Molecular and Functional Characterization of Clathrin- and AP-2-binding Determinants within a Disordered Domain of Auxilin*

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Uncoating of clathrin-coated vesicles requires the J-domain protein auxilin for targeting hsc70 to the clathrin coats and for stimulating the hsc70 ATPase activity. This results in the release of hsc70-complexed clathrin triskelia and concomitant dissociation of the coat. To understand the complex role of auxilin in uncoating and clathrin assembly in more detail, we analyzed the molecular organization of its clathrin-binding domain (amino acids 547–813). CD spectroscopy of auxilin fragments revealed that the clathrin-binding domain is almost completely disordered in solution. By systematic mapping using synthetic peptides and by site-directed mutagenesis, we identified short peptide sequences involved in clathrin heavy chain and AP-2 binding and evaluated their significance for the function of auxilin. Some of the binding determinants, including those containing sequences 67DPF and 68WDW, showed dual specificity for both clathrin and AP-2. In contrast, the two DLL motifs within the clathrin-binding domain were exclusively involved in clathrin binding. Surprisingly, they interacted not only with the N-terminal domain of the heavy chain, but also with the distal domain. Moreover, both DLL peptides proved to be essential for clathrin assembly and uncoating. In addition, we found that the motif 720NWQ is required for efficient clathrin assembly activity. Auxilin shares a number of protein-protein interaction motifs with other endocytic proteins, including AP180. We demonstrate that AP180 and auxilin compete for binding to the α-ear domain of AP-2. Like AP180, auxilin also directly interacts with the ear domain of β-adaptin. On the basis of our data, we propose a refined model for the uncoating mechanism of clathrin-coated vesicles.

Clathrin-coated vesicles are involved in numerous membrane transport processes. After their formation, they rapidly shed their protein coats to allow fusion of the vesicle membranes with endosomes and to recycle the coat components. The disassembly of the clathrin coat is mediated by the molecular chaperone hsc70 and requires a cofactor of the DnaJ protein family known as auxilin (1). Auxilin is a neuron-specific protein involved in the removal of the clathrin coat from endocytosed synaptic vesicle membranes and thus in the recycling of synaptic vesicles (1–3), whereas the homolog auxilin-2, also referred to as cyclin G-associated kinase, is more broadly distributed (4, 5). Both proteins contain a C-terminal J-domain preceded by a centrally located clathrin-binding domain and an N-terminal phosphatase- and tensin-like domain with unknown function. In addition to these domains, auxilin-2 also possesses an N-terminal Ark-type kinase domain that has been shown to autophosphorylate auxilin-2 and to phosphorylate the μ-subunit of the adaptor protein complexes AP-1 and AP-2 (4).

According to the current model for the uncoating of clathrin-coated vesicles, it is believed that auxilin first binds to clathrin coats and then recruits hsc70 in an ATP-dependent reaction via its J-domain. The interaction with the J-domain stimulates the ATPase activity of hsc70, thereby stabilizing the binding of hsc70 to clathrin. hsc70 interacts stoichiometrically with the legs of the clathrin triskelia, whereas auxilin functions catalytically in this process. The association of hsc70 with clathrin results in the release of clathrin triskelia by disrupting clathrin-clathrin interactions. Apart from guiding hsc70 to its clathrin substrate, the clathrin-binding domain of auxilin (amino acids 547–813) functions as a clathrin assembly protein in vitro when added in stoichiometric amounts to the heavy chain (2).

Like numerous other endocytic proteins, auxilin directly interacts with both clathrin and AP-2. The clathrin-binding domain contains three DPF sequences. This motif has previously been shown for other endocytic proteins to play a role in the interaction with AP-2 (6). However, auxilin lacks typical clathrin box-binding sequences, which mediate the interaction of a number of endocytic proteins with the N-terminal domain of clathrin (clathrin-TD).1 Two types of clathrin box motifs have been described so far. The clathrin box I motif follows the consensus sequence L(L/I)/D(E/N)XLF/DD/E(7), whereas the clathrin box II motif is defined by the motifs WDWP and LMDLA (8). Instead, two DLL sequences that have previously been implicated in the clathrin assembly function of AP180 (9) are present in the clathrin-binding domain of auxilin. These motifs are tandemly arranged within this region with the putative AP-2-binding DPF sequences. The affinity for the ear domain of α-adaptin seems to be correlated with the number of DP/FW peptides, which often occur in multiple copies in the sequence of many accessory proteins (6). An alternative binding motif for the α-ear domain was recently defined by the consensus sequence FXDXF (10). In the case of AP180 and amphiphasin, the FXDXF motifs seem to contribute to the binding of the α-ear domain more than the DPF sequences (11).

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1 The abbreviations used are: clathrin-TD, clathrin N-terminal domain; Trx, thioredoxin; Aux, auxilin; GST, glutathione S-transferase; MES, 2-(N-morpholino)ethanesulfonic acid.
12). However, this motif is not present in auxilin. The α-ear domain of AP-2 consists of a β-sandwich structure and a C-terminal platform domain that are both engaged in protein-protein interactions. The binding sites of the DPF and FXDXF peptides overlap on the α-ear platform, with the latter motif covering an extended site on this subdomain (13). Despite the low sequence identity, the respective ear domains of the α- and β-subunits of AP-2 display a high structural similarity. A subset of DP(F/W) motif-containing proteins, e.g., AP180, epsin-I and eps15, binds not only to the α-ear domain, but also to the β-ear domain of AP-2 (14).

The clathrin heavy chain is organized into a globular N-terminal domain, rod-like distal and proximal domains, and a trimerization region near the C terminus. The N-terminal domain is connected to the distal domain by a flexible linker and can be cleaved off from assembled clathrin by digestion with trypsin without destabilizing the cages. Because auxilin is able to stimulate the hsc70-dependent dissociation of such truncated clathrin cages, it has been suggested that the interaction of auxilin with the proximal or distal domain of clathrin is sufficient for its cofactor activity (1). However, we previously found that auxilin also interacts with clathrin-TD. The clathrin-binding domain of auxilin (amino acids 547–813) is bipartite and consists of two subdomains comprising either predominantly clathrin N-terminal or distal domain interaction sites (15). Recently, Ma et al. (16) constructed a chimeric protein from a C-terminal fragment of murine AP180 (residues 325–813) and the J-domain of auxilin. With this chimera, they were able to demonstrate that binding of a J-domain protein to the N-terminal domain of the clathrin heavy chain is sufficient to recruit hsc70 and to facilitate the dissociation of clathrin cages to a certain degree.

To characterize the overall structural organization of the clathrin-binding domain, we employed CD spectroscopy, which showed that this segment of auxilin is intrinsically disordered. This finding formed the basis for further dissections of the clathrin-binding domain and for defined mutations within putative protein-protein interaction motifs. We found that the two short sequences 636WDW and 674DPF are utilized for the interaction with the α-ear domain of AP-2 and clathrin-TD. Moreover, the 674DPF peptide can also interact with the clathrin distal domain. The 726NWQ sequence was recognized as important for efficient clathrin assembly by auxilin.

EXPERIMENTAL PROCEDURES

Antibodies—The primary antibodies used in this study were the monoclonal antibody 100/4 (2), which is directed against auxilin, and a monoclonal antibody recognizing the His6 epitope (QIAGEN, Hilden, Germany). Antibody binding was detected with goat anti-mouse IgG conjugated to horseradish peroxidase (ICN, Aurora, OH) using Renaissance Enhanced luminescent substrate (PerkinElmer Life Sciences). Alternatively, we detected thioredoxin (Trx/His6) tags coded by pET32 series vectors (Merck Biosciences, Darmstadt, Germany) with horseradish peroxidase-conjugated protein S (Merck Biosciences).

DNA Constructs—All auxilin constructs used in this study were generated from the DNA of auxilin (Aux)-(547–902) cloned into the pGEX-4T2 vector (17). The DNA fragment of Aux-(547–715) was amplified by PCR and inserted into the BamHI and SacI sites of pQE-31(QIAGEN). Cloning of Aux-(715–902) into the pET32a vector was described previously in (15). However, for this work we transferred the insert DNA from pET32a into pQ920 by recombination with NcoI and Sall and ligation between the BamHI and Sall sites of the pQE-30 vector after blunting the NcoI and BamHI overhangs with Klenow polymerase. The construct of Aux-(715–902) in pET32a was used to create Aux-(715–776) and Aux-(778–902). The latter fragment was excised with PvuII and EcoRI and inserted into the EcoRV and EcoRI sites of pET32a, whereas Aux-(715–776) was obtained by digestion with NcoI and PvuII and ligated between the NcoI and filled-in EcoRI sites of the pET32a vector. To generate Aux-(715–813), the DNA of Aux-(715–902) was cleaved with BamHI and introduced into the pQE-30 vector that was linearized with the same enzyme. Aux-(547–618) was excised from Aux-(547–715) in pET32a using SpI and NcoI and inserted into the NcoI and EcoRV-cleaved pET32a vector. The resulting SpI overhangs were blunted with mung bean nuclelease. The Aux-(619–738) fragment was obtained with SpI, subcloned into pUC57 (MBI Fermentas, St. Leon-Roth, Germany), excised with SalI and HindIII, and inserted into the same restriction enzyme sites of the pQE-31 vector. This plasmid was then digested with SalI and Stul, treated with Klenow polymerase, and religated.

Constructs of auxilin mutants were obtained with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) utilizing Aux-(547–715), Aux-(547–715), Aux-(715–902), and Aux-(619–738) as templates. Mutagenic primers were used to convert the DPF and DLL sequences into APA and ALA, respectively. Finally, the DWFV and 726NWQ, and all other residues were mutated into their respective bases. To generate the DPF peptide 674DPFADLG, the oligonucleotides 5′-GATCCGGATCTTTCGGTACGACGCTGGAGCCTTAAC and TCGAGTTAACGGCCCAAGTTCAGCGAAGGATCG-3′ were melted at 95 °C for 2 min, annealed at 75 °C for 5 min, and cooled down to 4 °C at a rate of 0.6 °C/min. The double-stranded DNA was ligated with the pET32a expression vector that was linearized with BamHI and SalI. Restriction enzymes and modifying enzymes were obtained from Roche Applied Science (Mannheim, Germany, New England Biolabs Inc. (Frankfurt, Germany), or MBI Fermentas. Verification of the engineered plasmids was performed through DNA sequencing. The point mutation T562A was detected for Aux-(547–715) and mutant constructs derived from this fragment.

Auxilin fragments used in clathrin binding assays were initially cloned into the pQE-31 vector for overexpression in M15 bacteria (QIAGEN). The expression and purification protocol was described previously (15). The Aux-(547–715) mutant with 715DPF replaced with APA or with 715DLL replaced with ALA was extracted from inclusion bodies according to the recommendations given by QIAGEN. The auxilin fragments Aux-(547–715), Aux-(715–902) were further purified by ion exchange chromatography in 25 mM Tris-HCl (pH 8.0) using a Mono Q column (Amersham Biosciences, Freiburg, Germany) that retained the contaminations quantitatively while the desired proteins were collected in the flow-through fraction. GST fusion proteins were thrombin (ICN)-digested to remove the GST moiety. hsc70 was prepared from porcine brains according to the protocol for bovine hsc70 (19). Clathrin was extracted from pig brain clathrin-coated vesicles (20) using 0.5 M Tris-HCl (pH 7.0), and assembly into cages was induced by dialysis against buffer A (100 mM MES, 1 mM EDTA, 0.5 mM MgCl2, and 2 mM CaCl2 (pH 6.4)).

Limited Trypsin Digestion of Clathrin Cages—To remove the N-terminal domains from the clathrin heavy chains, clathrin cages (1.5 mg/ml) were mildly digested with a final concentration of 0.5 mg/ml trypsin (porcine pancreas, Worthington) in a total reaction volume of 516 μl in buffer A containing 1 mM CaCl2. After incubation for 15 min on ice and for 50 min at 10 °C, the reaction was terminated with 1.3 mg of soybean trypsin inhibitor/mg of protease. Trypsin-digested cages were separated by ultracentrifugation for 20 min at 126,000 × g from the released N-terminal domains and the trypsin. The cages were washed once with 300 μl buffer B, pelleted by ultracentrifugation, and resuspended in 90 μl of buffer B (25 mM HEPES, 12.5 mM potassium acetate, and 5 mM magnesium acetate (pH 7.1)) containing the inhibitor at ~0.2 mg/ml.

Limited Trypsin Digestion of Clathrin-coated Vesicles— Pig brain clathrin-coated vesicles were mildly digested with trypsin to deplete endogenous auxilin. 20 μl of clathrin-coated vesicles prepared as described (21) were incubated with 200 μg/ml trypsin in a total reaction volume of 500 μl to inactivate the trypsin, 20 μg/ml soybean trypsin inhibitor was added to the reaction mixture. The vesicles were pelleted by ultracentrifugation, washed once with phosphate-buffered saline (2.7 mM KCl, 137 mM NaCl, 1.9 mM KH2PO4, and 8.2 mM

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Na₂HPO₄ (pH 7.0), and resuspended in 200 μl of buffer B containing the inhibitor at 5 μg/ml. This treatment quantitatively inactivated the associated auxilin, whereas many other components of clathrin-coated vesicles such as AP180 and clathrin heavy and light chains were not affected. Only the α-subunit of AP-2 was sensitive to degradation, but at least 20% of the starting material remained completely intact under the digestion conditions used.

Cage Binding—1.4 × 10⁻¹⁰ mol of the recombinant auxilin fragments were incubated for 30 min on ice with 6.8 × 10⁻¹¹ mol of clathrin assembled into cages in a total volume of 50 μl of buffer B. After centrifugation at 125,000 × g for 30 min, the clathrin pellets were resuspended in 0.5 M Tris-HCl (pH 7.0) in a final volume that corresponded to that of the supernatants. In all subsequent experiments, pelleted and supernatant fractions analyzed by SDS-PAGE were directly comparable to each other. Protein bands were quantified from digitalized images using NIH Image Version 1.62 software (National Institutes of Health, Bethesda, MD).

Clathrin Assembly—Assembly experiments were performed as described previously (15).

Uncoating Assay—The cofactor function of recombinant auxilin fragments was tested in an uncoating reaction that was carried out for 15 min at 25°C with auxilin-depleted clathrin-coated vesicles (∼0.9 μM clathrin); 3.2 μM hsc70; and an ATP-regenerating system containing 2 mM ATP, 5 mM creatine phosphate, 1 mM dithiothreitol, and 5 units/ml creatine kinase in a total volume of 50 μl of buffer B. hsc70 was preincubated for 50 min on ice with the ATP-regenerating mixture and then added to the vesicles and increasing stoichiometric concentrations of auxilin fragments. The released clathrin was separated from intact cages by ultracentrifugation. Trypsin-digested clathrin cages were dissociated under the same conditions as clathrin-coated vesicles using 1.4 μM clathrin and 66 nm recombinant auxilin fragments.

Full-down Experiments—Binding and competition experiments were performed as described (15) with the following modifications. GST-α ear domain and GST-TD fusion proteins precoated to glutathione-Sepharose beads were incubated for 1 h on ice in a reaction volume of 50 μl of buffer B with 2 × 10⁻¹⁰ mol of the recombinant auxilin fragments. For competition experiments, the beads were diluted 1:3 with Sepharose CL-4B, and 1.5 × 10⁻⁸ mol of the recombinant auxilin fragments were mixed with an equimolar amount or a 2-, 5-, or 10-fold molar excess of either His₅-Aux-(328–382) or the distal domain of clathrin.

Overlay Assay on an Auxilin Peptide Array—Auxilin-derived peptide fragments 15 amino acids in length were chemically synthesized in situ on a cellulose membrane as described previously (21, 22). The sequence of each peptide overlapped with that of the following one by 12 residues spanning amino acids 570–789. In an overlay assay, biotinylated recombinant proteins of the α ear domain and GST-TD fusion proteins precoated to glutathione-Sepharose beads were incubated for 1 h on ice in a reaction volume of 50 μl of buffer B with 2 × 10⁻¹⁰ mol of the recombinant auxilin fragments. For competition experiments, the beads were diluted 1:3 with Sepharose CL-4B, and 1.5 × 10⁻⁸ mol of the recombinant auxilin fragments were mixed with an equimolar amount or a 2-, 5-, or 10-fold molar excess of either His₅-Aux-(328–382) or the distal domain of clathrin.

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the spectra of the mixtures and the combined spectra of the individual components (Fig. 2, D and E). Thus, auxilin apparently does not gain structure upon interaction with clathrin-TD. Similar observations were previously obtained for the interaction between the DPW domain of epsin-1 and the ear domain of AP-2 (13). However, both sets of results need to be viewed with caution because it cannot entirely be ruled out that, under the conditions used, a low affinity interaction between the fragments may not form a sufficient amount of complex to generate a detectable signal. Nevertheless, we think that the chosen protein concentrations should be high enough to enable an efficient interaction.

**Heat Stability of the Clathrin-binding Domain of Auxilin**

The disordered regions of epsin-1 and AP180 are highly soluble and resistant to irreversible denaturation upon heating to 100 °C (18). This can be explained by the lack of a compact fold of the polypeptide chain, even under physiological conditions.

**TABLE I**

| Contents of secondary structure elements in recombinant auxilin fragments |
|---|
| Auxilin fragment | α-Helix | β-Sheet | Turn | Random coil |
|---|---|---|---|---|
| His8-Aux-(547–715) | 5 | 15 | 10 | 69 |
| His8-Aux-(715–813) | 5 | 13 | 9 | 72 |
| His8-Aux-(715–902) | 17 | 12 | 13 | 59 |
| His8-Aux-(547–715) + TFE | 17 | 12 | 13 | 59 |
| His8-Aux-(715–813) + TFE | 24 | 12 | 14 | 50 |
The organization of the clathrin-binding domain into two subdomains became evident by the different affinities of the wild-type constructs His<sub>6</sub>-Aux-(547–715) and His<sub>6</sub>-Aux-(715–902) for clathrin cages. The latter fragment is predominantly involved in binding to the distal or proximal domain of the heavy chain, whereas the former preferentially associates with the N-terminal domain (see also Ref. 15). Yet, His<sub>6</sub>-Aux-(547–715) retained a weak affinity for truncated clathrin cages; and likewise, binding of His<sub>6</sub>-Aux-(715–902) was affected by the removal of the N-terminal domain from cages, implying an interaction with clathrin-TD (Fig. 5C).

Mutations in motifs 579DPF, 674DPF, and 591DLL reduced the association of His<sub>6</sub>-Aux-(547–715) with assembled clathrin (Fig. 5C). A further reduction in binding to the cages was observed when the 636WDW sequence was mutated, suggesting that these residues contribute to the epitope for the antibody. Our finding was confirmed by binding of monoclonal antibody 100/4 to the auxilin peptide arrays (data not shown). Therefore, these sequences seem to be exclusively responsible for the interaction with clathrin.

Auxilin fragments failed to be detected by monoclonal antibody 100/4 when the 636WDW sequence was mutated, suggesting that the three DPF motifs are not equivalent because mutation of 674DPF had a much stronger negative effect on binding to the α-ear domain than the elimination of 579DPF. We observed a similar effect on binding of auxilin constructs to clathrin-TD (Fig. 5C). The association with the α-ear domain of AP-2 was not affected, however, when the DLL peptides were altered (data not shown). Therefore, these sequences seem to be exclusively responsible for the interaction with clathrin.

Identification of Binding Motifs for Clathrin and AP-2—

The clathrin-binding domain of auxilin contains three DPF and two DLL binding motifs, which have previously been shown to be involved in the interactions of other proteins with AP-2 and clathrin, respectively. However, it is not known whether they fulfill a similar function in auxilin. We started with a broad approach to identify the sequences responsible for the protein interactions of auxilin by using arrays of auxilin peptides synthesized in situ on cellulose membranes. Each peptide was a 15-mer that overlapped with the following one by 12 residues. The arrays covered the segment of amino acids 570–789 of auxilin. The membrane strips were probed with recombinantly expressed clathrin-TD and the α-ear domain of AP-2 in overlay assays. The experiments revealed a surprisingly large number of interactions between auxilin peptides and the clathrin and AP-2 probes (Fig. 4). Intriguingly, in most cases, the interaction sites for clathrin-TD and the α-ear domain of AP-2 seemed to overlap. Only sequence 591DLL appeared to interact exclusively with clathrin-TD. All other sequences considered to be involved in clathrin or AP-2 binding such as the three DPF peptides and the 636WDW sequence, which might be distantly related to clathrin box II motifs, associated with both clathrin-TD and the α-ear domain of AP-2. In addition to these interactions, clathrin-TD and the α-ear domain also bound to peptides showing no similarities to previously described motifs for clathrin or AP-2 binding. One set of such peptides contains the short sequence 735WQ; another one includes the dipeptide 750FS, which is also conserved in auxilin-2. The common denominator seems to be a large hydrophobic residue such as Trp, Phe, or Leu.
the affinity of the wild-type construct was already low (Fig. 5C).

A combined mutation of the 605DPF and 674DPF sequences did not seem to further reduce binding of the auxilin fragment to clathrin cages compared with the single mutation of 674DPF. This reflects the finding that the 605DPF motif did not play a major role in the interaction with GST-TD in the pull-down experiments (Fig. 5A). Our mutation analysis singled out the 674DPF sequence as a most versatile protein-protein interaction module. Its alteration not only affected the binding of auxilin to clathrin-TD and the \( \beta/H9251 \)-ear domain of AP-2 in pull-down experiments, but also reduced the interaction of His\(_6\)-Aux-(619–738) with clathrin cages lacking the N-terminal domains (Fig. 5C). This result indicates that the 674DPF motif is directly or indirectly involved in the association of His\(_6\)-Aux-(619–738) with the distal or proximal domain of the clathrin heavy chain. Thus, the 674DPF motif is engaged in multiple interactions of auxilin with clathrin and AP-2.

A particularly strong effect on binding of several auxilin fragments to clathrin cages was observed upon mutation of the two DLL peptides within the region of amino acids 547–910, especially of the sequence 781DLL (Fig. 5C). This could be seen even with cages from which the N-terminal domains had been removed. Taken together with the results from pull-down experiments with GST-TD, this suggests that both DLL motifs have the potential to bind to the N-terminal domain as well as to the distal or proximal domain of the clathrin heavy chain. Thus, the 674DPF motif is engaged in multiple interactions of auxilin with clathrin and AP-2.

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Because the 674DPF peptide is involved in interactions with both the α-ear domain of AP-2 and truncated clathrin cages, we predicted that the α-ear and clathrin distal domains might also compete for an interaction with auxilin. To test this, we incubated His6-Aux-(619–738) with GST-α-ear domain and added increasing amounts of a recombinant clathrin distal domain fragment (Trx/His6-tagged clathrin distal domain) to the assay. The experiment clearly demonstrates that the auxilin fragment...
was displaced from the α-ear domain of AP-2 by an excess of the distal domain of clathrin, indicating that both interactions exclude each other (Fig. 1B).

Role of Clathrin-binding Motifs in the Assembly Activity of Auxilin—When present in stoichiometric amounts relative to clathrin, auxilin promotes polymerization of clathrin triskelia to cages in vitro (2). To gain insight into the molecular requirements for the assembly activity of auxilin, we examined the effects of the mutant binding motifs on the polymerization of clathrin into cages. We added a 2-fold molar excess of different auxilin fragments and mutant proteins to purified clathrin and separated assembled clathrin cages from free clathrin triskelia by ultracentrifugation. The His$_6$-Aux (547–715) fragment could only poorly assemble clathrin into cages, whereas the C-terminal fragment His$_6$-Aux (715–902) was almost as efficient as Aux (547–910) (Fig. 8A). Knocking out the DLL motifs strongly reduced the ability of the recombinant auxilin fragments to induce clathrin polymerization. It had been shown previously that elimination of the DLL motifs in AP180 also reduces the assembly activity of this protein (9). Mutations of the DPF motifs in auxilin were much less effective. Unexpectedly, however, the substitution of $^{726}$NWQ with AAA resulted in a reduction of the assembly activity of His$_6$-Aux (715–902) by $>40\%$, whereas mutation of $^{753}$PS had only a slight effect. Therefore, the most important sequences involved in clathrin assembly seem to be $^{726}$NWQ and $^{781}$DLL.

However, the existence of only one of the latter motifs in a fragment proved insufficient to promote clathrin polymerization because neither of the fragments Trx/His$_6$-Aux (715–776) and Trx/His$_6$-Aux (778–902) was effective in an assembly experiment, although both fragments were still capable of interacting with GST-TD and trypsin-digested cages (Figs. 6 and 8B).

Similarities between the Clathrin-binding Domains of AP180 and Auxilin—Both auxilin and AP180 interact with clathrin-TD as well as with the α-ear domain of AP-2 and are able to support the polymerization of clathrin to cages. Many of the short peptide sequences that we have identified for these interactions in auxilin are also present in AP180. We already showed that auxilin and AP180 compete for clathrin-TD (15). Therefore, we asked whether both proteins bind to the same sites on AP-2. This was tested by incubating His$_6$-Aux (547–715) with GST-α-ear domain in the presence of increasing amounts of His$_6$-AP180 (328–896). A 2-fold molar excess of AP180 displaced the auxilin fragment by more than half. Thus, we conclude that AP180 and auxilin also compete for binding to AP-2, indicating that they interact with the same site on the α-ear domain (Fig. 9B).

It was previously demonstrated that AP180 can associate via its DPF motifs not only with the α-ear domain, but also with the β$_2$-ear domain of AP-2 (14). This raised the question of whether the same would be true for auxilin. Therefore, we performed a GST pull-down experiment and showed that the β$_2$-ear domain bound also directly to auxilin (Fig. 9B).

Role of Interactions with Clathrin and AP-2 in the Uncoating Activity of Auxilin—Because we observed strong effects on the interaction of auxilin with clathrin and AP-2 after mutating putative binding motifs, it was of interest to see how the elimination of these motifs influenced the ability of the auxilin fragments to stimulate the uncoating of clathrin-coated vesicles. To address this question, we used clathrin-coated vesicles mildly treated with trypsin to inactivate endogenous auxilin. Although clathrin was unaffected under the chosen conditions, we could ensure that a significant amount of α-adaptin was not degraded by the protease. The trypsin-treated coated vesicles were incubated with hsc70, an ATP-regenerating system, and wild-type or mutant fragments of auxilin. Intact coated vesicles were separated from released clathrin by ultracentrifugation.

Mutations of the $^{674}$DPF sequence in Aux (547–910) as well as the $^{726}$NWQ and $^{753}$PS sequences in His$_6$-Aux (715–902) had only a minor but detectable effect on the release of clathrin from the vesicle coats. However, the loss of motifs $^{591}$DLL and $^{781}$DLL led to a dramatic reduction in the uncoating activity of the fragments (Fig. 10, A and B). Therefore, both DLL sequences of His$_6$-Aux (715–902) play important roles in the hsc70-mediated dissociation of the clathrin coat. Interestingly, the N-terminal truncation of His$_6$-Aux (715–902), which quite effi-

![Fig. 6. Interaction of Trx/His$_6$-Aux (715–776) and Trx/His$_6$-Aux (778–902) with GST-TD and clathrin cages. The binding experiments were performed as described under “Experimental Procedures” and in the legend to Fig. 4. Equal aliquots of the supernatant (S) and the washed beads or pellet fractions (P) were resolved by SDS-PAGE and analyzed by immunoblotting. tryps., trypsin-digested.](Image 97x110 to 524x238)

![Fig. 7. A. binding of the Trx/His$_6$-tagged $^{674}$DPFADLG$^{680}$ sequence of auxilin to immobilized GST-TD and GST-α-ear domain as well as to intact or truncated clathrin cages. The binding experiments were performed as described under “Experimental Procedures” and in the legend to Fig. 4. Equal aliquots of the supernatant (S) and the washed beads or pellet fractions (P) were resolved by SDS-PAGE and analyzed by immunoblotting. B, competition of the distal domain of the clathrin heavy chain (Trx/His$_6$-tagged clathrin distal domain (DD)) and the GST-α-domain for binding to His$_6$-Aux (619–738). The pull-down experiments were carried out on ice for 1 h with $1.5 \times 10^{-10}$ mol of the recombinant auxilin fragment and increasing concentrations of the clathrin distal domain (either equimolar or a 2-, 5-, or 10-fold molar excess over auxilin). Note that the distal domain of clathrin inhibited the association of the auxilin fragment with the ear domain of α-adaptin by competitively interacting with the same binding site. tryps., trypsin-digested.](Image 104x303 to 260x387)
ciently functions in the coat disassembly, to Trx/His6-Aux-(778–902) resulted in an almost complete loss of uncoating activity (Fig. 10C). Therefore, the J-domain and the 781DLL motif are essential, but not sufficient, for coat dissociation induced by Aux-(715–902) in the presence of hsc70. The segment between amino acids 715 and 778 seems to make an important contribution to the cofactor function of auxilin in this assay.

Clathrin cages with the N-terminal domains removed by trypsin digestion can be disassembled in vitro by hsc70 in the presence of auxilin (1). We tested whether the mutations in Aux-(547–910) would affect the disassembly of such cages. Again, alterations in the DLL motifs resulted in a strong reduction of the ability of the auxilin fragment to mediate the release of clathrin triskelia from the cages, whereas mutation of 674DPF had only a small effect (Fig. 10D). This result confirms that both DLL sequences in Aux-(547–910) are essential for a productive interaction of auxilin with the proximal and distal domains of clathrin and its cofactor activity in coat dissociation.

**DISCUSSION**

Our previous studies (15) had shown that the clathrin-binding domain of auxilin (amino acids 547–813) contains multiple
interaction sites for the two major coat components clathrin and AP-2. This raised a number of questions about the nature of these interactions and their significance for the uncoating of clathrin-coated vesicles by hsc70. Therefore, it was of interest to perform a detailed analysis of the molecular organization of this domain to determine what kind of motifs are involved in the interactions and how they are arranged within the domain.

We investigated the structural organization of the clathrin- and AP-2-binding domain of auxilin by CD spectroscopy and observed that this region is almost completely disordered and has a high content of random-coil conformation. Similar structural properties were previously reported for the clathrin- and AP-2-binding segments of epsin-1 and AP180 (13, 18). Although auxilin has an increased helical content in solvents favorable to helix formation, we were not able to observe that this structure is induced upon incubation with clathrin-TD. Thus, it seems that the central region of auxilin remains overall disordered, even when associated with its interaction partners.

All of the identified binding sequences are tandemly arranged within this domain and consist of short linear peptides. A flexible clathrin-binding domain could enable auxilin to engage several interaction partners simultaneously, thus allowing for cooperative binding to clathrin and AP-2. Many endocytic proteins contain multiple copies of the DP(F/W) sequence, and the strength of the interaction with AP-2 seems to correlate with their number (6). Using mutant fragments of auxilin with successively eliminated DPF motifs, we could now show that these motifs bind cooperatively to the α-ear domain of AP-2. The observation that the clathrin-binding domain is intrinsically disordered and contains multiple, alternating short interaction motifs also explains why it was possible to divide the clathrin-binding domain into several shorter fragments that retained clathrin- and AP-2-binding activities.

We also identified novel binding sequences for clathrin and AP-2 that do not seem to be related to previously described motifs. These are sequences $\text{W}636\text{WDW}$, $\text{W}726\text{NWQ}$, and $\text{W}753\text{FS}$. Moreover, we noted that the $\text{W}579\text{DPF}$, $\text{W}674\text{DPF}$, and $\text{W}636\text{WDW}$ motifs were able to engage both AP-2 and clathrin and thus represent binding sequences with dual specificity (an overview of the identified binding determinants and their interaction partners is given in Fig. 11). Similar binding characteristics were previously assigned to the PWDLW binding sequence in human amphiphysin-2 (8).

To what extent interactions with binding sequences of multiple specificities can impede each other became apparent from our competition studies. As we had previously shown for the N-terminal domain (15), the distal domain of the clathrin-heavy chain was able to interfere with the binding of His$_6$-Aux-(619–738) to the α-ear domain of AP-2. Furthermore, the α-ear domain of AP-2 was also able to displace His$_6$-Aux-(547–715)
from clathrin-TD. The design of a clathrin-binding domain with multiple low affinity monospecific and dual specific interaction sequences arranged along a flexible polypeptide chain and with a high content of serine and threonine residues would be perfectly suited for regulation by phosphorylation. Computational analysis of the auxilin sequence using the PROSITE Database (27) predicts, for instance, sequence 686SQQD, which overlaps with the 501DLL peptide, to be a potential substrate for casein kinase-2. This enzyme has previously been shown to be present in the protein coats of clathrin-coated vesicles (28, 29). Such phosphorylation sites within or in close proximity to binding determinants could be utilized to selectively raise or lower the affinity of auxilin for one or the other interaction partner. However, so far, we do not know whether auxilin is a target for kinases.

Our study has shown that the interaction of auxilin with clathrin is not mediated by binding sequences of the canonical clathrin box I and II types that have been implicated in the association with clathrin-TD. Instead, auxilin utilizes clathrin-binding determinants that include the above-mentioned sequences 636VDW, 573DPF, and 674DPF. Furthermore, the clathrin-binding domain contains two repeats of the DLL sequence that are involved in the interactions with clathrin. For AP180, this motif was implicated in the assembly of clathrin, presumably by cross-linking the N-terminal domains of three adjacent triskelia (9). In fact, auxilin was originally also identified as a clathrin assembly protein (2) before its role in the uncoating of clathrin-coated vesicles was recognized. Our data confirm the role of the DLL sequences in the assembly function of auxilin, but they also show that the assembly mechanism involves not only their association with the N-terminal domains, but also binding to the distal domains of the clathrin heavy chain. This conclusion is based on our previous finding (15) that the clathrin-binding domain of auxilin can be further divided into two functionally distinct subdomains represented by the two auxilin fragments His<sub>n</sub>-Aux-(547–715) and His<sub>n</sub>-Aux-(715–902). The C-terminal auxilin fragment, which is most efficient in clathrin assembly, seems to interact preferentially with the distal domains of the clathrin heavy chain and associates only poorly with the globular N-terminal domain. In contrast, His<sub>n</sub>-Aux-(547–715), which only poorly supports assembly, displays complementary binding specificities with a preference for clathrin-TD (15). Therefore, we propose that the interaction of auxilin with the distal domain of clathrin is essential for driving efficient clathrin assembly.

Direct evidence for involvement of the DLL peptides in binding to the N-terminal and distal domains of clathrin was obtained from mutation analysis. The ability of auxilin to polymerize clathrin was reduced upon substitution of the DLL sequences with ALA. Furthermore, the elimination of these motifs resulted in impaired binding to trypsin-digested cages lacking the N-terminal domains and the clathrin light chains. However, our data also indicate that the presence of one DLL motif is not sufficient for assembly function because Trx/His<sub>n</sub>-Aux-(778–902), which contains the 781DLL sequence, displayed no clathrin assembly activity. In contrast, His<sub>n</sub>-Aux-(715–902) efficiently promoted clathrin assembly. This fragment contains the 726NWQ peptide, which was considered a candidate for a novel clathrin-binding motif on the basis of the peptide array overlay assay. We demonstrated that the 726NWQ sequence is involved in the assembly activity of auxilin. Its elimination from His<sub>n</sub>-Aux-(715–902) significantly reduced the ability of this fragment to promote clathrin polymerization, but did not abolish it completely. Taken together, the 726NWQ sequence synergistically supports the 781DLL peptide in clathrin assembly. Evidence for a role of the 726NWQ sequence in this function is also indirectly provided by the failure of an auxilin fragment ranging from amino acids 728 to 910 to drive clathrin assembly (16).

Quantitative clathrin assembly studies using auxilin fragments with systematically mutated putative binding sequences revealed a small, but significant effect when the 674DPFADL sequence was changed to APAADL. A related sequence (885DPFADL) occurs together with 726NWQ in a C-terminal fragment of AP180 (C16) that was shown by Morgan et al. (9) to process moderate clathrin assembly activity, although it lacks DLL motifs. Thus, it seems that different endocytic proteins utilize similar combinations of identical or related short binding sequences to promote clathrin polymerization.

Taking into account the number of clathrin interaction sites within the clathrin-binding domain of auxilin, a correct spacing seems to be a further requirement for efficient binding and assembly activity. This could explain why binding motifs for clathrin-TD within His<sub>n</sub>-Aux-(547–715) can only poorly assemble clathrin, whereas they might be important in the context of the entire clathrin-binding domain of Aux-(547–910). This idea is supported by the clathrin-binding studies with deletion mu-
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