Amphiphysin 1 and 2 are proteins implicated in the recycling of synaptic vesicles in nerve terminals. They interact with dynamin and synaptojanin via their COOH-terminal SH3 domain, whereas their central regions contain binding sites for clathrin and for the clathrin adaptor AP-2. We have defined here amino acids of amphiphysin 1 crucial for binding to AP-2 and clathrin. Overexpression in Chinese hamster ovary cells of an amphiphysin 1 fragment that binds both AP-2 and clathrin resulted in a segregation of clathrin, which acquired a diffuse distribution, from AP-2, which accumulated at patches also positive for Eps15. These effects correlated with a block in clathrin-mediated endocytosis. A fragment selectively interacting with clathrin produced a similar effect. These results can be explained by the binding of amphiphysin to the NH2-terminal domain of clathrin and by a competition with the binding of this domain to the β-subunit of AP-2 and AP180. The interaction of amphiphysin 1 with either clathrin or AP-2 did not prevent its interaction with dynamin, supporting the existence of tertiary complexes between these proteins. Together with previous evidence indicating a direct interaction between amphiphysin and membrane lipids, these findings support a model in which amphiphysin acts as a multifunctional adaptor linking the membrane to coat proteins and coat proteins to dynamin and synaptojanin.

Synaptic vesicles are the specialized organelles that store and secrete fast-acting neurotransmitters at synapses. After exocytotic fusion with the presynaptic plasmalemma, components of the synaptic vesicle membrane are rapidly re-internalized by endocytosis and reused for the generation of new, neurotransmitter-filled, synaptic vesicles. This membrane recycling is crucial for the proper functioning of synapses during prolonged high frequency stimulation (1).

Strong evidence implicates clathrin-mediated endocytosis in synaptic vesicle reformation. Recent studies show that in addition to the major components of clathrin coats, several other accessory proteins are implicated in this endocytic reaction (1). Two such proteins are amphiphysin 1 and 2, which are members of a protein family conserved from yeast to man (2–4). Yeast homologues of amphiphysin, Rvs167 and Rvs161, have been implicated in endocytosis, actin function, and signaling (5, 6). In mammals, both amphiphysin 1 and 2 are highly expressed in the nervous system, where they are concentrated at synapses and further concentrated on endocytic intermediates implicated in synaptic vesicle recycling (7–11). Amphiphysin 1 and 2 have a similar structure that includes several main domains.

The NH2-terminal region is the domain of amphiphysin most conserved in evolution. It contains amino acid stretches predicted to form coiled-coil domains that mediate the formation of hetero- and homodimers, in agreement with the reported heterodimerization of Rvs161 and Rvs167 in yeast (6, 11–13). This region also contains a lipid binding site that accounts for the property of amphiphysin to bind liposomes and to evaginate them into narrow tubules (14). It has been speculated that this property may reflect a role of amphiphysin in the process of membrane invagination during the generation of an endocytic bud.

The COOH-terminal domains of amphiphysin 1 and 2 contain an SH3 domain and bind two main proteins in brain, synaptojanin 1, and dynamin 1 (7, 15, 16). Both these proteins are concentrated in nerve terminals and are colocalized with amphiphysin on endocytic intermediates (17, 18). Dynamin 1 is a GTPase crucially required for the fission and detachment of endocytic buds from the plasma membrane (19, 20). Although its precise mechanism of action remains unclear, its action is thought to involve its recruitment and oligomerization at the neck of endocytic buds (17, 19, 20). Synaptojanin 1 is a polyphosphoinositide phosphatase that has also been implicated in synaptic vesicle reformation (16, 21). The physiological importance of the SH3-mediated interactions of amphiphysin is strongly supported by the potent inhibition of clathrin-mediated endocytosis produced by disruption of such interactions in living cells (22, 23). Based on results of peptide microinjection in a giant synapse followed by electron microscopic analysis, disruption of the SH3 domain-mediated interactions of amphiphysin arrests the endocytic reaction at the stage of deeply invaginated clathrin-coated pits (23). These findings, complemented by the demonstration that amphiphysin and dynamin can co-assemble into rings on lipid tubules, have led to the speculation that amphiphysin and dynamin may be functional partners in the fission reaction (14).

The central region of amphiphysin 1 and 2 binds clathrin and AP-2 (12, 24). Such interaction could provide a mechanism through which amphiphysin can be recruited to clathrin coats, where, in turn, it may participate in the recruitment of dynamin. A first characterization of the clathrin binding site in amphiphysin 2 has been reported (24). To obtain further in-
sight into the physiological function of the binding of amphiphysin to clathrin and AP-2, we have now mapped the clathrin and AP-2 binding sites of amphiphysin 1 and defined the amino acid crucial for binding by site-directed mutagenesis. To test whether these binding sites play a crucial role in vivo, we expressed amphiphysin fragments that contain both or either one of the two sites and tested their effect on clathrin coat function. Finally, we have examined whether binding to AP-2 and clathrin may occur simultaneously, as predicted by the hypothesis that amphiphysin plays a role in the recruitment of fission factors.

MATERIALS AND METHODS

Antibodies—Monoclonal antibodies Bioreagents were purchased from Affinity Bioreagents (anti-α-adaptin (APG) and anti-clathrin (X22)) and Sigma (anti-β-adaptin). A mouse hybridoma producing a monoclonal antibody against clathrin (TDI) was obtained from ATCC. Polyclonal antibodies to α-adaptin and γ-adaptin were kindly provided by Dr. M. Robinson (University of Cambridge, United Kingdom). Polyclonal antibodies against amphiphysin (25), dynamin (15), Eps15 (26), and monoclonal antibodies against AP180 and dynamin were generated in our laboratory.

Site-directed Mutagenesis and cDNA Cloning—Mutations were introduced in the A1 (amino acids 262–405) fragment of human amphiphysin 1 cloned into pGEX-6p-1 vector (Amersham Pharmacia Biotech) as BamHI/EcoRI fragments. The nucleotide sequences of the constructs were verified by DNA sequencing. The NH2-terminal domain of clathrin (amino acids 1–579) in pGEX2 vector (27) was kindly provided by Dr. J. Keen (Thomas Jefferson University). The expression of GST fusion proteins was induced with 0.25 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 3 h in SB medium (35 g/liter trypotide, 20 g/liter yeast extract, 5 g/liter NaCl, pH 7.5) supplemented with 200 μg/ml carbenicillin at A500 = 0.8. Bacterial cells were lysed by sonication in the lysis buffer (300 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton X-100) containing 5 mM EDTA, 2 μg/ml aprotinin, 0.5 mM Pefabloc, and spun at 35,000 × g. Supernatants were incubated with 1–2 ml of glutathione-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C on a rotating wheel. Beads were then washed twice with lysis buffer in a batch, transferred into 10-ml columns, and washed with 3 column volumes of lysis buffer and lysis buffer without Triton X-100. The GST fusion proteins were eluted with 15 mM glutathione, desalted in HBS (20 mM HEPES, 150 mM NaCl, pH 7.5), and stored in aliquots at −70 °C. The removal of GST from fusion proteins with PreScission protease (Amersham Pharmacia Biotech) was performed according to manufacturer's instructions.

RESULTS

Fragments of the central portion of amphiphysin 1 were expressed as GST fusion proteins and used for affinity purification from a Triton X-100 extract of rat brain. Bound proteins were analyzed by SDS-PAGE and Coomassie Blue staining. As described previously, a fragment comprising amino acids 262–405 of amphiphysin 1 (fragment A1) bound both clathrin and the clathrin adaptor AP-2 (Fig. 1A), whereas subfragments of this region bound selectively clathrin or AP2 (12). Further deletions of these fragments allowed us to narrow down the regions of amphiphysin 1 critical for the interaction with these proteins (Fig. 1 and Ref 12).

The core of the AP-2 binding site, i.e. the region necessary and sufficient for binding to AP-2, was mapped to amino acids 322–340 (Fig. 1 and 2). The presence of the additional 23 amino acids at the COOH-terminal end of this fragment (up to amino acid 363) enhanced AP-2 binding, suggesting a role of this region in the physiological interaction. Such a region includes the consensus DF/F/W (Fig. 2), which is present in the AP-2 binding region of several AP-2-binding proteins. The same region, however, was not sufficient by itself for detectable binding of AP-2 (Fig. 1A, A8). Interestingly, the effect of truncations of this region on the AP-2 binding was not linear. Although all constructs shorter than A11 (amino acids 322–363) bound less AP-2 (Fig. 1, B and C), the shorter construct A15 (amino acids 322–346) bound more AP-2 than the longer A14 (amino acids 322–355). This observation may reflect the importance of the sequence downstream of the core region of the binding site for maintaining the correct conformation or surface accessibility.

The clathrin binding site was mapped using amino acids 347–386 (Figs. 1 and 2). This portion of the molecule includes the sequence LLDDL, that fits a previously proposed clathrin binding consensus sequence. Although an extremely weak binding of clathrin was observed with a construct containing only the putative consensus sequence (see for example construct A11,
A17, and A18 in Fig. 1), efficient binding of clathrin required the presence of downstream residues up to amino acid 386 (see construct A10 in Fig. 1).

To confirm and complement these results, we performed site-directed mutagenesis. Wild type charged residues were mutated to residues of the opposite charge, and hydrophobic residues were mutated to hydrophilic residues (Fig. 2A). To disrupt the AP-2 interaction we targeted the amino acid sequence FFED (amino acids 323–326), which appeared to be essential for binding of AP-2 based on deletion analysis (compare fragment A16 and A18 in Fig. 1). To disrupt clathrin binding, we chose the sequence DLD in the putative clathrin binding consensus and the WD residues in the downstream sequence that cooperate in clathrin binding. These two residues were chosen because of their selective conservation in amphiphysin 1 and 2. The clathrin and AP-2 binding properties of A17, and A18 in Fig. 1), efficient binding of clathrin required the presence of downstream residues up to amino acid 386 (see construct A10 in Fig. 1).

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of the mutant constructs were compared with those of the parental wild type construct A1 in affinity chromatography experiments (Fig. 2B). Mutant proteins with an altered AP-2 binding site (A1/SSR and A1/SKR) lost their ability to bind AP-2 but still bound clathrin. Conversely, mutations in the two sites that contribute to clathrin binding (A1/HSR and A1/SR) severely impaired binding of clathrin (more than 20-fold) without affecting the interaction with AP-2. The combined mutation (A1/HSR/SR) completely abolished clathrin binding. Finally, the protein with mutations in both the clathrin and the AP-2 binding sites (A1/HSR/SR/SSR) did not bind either clathrin or AP-2.

Previously we observed that overexpression in CHO cells of an amphiphysin fragment that comprises both the clathrin and the AP-2 binding sites, originally designated as B/C domain (amino acids 250–588), resulted in a block of receptor-mediated endocytosis of transferrin (12). We have now observed that this fragment drastically disrupts the partial co-localization of clathrin with AP-2. Although in untransfected cells AP-2 is colocalized with a pool of clathrin at “hot spots” at the cell periphery, in cells overexpressing the B/C domain a complete dissociation of two immunofluorescence patterns was observed. Clathrin immunoreactivity completely redistributed to diffuse fluorescence (Fig. 3, see also Ref. 12), whereas AP-2 coalesced into large aggregates at the cell periphery (Fig. 3, middle panels). These patches were also positive for Eps15 (28) (Fig. 3, bottom panels), an AP-2-binding protein. In addition, a mislocalization of the AP-1 clathrin adaptor was also observed, as demonstrated by a redistribution of its γ-adaptin subunit from the central region of the cell to large dots scattered throughout the cytoplasm (not shown). Similar results were obtained with an HA-tagged version of the A1 construct (Fig. 4, A–D), which is a shorter fragment of the B/C domain that still contains both binding sites (Fig. 1B).

To gain further insight into the distinct roles of the clathrin and AP-2 binding sites in these effects, we overexpressed in CHO cells HA-tagged constructs corresponding to the A1 fragment in which either one of the two sites had been selectively disrupted by site-directed mutagenesis. Overexpression of the fragment that binds clathrin but not AP-2 (fragment A1/SSR of Fig. 2) resulted in a block of the receptor-mediated endocytosis of transferrin (not shown) and in a redistribution of clathrin and AP-2 very similar to that observed for the wild type A1 fragment (Fig. 4, I–L). The construct, which binds AP-2 but not clathrin (fragment A1/HSR/SR of Fig. 2), also produced a block in transferrin internalization (not shown) and a partial redistribution of AP-2 into peripheral patches (Fig. 4, E–H). This construct did not, however, significantly perturb the localization of clathrin.

It was shown that several clathrin-interacting proteins including β-subunits of adaptor complexes (29, 30) arrestin (27), AP-180 (31), and epsin (32) bind to the NH2-terminal domain of clathrin and that these proteins share the putative clathrin binding consensus (LLDLD) that is also present in amphiphysin (Ref. 24 and see above). The results of transfection experiments obtained with the clathrin-binding constructs suggest that saturation of the amphiphysin binding sites in clathrin may compete the interactions mediating clathrin recruitment to coated pits. The interaction between clathrin and β2 is thought to play a critical role in clathrin coat assembly at the cell surface. We tested therefore whether amphiphysin 1 binds to the NH2-terminal domain of clathrin and whether it competes other interactions.

The fragment comprising amino acids 1–579 of the heavy chain of rat clathrin was used to affinity purify interacting proteins from a rat brain membrane extract, and the bound material was analyzed by Coomassie Blue staining and Western blotting following separation by SDS-PAGE. AP-2, AP180, and amphiphysin 1 were selectively retained by the beads (Fig.
Amphiphysin 1 and Clathrin Coat Function

FIG. 5. Amphiphysin binds the NH$_2$-terminal domain of clathrin heavy chain. GST-clathrin (amino acids 1–579 of clathrin heavy chain (27)) (100 µg) immobilized on glutathione-Sepharose beads was used for affinity purification from rat brain detergent extract (10 mg/ml protein). The material specifically retained on the beads was separated by SDS-PAGE and analyzed by Coomassie Blue staining (left) or by Western blotting using antibodies against AP180, amphiphysin 1, α-adaptin (not shown), and β-adaptin (right). The bands recognized by the antibodies co-migrated precisely with the Coomassie blue-stained bands indicated by arrows in the left panel. The amphiphysin 2 band is not visible due to its co-migration with the GST fusion protein.

5). Next, we analyzed if the addition of recombinant full-length amphiphysin 1 to the incubation mixture competes with binding of other proteins. Although the interaction of clathrin with AP180 was completely inhibited by exogenous amphiphysin, the interaction with AP-2 was only decreased by approximately 50%, as demonstrated by blotting for its β2-adaptin (Fig. 6, panel A) and α-adaptin (not shown) subunits. However, the interaction with AP-2 was efficiently competed by a short fragment of amphiphysin 1, which includes the clathrin binding site only (fragment A5 of Fig. 1B) (Fig. 6, panel B). The incomplete competition by full-length amphiphysin 1 may have two explanations. One is that in the presence of amphiphysin 1 the direct interaction between clathrin and AP-2 is replaced by indirect interaction mediated by amphiphysin. The second is that amphiphysin 1, known to dimerize through its coiled-coil domain (39, 40), partially overlaps with the clathrin binding site and that competition (5 mg) by the GST-NH$_2$-terminal fragment of clathrin (30 µg) in the absence or presence of 150 µg of amphiphysin 1 fragment A5 (Fig. 1B). For these experiments, GST was removed from the fusion protein of fragment A5 by cleavage with PreScission protease.

DISCUSSION

We have demonstrated that clathrin and the clathrin adaptors AP-2 and AP180 bind to distinct but adjacent binding sites in the central region of amphiphysin 1 and have determined the amino acids critical for these two distinct interactions. As predicted, these amino acids are conserved in the sequence of amphiphysin 1 and 2, which share the property of binding clathrin and AP-2. The property of binding the two major components of the clathrin coat is shared by several other proteins that participate in clathrin-mediated endocytosis, including epsin (32), AP180 (34), and β-arrestin (35). Furthermore, in epsin, as in amphiphysin, the binding sites for AP-2 and clathrin have a tandem arrangement (32), suggesting that this close proximity is physiologically important.

In amphiphysin 1, the binding site for AP-2 is located upstream of the clathrin binding site and is centered around a sequence that is similar in mammalian amphiphysin 1 and 2: S/F/L/F/E/D/N/T/FVP/E/D. A downstream sequence that partially overlaps with the clathrin binding site and that contains one copy of the amino acid sequence DPF (DPL in amphiphysin 2) increases the affinity of the interaction with AP-2. Amphiphysin 1 and 2 bind AP-2 via the ear domain of its α-adaptin subunit (33), i.e., via the same domain involved in AP-2 binding to AP180 (34), Eps15 (36, 37), and epsin (26). It is therefore noteworthy that the sequence DPF/E/F/W is found in multiple copies in Eps15, AP180, and epsin. In the cases of epsin and Eps15, these repeats were shown to be located in the AP-2 binding region. The DPF motif of amphiphysin 1 is essential for the strength of the interaction but is not sufficient for AP-2 binding. According to the map of the amphiphysin 2 (BIN1) gene, the core of the AP-2 binding site (32$^{33}$FFED) and the sequences adjacent to DP/L/F are located on different exons (12A and 12B). Interestingly, several tumor cell lines express an isoform of amphiphysin 2 that contains the core of AP-2 binding site (exon 12A) but not the sequence DPL and a portion of the clathrin-interacting domain (exons 12B and 12C) (38).

The crystal structure of the amphiphysin binding portion of AP-2, i.e., the ear domain of α-adaptin, was recently reported (39, 40). These studies demonstrated a competition between amphiphysin, Eps15, epsin, and AP180 for binding to this domain (39, 40). Accordingly, the AP-2 binding fragments of amphiphysin 1 competed the binding of epsin and AP180 to AP-2. Therefore, although the sequences of AP-2 binding sites in these proteins are different, they recognize the same struc-

$^2$ V. I. Slepnev, G.-C. Ochoa, M. H. Butler, and P. De Camilli, unpublished observation.
tural motif in α-adaptin. However, the affinity of interaction is different and is higher for epsin and Eps15 than for amphiphysin and AP180 (39, 40).

The clathrin binding domain is located between amino acids 347 and 386 of human amphiphysin 1, in good agreement with the mapping of the clathrin binding domain in amphiphysin 2. Within this region, two amino acid stretches contribute to clathrin binding. The first stretch includes the sequence LL-DDLD, which is highly conserved in amphiphysin 2 and which fits the proposed consensus binding motif for clathrin binding. The amount of dynamin in the supernatant of the binding reaction (5) and bound to beads (B) was detected by Western blotting. Panel B, presence of amphiphysin induces the recruitment of dynamin to the GST fusion protein of the NH2-terminal fragment of clathrin. Shown is Coomassie Blue staining (top) and Western blotting (bottom) of the material purified from rat brain membrane extract (5 mg) by GST-NH2-terminal fragment of clathrin (30 μg) in the absence or presence of recombinant amphiphysin 1 (100 μg). Endogenous clathrin-binding proteins are not detectable in the middle lane of the Coomassie Blue-stained gel due to lower concentrations of the extract and GST fusion protein in this experiment compared with Fig. 5.

A

B

FIG. 7. Amphiphysin 1 can bind simultaneously to dynamin and either clathrin or AP-2. Panel A, amphiphysin mediates binding of dynamin 1 to the GST-ear domain of α-adaptin. The interaction between purified recombinant dynamin 1 and bead-immobilized GST-ear of α-adaptin was assayed in the presence or absence of purified recombinant amphiphysin 1. The amount of dynamin in the supernatant of the binding reaction (S) and bound to beads (B) was detected by Western blotting. Panel B, presence of amphiphysin induces the recruitment of dynamin to the GST fusion protein of the NH2-terminal fragment of clathrin. Shown is Coomassie Blue staining (top) and Western blotting (bottom) of the material purified from rat brain membrane extract (5 mg) by GST-NH2-terminal fragment of clathrin (30 μg) in the absence or presence of recombinant amphiphysin 1 (100 μg). Endogenous clathrin-binding proteins are not detectable in the middle lane of the Coomassie Blue-stained gel due to lower concentrations of the extract and GST fusion protein in this experiment compared with Fig. 5.

In conclusion, our present findings provide additional evidence for a role of amphiphysin as a pleotropic adaptor linking the lipid bilayer (14) to intrinsic components of the clathrin coat and to accessory factors of such coat. Moreover, we have shown that fragments of amphiphysin that encode the clathrin and AP-2 binding sites are enriched in Eps15. One would expect competition of ear domain binding to Eps15 by the overexpressed amphiphysin fragment.

Finally, we have shown that binding of amphiphysin 1 to either AP-2 or clathrin is not mutually exclusive with its binding to dynamin via the COOH-terminal SH3 domain. Using purified recombinant proteins, we have shown that amphiphysin 1 can mediate the previously described interaction between AP-2 and dynamin (12, 22, 33). Similarly, the binding of amphiphysin 1 to clathrin resulted in co-purification of dynamin from the brain extract. Amphiphysin forms dimers and heterodimers. Irrespective of whether these tertiary complexes between coat components, amphiphysin, and dynamin are mediated by distinct subunits within the amphiphysin dimer or by the same subunit, our findings provide a clear biochemical demonstration that amphiphysin acts as an adaptor between the clathrin coat and its SH3 domain interactors.

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