on a surface such as a Sepharose matrix or a membrane. When triskelia assemble into a clathrin cage three terminal domains from different triskelia congregate underneath each vertex. They are even close enough to become connected by a single extended polypeptide chain with multiple clathrin binding motifs (36). It was recently suggested that AP180 might function as an assembly protein by bundling the TDs of three triskelia which then nucleate the assembly of an extended lattice (29).

The TD folds into a seven-bladed β-propeller (37). The groove between blade 1 and 2 associates with proteins bearing a clathrin box motif that is characterized by the sequence L<sub>Φ</sub>ADV (Φ representing a bulky hydrophobic residue and X any polar residue) (38). More recently additional sequence motifs were described that do not fit the clathrin box motif but clearly interact with the TD in pull-down experiments. Examples are the peptide motifs LMDLADV found in epsin 1 (33) and LPWDLWTT and IPWDLWEP, found in amphiphsin I and II (39). The TD-binding characteristics of the peptide LPWDLWTT was analyzed in depth, and it was concluded that for optimal clathrin binding a central acidic residue should be preceded in position −1 by a Trp residue and followed in position +2 also by a Trp residue (40). Because the structural features of these clathrin binding motifs differ from that of the conventional clathrin box motif L<sub>Φ</sub>ADV it is referred to as type II clathrin binding sequence. The epsin 1 sequence LMDLADV, which has a low affinity for the clathrin TD, is considered a variant of the type II motif. The structural differences between type I and type II sequences suggest the existence of a second protein ligand binding site on the TD, which might be constituted by the groove between blades 4 and 5 (38).

A principal TD-binding element resides within the auxilin 1 segment 547–714 and a low affinity site in segment 715–901. A third fragment, auxilin 1-(619–738), which partly overlaps with both of the former, binds almost as efficiently as the fragment 547–714 suggesting that an important TD site might lie between residues 619–738 presumably within segment 619–714. Auxilin 1 lacks a typical clathrin box type I motif, but contains sequences which are related to the type II motif. These are L<sub>625</sub>AWDWHL<sub>639</sub> and possibly L<sub>677</sub>TDLPF<sub>676</sub>, respectively. Our observation that auxilin 1 and AP180 compete for binding to the clathrin TD suggests that they might either bind to the same or to overlapping sites on the TD. The sequence motif DLL/F, which occurs 10 times in AP180, was recently proposed to mediate AP180 binding to the clathrin TD (29). This tripeptide is also found four times in auxilin 1, twice within segment 547–910. However, we do not think that this motif is important in auxilin binding to the TD because segment 619–738, which is readily pulled down by the TD, lacks it while fragment 715–901, which associates only poorly with the TD, contains one.

Exposed DPF motifs are known to be essential for the interaction of proteins with the α-appendage domain of AP-2 (13, 41, 42). This tripeptide occurs three times in auxilin 1. Auxilin 1-(547–714) has two that start with Asp<sub>657</sub> and Asp<sub>660</sub>, respectively, and auxilin 1-(619–738) has one (position 674). This explains their association with the α-appendage domain. The type II TD binding motif PWDLW of amphiphsin II also associates with cytosolic AP-1 and AP-2. A direct interaction with the α- and β<sub>2</sub>-appendage domains of AP-2 was demonstrated.
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FIG. 9. Uncoating of trypsin-treated clathrin-coated vesicles with hsc70 and recombinant auxilin 1 fragments. Clathrin-coated vesicles from pig brain were mildly digested with trypsin to remove endogenous auxilin 1. A, immunoblots of trypsin-treated coated vesicles show that immunoreactivity of auxilin 1 with mAb 100/4 was already lost upon digestion with 0.3 μg of trypsin. Neither the clathrin heavy chain nor the light chains or the β3-adaplin were significantly affected even under the harshest digestion conditions employed. Uncoating of clathrin-coated vesicles. Untreated or trypsin-treated coated vesicles were incubated in the presence of hsc70, ATP-regenerating mixture, and, where indicated, with recombinant auxilin 1-(547–910) and then pelleted by ultracentrifugation to separate released clathrin from the membrane fraction. Aliquots of supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE, and protein was stained with Coomassie Blue. C, uncoating of trypsin-treated clathrin-coated vesicles with the recombinant auxilin 1 fragments (547–714). The assay was performed as described in B. Additions of auxilin 1 fragments are indicated on top of the gel. Note that only the two J-domain-containing fragments 547–910 and 715–901 support the release of clathrin.

(40). The competition between the TD and the α-appendage for binding to the auxilin 1 fragment 619–738 suggests that the type II-related peptide motif \textit{K}E\textit{T}LDP\textit{K} might be directly involved in binding both the TD of clathrin and the α-appendage domain of AP-2.

Like auxilin 1-(547–910) the fragments 619–738 and 715–901 also interact with cages that lack the TD. In contrast, the principal binding site of auxilin 1-(547–714) on clathrin is probably the TD because binding of this fragment is reduced by the removal of the TD. The observation that all fragments retain clathrin-assembly promoting activities suggest that they are capable through perhaps multiple binding sites to cross-link triskelia and thereby stabilize the assembled state. This might either work through tandemly arranged sites along a single polypeptide chain or through self-association of fragments that possess only one binding site.

So far the only known function of auxilin 1 is its role in the uncoating reaction. For this function the small segment 715–901, which binds preferentially to the distal clathrin leg, appears to be sufficient. This raises the question why auxilin 1 binds also the TD and the α-appendage domain. One possible explanation is that multiple low affinity interactions might allow the auxilins to discriminate between free and polymerized triskelia. Likewise an additional low affinity binding site on the α-appendage domain of AP-2 would also serve the purpose of discriminating between free- and coat-associated triskelia. Moreover, redundant auxilin binding sites on the clathrin heavy chain would ensure binding of auxilins even in the presence of potentially competing clathrin binding proteins. Alternatively, auxilins and hsc70 might also be involved in multiple functions that precede and follow clathrin dissociation. It is conceivable that different stages of the internalization process require auxilin 1 and hsc70 at distinct sites on the triskelia or AP-2. For example, it is possible that auxilins might be recruited via their PTEN-like domain to the membrane where they could assist in the assembly of coated pits. By activation of their J-domain hsc70 might be then utilized to alter protein-protein interactions during budding and at a later stage for the uncoating of transport vesicles. The puzzling competition between TD and α-appendage for binding to one of the auxilin 1 segments is suggestive of a scenario in which highly dynamic protein-protein interactions that lie at the very core of the endocytic machinery, are formed hierarchically and sequentially.

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