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Epsin Binds to Clathrin by Associating Directly with the Clathrin-terminal Domain

EVIDENCE FOR COOPERATIVE BINDING THROUGH TWO DISCRETE SITES*

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Epsin is a recently identified protein that appears to play an important role in clathrin-mediated endocytosis. The central region of epsin 1, the so-called DPW domain, binds to the heterotetrameric AP-2 adaptor complex by associating directly with the globular appendage of the α subunit. We have found that this central portion of epsin 1 also associates with clathrin. The interaction with clathrin is direct and not mediated by epsin-bound AP-2. Alanine scanning mutagenesis shows that clathrin binding depends on the sequence ^{257}LMDLADV located within the epsin 1 DPW domain. This sequence, related to the known clathrin-binding sequences in the adaptor β subunits, amphiphysin, and β-arrestin, facilitates the association of epsin 1 with the terminal domain of the clathrin heavy chain. Unexpectedly, inhibiting the binding of AP-2 to the GST-epsin DPW fusion protein by progressively deleting DPW triplets but leaving the LMDLADV sequence intact, diminishes the association of clathrin in parallel with AP-2. Because the β subunit of the AP-2 complex also contains a clathrin-binding site, optimal association with soluble clathrin appears to depend on the presence of at least two distinct clathrin-binding sites, and we show that a second clathrin-binding sequence ^{480}LVDLD, located within the carboxyl-terminal segment of epsin 1, also interacts with clathrin directly. The LMDLADV and LVDLD sequences act cooperatively in clathrin recruitment assays, suggesting that they bind to different sites on the clathrin-terminal domain. The evolutionary conservation of similar clathrin-binding sequences in several metazoan epsin-like molecules suggests that the ability to establish multiple protein-protein contacts within a developing clathrin-coated bud is an important aspect of epsin function.

Endocytosis occurs primarily at specific regions of the plasma membrane coated on the cytoplasmic surface with the AP-2 adaptor complex and clathrin. The ordered polymerization of clathrin into a polyhedral coat is thought to mechanically introduce curvature into the underlying membrane and thereby drive the formation of clathrin-coated vesicles. Because AP-2 and clathrin are the major protein components on clathrin-coated vesicles that bud from the cell surface, much work over the past decade has centered on carefully dissecting the specific roles that these proteins play in ordered coat assembly and the protein sorting process. It has become clear, however, that multiple factors in addition to clathrin and AP-2 are critical in regulating and coordinating endocytic events. Some additional molecules that have been demonstrated to affect endocytosis include dynamin (1, 2), amphiphysin (3, 4), eps15 (5–7), β-arrestin (8), epsin (9), intersectin/Ese (10, 11), synaptojanin (12), POB1 (13), and polyphosphoinositides (14–18).

Although dynamin and synaptojanin each exhibit hydrolytic (GTPase and inositol-5-phosphatase, respectively) activity, the other endocytic proteins appear to be primarily involved in establishing an extensive array of complex protein-protein interactions that appear necessary to construct a clathrin-coated vesicle. For example, amphiphysin is able to interact with dynamin, synaptojanin, AP-2, and clathrin in a phosphorylation-dependent manner (19, 20). Likewise, eps15 interacts with AP-2 (21), the 170-kDa long splice isoform of synaptojanin 1 (22), epsin (9), and intersectin/Ese (10, 11). AP180, another clathrin coat component, binds to both AP-2 and clathrin. Thus, an emerging consensus from these more recent studies is that highly coordinated, multivalent protein-protein interactions are likely to contribute to the dynamics of AP-2-dependent clathrin coat formation that occurs during rapid endocytosis at the plasma membrane.

Epsin was recently identified in screens for proteins that interact with the eps15 homology (EH) domain of eps15 (9, 23, 24). The carboxyl-terminal portion of epsin 1, the so-called NPF domain, contains three repeats of the tripeptide motif asparagine-proline-phenylalanine (NPF) (9). These repeats facilitate binding to the eps15 homology (EH) domains of not only eps15 but also to the eps15 homology (EH) domains of the recently identified intersectin/Ese protein family (10, 11) and POB1 (13). The central region of the epsin 1 molecule binds to the α subunit of AP-2, in an apparently phosphorylation-dependent manner (9). In fact, epsin was first discovered as a novel binding partner of the globular appendage domain of the AP-2 α chain (25). This report describes the ability of epsin 1 to also interact directly with clathrin and identifies linear sequences within the epsin 1 (and epsin 2) primary structure that are responsible for this association. One binding site is adjacent to but distinct from the AP-2-binding site located within the central portion of the protein. A second site is upstream of the first NPF repeat within the carboxyl-terminal NPF domain. Thus, like AP-2, clathrin, amphiphysin, eps15, and β-arrestin, epsin is also capable of forming a multivalent protein network that likely influences the endocytic process. In addition, the potential relevance of related clathrin-binding sequences present in

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The amino acid sequences of these proteins can be accessed through NCBI Protein Database under NCBI accession numbers AF096269 and AAF05113.

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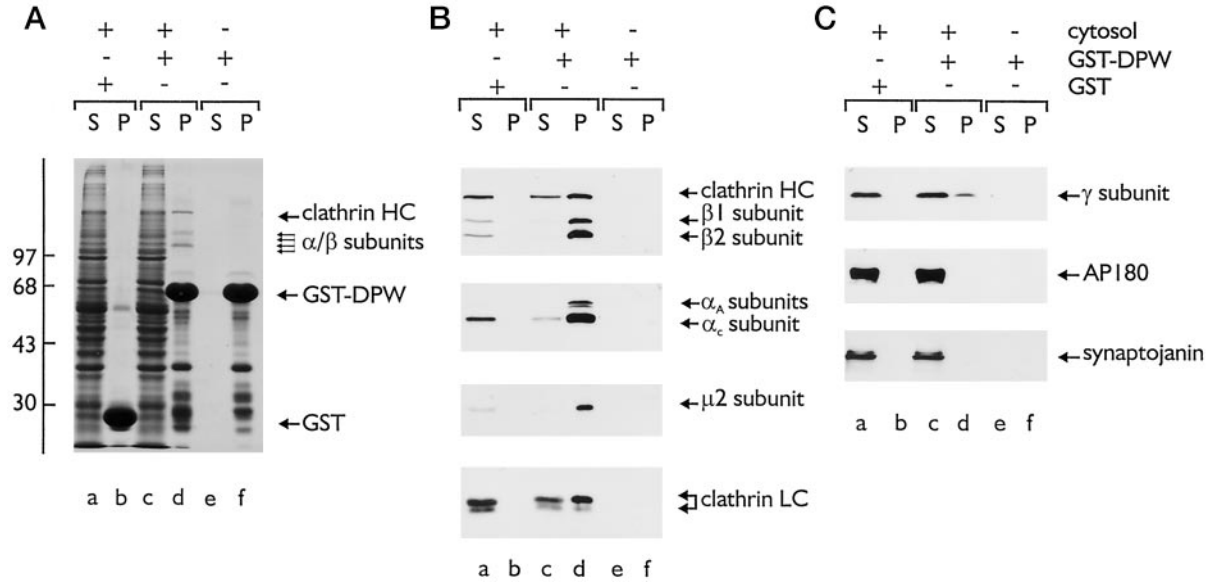

FIG. 1. **Association of adaptors and clathrin with the DPW domain of epsin 1.** Approximately 200 μg of either GST (*lanes a* and *b*) or GST-DPW (*lanes c–f*) was first immobilized on 50 μl of packed glutathione Sepharose and then mixed with either buffer (*lanes e* and *f*) or with rat brain cytosol to give a final concentration of ~7.5 mg/ml (*lanes a–d*). After incubation at 4 °C for 60 min, the Sepharose beads were recovered by centrifugation and then washed. Aliquots corresponding to 1/200 of each supernatant (*S*) and 1/15 of each pellet (*P*) were resolved by SDS-PAGE and either stained with Coomassie Blue (*A*) or transferred to nitrocellulose (*B* and *C*). Portions of the blots were probed with a mixture of the anti-clathrin heavy chain (HC) mAb TD.1 and the anti-β1/β2 subunit mAb 100/1, or the anti-AP-2 α subunit mAb 100/2, or anti-AP-2 μ2 subunit antiserum, or the anti-clathrin light chain (LC) mAb Cl 57.3, or the anti-AP-1 γ subunit antibody AE/1, or an anti-AP180 mAb, or anti-synaptojanin antibody RV/2. The positions of the molecular mass standards (in kDa) are indicated on the *left*, and only the relevant portion of each blot is shown.

several additional proteins that participate in clathrin-coat assembly is also discussed.

EXPERIMENTAL PROCEDURES

Antibodies—The anti-α subunit mAb[1] 100/2 and the anti-β1/β2 subunit mAb 100/1 were generously provided by Ernst Ungewickell. Polyclonal anti-μ2 subunit antiserum was a gift from Juan Bonifacino. The affinity-purified anti-AP-1 γ subunit antibody, AE/1, has been described elsewhere (26). The anti-AP-2 α subunit mAb AP.6, the clathrin heavy chain mAb X22, and mAb TD.1, which recognizes the clathrin-terminal domain, were kindly provided by Frances Brodsky, and the antibody specific for the clathrin light chain neuronal-specific insert, mAb Cl57.3, was from Reinhard Jahn. Polyclonal anti-epsin antibodies from rabbits injected with residues 229–575 of rat epsin 1 fused to glutathione *S*-transferase (GST) were affinity purified from serum on either purified epsin DPW domain immobilized on nitrocellulose or on histidine-tagged epsin 1 coupled to CNBr-activated Sepharose 6MB (Amersham Pharmacia Biotech). Polyclonal anti-peptide (RVDASDEDRISEVRKVLC) antibodies against synaptojanin were also raised in rabbits and affinity purified on the immunizing peptide immobilized on CNBr-activated Sepharose 4B. The pan-arrestin mAb F4C1 was kindly provided by Larry Donoso, whereas the polyclonal anti-β-arrestin serum was a gift from R. Lefkowitz. Monoclonal antibodies specific for amphiphysin, AP180, and the γ subunit of AP-1 (mAb 88) were purchased from Transduction Laboratories.

cDNA Constructs and Mutagenesis—A cDNA encoding residues 229–407 of rat epsin 1 cloned into pGEX-4T-1 (GST-DPW) was kindly provided by Pietro de Camilli. A construct containing residues 1–579 of the bovine clathrin heavy chain cloned into pGEX-2T, was kindly provided by Jim Keen. Mutagenesis of epsin 1 was performed using the QuikChange system (Stratagene) with the GST-DPW plasmid as the template. The sense primers used were 5′-GGCAAGGAGGAGTCATCTGCCGCGGCTCTTGCTGACGTCTTC-3′ for the GST-DPW (^{257}LMD → AAA) substitution, 5′-GAGTCATCTCTTATGGATGCTGCCGCGGCCTTCACAACCCCAGCC-3′ for the GST-DPW (^{260}LADV → AAAA) substitution and 5′-GGAGGAGTCATCTCTTTTGGATCTTATGGACGCCTTAACAACCCCAGCCC-3′ for the GST-DPW (1 → 2) substitution. The GST-DPW(2 → STOP), (4 → STOP), (6 → STOP), and (8 → STOP) constructs were all generated by converting the relevant TGG tryptophan codon in the GST-DPW plasmid to a TAA stop codon using the QuikChange system. The GST-ETLLDLDF, GST-SSLMDLADV, and GST-AALVDLDS constructs were each prepared by annealing sense and antisense oligonucleotides and ligating the double-stranded product into *Eco*RI/*Xho*I-digested pGEX-4T-1. The pGEX plasmid encoding the final eight amino acids (RGYTLIDL) of *Saccharomyces cerevisiae* Ent1p fused to GST was kindly provided by Beverly Wendland (27). All of the constructs and mutations were confirmed by dideoxynucleotide sequencing.

Protein Expression and Purification—GST and the various GST fusion proteins were produced in *Escherichia coli* BL21 cells. The standard induction protocol entailed shifting log-phase cultures (A_{600} = ~0.6) from 37 °C to room temperature and adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 100 μM. After 3–5 h at room temperature with constant shaking, the bacteria were recovered by centrifugation at 8,000 rpm (JA-14 rotor) at 4 °C for 15 min and stored at −80 °C until used. GST fusion proteins were collected on glutathione Sepharose 4B after lysis of the bacteria in B-PER reagent (Pierce) and removal of insoluble material by centrifugation at 14,000 rpm (JA-20 rotor) at 4 °C for 15 min. Where necessary, proteins were cleaved with thrombin (Amersham Pharmacia Biotech) from GST while still immobilized on glutathione Sepharose. The conditions for the thrombin digestion were as recommended by the manufacturer, followed by addition of the irreversible thrombin inhibitor D-Phe-Pro-Arg chloromethyl ketone (Calbiochem) to a final concentration of 25 μM. To prepare the TD-Sepharose, thrombin-cleaved TD was coupled onto CNBr-activated Sepharose 4B to a density of ~5 mg/ml using standard procedures.

Rat brain cytosol was prepared from either fresh or frozen rat brains (PelFreez) exactly as described previously (28). Before use, the cytosol was adjusted to 25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, and 1 mM dithiothreitol and centrifuged at 245,000 × g_{max} (70,000 rpm, TLA-100.3 rotor) at 4 °C for 20 min to remove insoluble material. Clathrin-coated vesicles were prepared from fresh rat brain by standard procedures (29). For the preparation of purified clathrin, a crude coat extract was prepared from clathrin-coated vesicles using 1 M Tris-HCl, pH 7.0 followed by gel filtration in 500 mM Tris-HCl, pH 7.0 (30). Clathrin-containing fractions were pooled, the clathrin trimers were concentrated by addition of ammonium sulfate to 50% saturation, and then cages were assembled by dialysis against 100 mM MES-NaOH, pH 6.8, 2.5 mM $MgCl_2$, 1 mM EGTA, 3 mM $CaCl_2$ at 4 °C.

Binding Assays—The association of AP-2, clathrin, and other endo-

[1] The abbreviations used are: mAb, monoclonal antibody; GST, glutathione *S*-transferase; HC, heavy chain; LC, light chain; PAGE, polyacrylamide gel electrophoresis; TD, clathrin heavy chain-terminal domain; TGN, *trans*-Golgi network; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline.

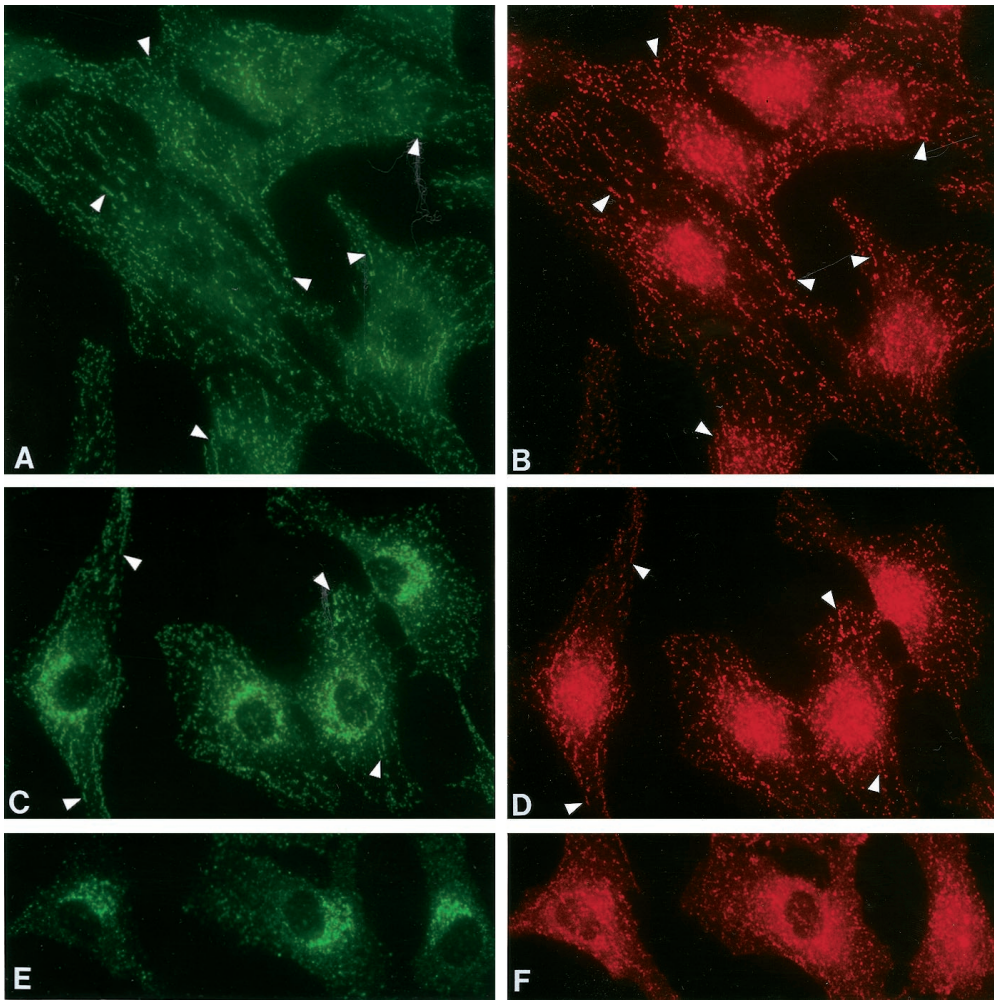

FIG. 2. **Steady-state distribution of epsin, AP-2, and clathrin in normal rat kidney cells.** Methanol-fixed (*A–D*) or formaldehyde-fixed (*E* and *F*) normal rat kidney cells were double labeled with affinity-purified antibodies against epsin (*B*, *D*, and *F*) and either mAb AP.6, against the α subunit of AP-2 (*A*) or mAb X22 directed against the clathrin HC (*C*) or mAb 88, against the γ subunit of AP-1 (*E*). *Arrowheads* point to representative examples of co-localization of epsin with AP-2- and clathrin-containing structures. Notice that in addition to the epsin found concentrated at clathrin bud sites on the cell surface, a pool of soluble epsin is also visible in the central region of each cell (*B*, *D*, and *F*). Careful inspection indicates that this staining pattern is, in general, quite distinct from that of the clathrin/AP-1 assembled at the TGN, however.

cytic accessory proteins with the various GST fusion proteins was assayed in 25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, and 1 mM dithiothreitol (assay buffer) in a final volume of 600 μl. Routinely, GST and the GST fusion proteins were first each immobilized on 50 μl of packed glutathione Sepharose to concentrations of ~2–6 mg/ml. The immobilized proteins were then washed and resuspended to 100 μl in assay buffer. Rat brain cytosol was added, and the tubes were then incubated at 4 °C for 60 min with continuous gentle mixing. The Sepharose beads were then recovered by centrifugation at 10,000 $\times$ g_{max} for 1 min, and 30-μl aliquots of each supernatant were removed and adjusted to 100 μl with SDS-PAGE sample buffer. After washing the Sepharose pellets four times each with ~1.5 ml of ice-cold PBS by centrifugation, the supernatants were aspirated, and each pellet was resuspended to a volume of 150 μl in SDS-PAGE sample buffer. Unless otherwise indicated, 10-μl aliquots, equivalent to 1/200 of each supernatant and 1/15 of each pellet, were loaded on the gels.

For the clathrin-cage binding experiments, preassembled cages were mixed with GST-DPW fusion proteins that had been centrifuged at 245,000 $\times$ g_{max} for 20 min to remove insoluble material. The binding assays were performed in 100 mM MES-NaOH, pH 6.8, 2.5 mM $MgCl_2$, 1 mM EGTA, 3 mM $CaCl_2$ in a final volume of 100 μl. After 20 min at room temperature, the cages were recovered by centrifugation at 245,000 $\times$ g_{max} for 10 min, and then equal aliquots of the supernatant and pellet fractions were analyzed by SDS-PAGE.

Electrophoresis and Immunoblotting—Samples were resolved on 10% polyacrylamide gels prepared with an altered acrylamide:bis-acrylamide (30:0.4) ratio stock solution. The decreased cross-linking generally improves resolution but also affects the relative mobility of several proteins, most noticeably AP180 and epsin 1. After SDS-PAGE, proteins were either stained with Coomassie Blue or transferred to nitrocellulose in ice-cold 15.6 mM Tris, 120 mM glycine. Blots were blocked overnight in 5% skim milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and then portions were incubated with primary antibodies as indicated in the individual figure legends. After incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG, immunoreactive bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech).

Immunofluorescence Analysis—Normal rat kidney cells were grown on 12-mm round glass coverslips in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cells were fixed either in methanol at −20 °C for 5 min or in 3.7% formaldehyde in PBS at room temperature for 15 min, washed with PBS, and then incubated in 10% normal goat serum, 0.2% saponin in PBS at room temperature for 30 min. The cells were then incubated with a mixture of affinity-purified anti-epsin 1 antibodies and mAb X22, mAb AP.6, or mAb 88 diluted in 10% normal goat serum, 0.05% saponin in PBS. After ~60 min at room temperature, the cells were washed three times with PBS and then incubated with a mixture of goat anti-mouse Alexa 488 and sheep anti-rabbit Alexa 596 (Molecular Probes) conjugates diluted 1:250 in 10% normal goat serum, 0.05% saponin in PBS. After ~30 min at room temperature, the cells were washed with PBS and mounted on Cytoseal.

RESULTS

The central portion of rat epsin 1 (residues 249–401) contains eight repeats of the tripeptide motif aspartic acid-proline-

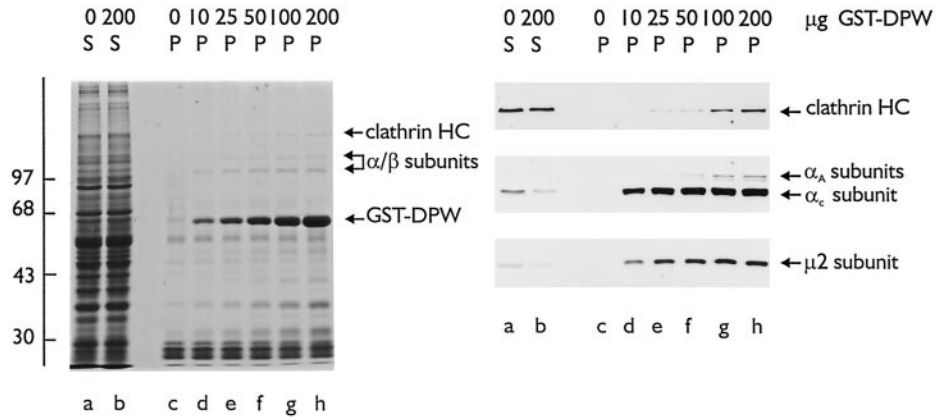

FIG. 3. **Dose-dependent binding of AP-2 and clathrin to GST-DPW**. Aliquots of 50 μl of packed glutathione-Sepharose were mixed alone (*lanes a* and *c*) or with 10 (*lane d*), 20 (*lane e*), 50 (*lane f*), 100 (*lane g*), and 200 μg (*lanes b* and *h*) of purified GST-DPW. After immobilizing the fusion protein, rat brain cytosol was added to each tube to a final concentration of ~7.5 mg/ml and incubated at 4 °C for 60 min. The Sepharose beads were then recovered by centrifugation and washed. Aliquots corresponding to 1/200 of the supernatant (*S*) resulting from the incubation with either 0 (*lane a*) or 200 μg (*lane b*) GST-DPW and 1/15 of each pellet (*P*) (*lanes c–h*) were resolved by SDS-PAGE and either stained with Coomassie Blue (*left panel*) or transferred to nitrocellulose. Portions of the blots were probed with the anti-clathrin HC mAb TD.1, the anti-AP-2 α subunit mAb 100/2, or anti-AP-2 μ2 subunit antiserum. The positions of the molecular mass standards (in kDa) are indicated on the *left*, and only the relevant portion of each blot is shown.

tryptophan (DPW) (9). This region, termed the DPW domain, interacts with the AP-2 adaptor complex by binding directly to the carboxyl-terminal appendage of the α subunit (9, 31, 32). After a GST-DPW fusion protein is incubated with rat brain cytosol, a group of ~100-kDa polypeptides associate with the sedimented glutathione-Sepharose (Fig. 1*A*, *lane d*). Immunoblotting with antibodies to the adaptor α and β subunits confirm the identity of these bands as the large subunits of AP-2 (Fig. 1*B*, *lane d* compared with *lanes b* and *f*). An anti-μ2 subunit antibody verifies the presence of this AP-2 subunit as well. The blots probed with the anti-adaptor β subunit antibody reveal that both the β1 chain of the AP-1 complex and the β2 chain of AP-2 are found with the GST-DPW fusion-protein pellet (Fig. 1*B*, *lane d*). In rat brain sections, some epsin 1 staining has been observed in the perinuclear region and, when full-length epsin 1 was overexpressed in Chinese hamster ovary cells, the protein was also found in clathrin-coated structures at the *trans*-Golgi network (TGN) (9). Because epsin binds to the carboxyl-terminal appendage of the α subunit of AP-2, targeting to the TGN could be via an analogous recognition site on the appendage domain of the γ subunit of the AP-1 adaptor complex. We therefore checked for the presence of AP-1 complexes bound to the epsin construct using an antibody against the γ subunit of AP-1 and, surprisingly, find that only limited amounts of the Golgi-adaptor associate with the GST-DPW (Fig. 1*C*, *lane d*). The steady-state distribution of epsin 1 in normal rat kidney cells also indicates that only minor amounts of epsin 1 are associated with the clathrin-coated structures forming at the TGN (Fig. 2). In cells double-labeled for epsin 1 and AP-2, the staining patterns are almost identical (*panels A* and *B*). Cells labeled for both clathrin and epsin 1 (*panels C* and *D*) show that although almost all the peripheral clathrin-positive structures also contain epsin, the compact perinuclear clathrin population on the TGN is largely devoid of co-localizing epsin. This is evident despite the diffuse perinuclear epsin signal that corresponds to the soluble form of the protein. Staining of the AP-1 adaptor complex, concentrated at the TGN, is again clearly distinct from the staining pattern of epsin 1 (*panels E* and *F*). Together, these experiments suggest that AP-1 and AP-2 do not associate with the DPW domain of epsin 1 with similar affinities and instead argue that the bulk of the β1 subunit bound to the GST-DPW is most likely explained by the promiscuity of β1 subunit incorporation into brain AP-2 complexes noted previously by us (28) and others (33, 34). The relative lack of epsin recruitment onto the TGN agrees well with the absence of eps15R on the clathrin coats assembling at the TGN (35).

Epsin 1 Binds to Soluble Clathrin Trimers—In addition to the subunits of the AP-2 heterotetramer that associate with the immobilized GST-DPW fusion protein, a prominent ~180-kDa protein is also found in the pellet (Fig. 1*A*, *lane d*). The binding of both AP-2 and the ~180-kDa protein is dependent on both the epsin portion of the fusion protein and cytosol as neither is found on the control GST Sepharose (*lane b*) or on GST-DPW Sepharose incubated without cytosol (*lane f*). To determine the identity of the large polypeptide, immunoblots were probed with antibodies against the clathrin heavy chain, AP180, and synaptojanin. Strong reactivity with the anti-clathrin antibody (Fig. 1*B*, *lane d*) indicates that this protein is likely the clathrin heavy chain. Unlike clathrin, neither AP180 nor synaptojanin are found in the pellet (*lane d*), although both proteins are seen in the cytosol fraction (Fig. 1*C*, *lanes a* and *c*), as expected. The simultaneous appearance of clathrin light chains in the pellet (Fig. 1*B*, *lane d*) confirms the association of clathrin with the DPW domain of epsin 1. Incubating brain cytosol with increasing amounts of the GST-DPW fusion shows that the association of clathrin with epsin is dose-dependent, as is the binding of AP-2 (Fig. 3, *lanes c–h*). It is important to note, however, that although clathrin binding is still detectable when 25 μg of the GST-DPW fusion is immobilized (*lane e*), the association of clathrin trimers with the GST-DPW appears significantly more sensitive to dilution than the binding of soluble AP-2 (*lanes d–h*).

Identification of a Clathrin-binding Sequence in Epsin—The sequence ^{257}LMDLADVF precedes the array of eight DPW repeats in the central portion of the rat epsin 1 molecule. A related sequence, ^{283}LLDLMDAL is found in rat epsin 2 (Table I). These short regions of alternating aliphatic hydrophobic and acidic residues are similar to the clathrin-binding sequences found in the β1, β2, and β3 subunits of the adaptor complexes (36, 37), in amphiphysin I and II (38) and in β-arrestin 1 and β-arrestin 2 (39) (Table I). To test whether this region of epsin 1 facilitates the association with clathrin, we separately mutated the ^{257}LMD or ^{260}LADV sequences to alanines in the context of the GST-DPW fusion protein. Either substitution causes a dramatic reduction in clathrin binding (Fig. 4*A*, *lanes d* and *e*) compared with the GST-DPW (*lane c*). By contrast, the association of AP-2 with the epsin mutants (*lanes d* and *e*) is

TABLE I
Characterized and putative clathrin-binding sequences in clathrin-coat-associated proteins

Protein		Sequence			Reference[a]
Rn[b] epsin 1	255	SS**L**MDLADVFT			this study
	478	AA**L**VDLDSLVS			
Rn epsin 2	281	TT**L**LDLMDALP			this study
	462	AA**L**VNLDSLVT			
Dm epsin	259	SH**L**LDLLDISL			
	542	SA**L**VNLDNLIK			
Ce epsin	385	SA**L**DDLLSLGV			
	524	SN**L**VNLDNLLG			
Rn amphiphysin I	349	ET**L**LDLDFDPF	380	LPWDLWTT	20, 38
Rn amphiphysin II	350	AS**L**LDLDFEPL	416	IPWDLWEP	38
Rn AP180	176	DA**L**LEFDVHPN			
	347	SD**L**LDLQPDFS			
Rn CALM	176	DA**L**LDFNVNSN			
	391	ND**L**LDLQQPTF			
Rn β1 subunit	626	GDLLGD**L**LNLDLGPP			36
Rn β2 subunit	626	GDLLGD**L**LNLDLGPP			36
Mm β3A subunit	827	DVF**L**LDLDDFNP			37
Hs β3B subunit	804	EIS**L**LDLEDFTP			37
Rn β-arrestin 1	374	TN**L**IELDTNDD			39
Rn β-arrestin 2	372	TN**L**IEFDTNYA			39
Sc Ent1p	447	RGYT**L**IDL[c]			27
Sc Ent2p	447	QGVS**L**IDL[c]			27

[a] Only reports showing that the sequences presented here (or highly related sequences from a different species) are directly responsible for clathrin binding are cited.
[b] *Rn*, *Rattus norvegicus*; *Dm*, *D. melanogaster*; *Ce*, *C. elegans*; *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*; *Sc*, *S. cerevisiae*.
[c] Indicates the natural carboxyl terminus of the protein.

indistinguishable from native fusion protein (*lane c*). This rules out the possibility that clathrin simply associates with epsin only indirectly by interacting solely with the bound AP-2 adaptor. It also argues against gross misfolding of the mutant fusion proteins.[2] Rather, the DPW domain of epsin 1 displays an independent clathrin-binding site. To examine whether the related sequence in epsin 2 (Table I) also has the capacity to bind to clathrin triskelia, we mutated the ^{257}LMDLADVF sequence in epsin 1 to the epsin 2 sequence LLDLMDAL. This substitution does not change the ability of the GST-DPW fusion (GST-DPW(1 → 2)) to recruit soluble clathrin trimers (Fig. 4*B*), suggesting that epsin 2 is also likely to be able to associate with clathrin as well. Switching the epsin 1 clathrin-binding sequence for that of epsin 2 has no affect on the ability of the DPW fusion protein to bind to AP-2, confirming that the two binding sites are independent of each other.

Epsin Binds to the Terminal Domain of the Clathrin Heavy Chain—The clathrin-binding sequence ^{818}LLDLD, located within the carboxyl-terminal segment of the β3A subunit of the human AP-3 adaptor complex, facilitates direct association with the terminal domain of the clathrin heavy chain (37). Similarly, the structurally related sequence ^{374}LIEFE in bovine β-arrestin 2 (arrestin3), located near the carboxyl terminus, binds directly to the clathrin-terminal domain (40). The terminal domain folds into a seven-bladed β-propeller structure (41), and the β-arrestins bind to a cluster of hydrophobic and basic residues projecting off the c- and d-β strands of blades 1 and 2 (40, 41). The similarity of the clathrin-binding sequences (Table I) lead us to assess whether epsin 1, like the adaptor β subunits and the β-arrestins, can interact with the terminal domain directly. When GST-TD coupled to glutathione Sepharose is incubated with rat brain cytosol, we find that AP-2 (α and μ2 subunits), AP180, and amphiphysin I and II all bind to the fusion protein and are found in the pellet fraction (Fig. 5*A*, *lane d*). This demonstrates that the clathrin-binding sequences in these proteins (Table I) appear to facilitate association with the terminal domain. Epsin 1 also translocates onto the expressed terminal-domain matrix but seems to bind significantly less avidly than the other proteins (*lane d*). Unexpectedly, in this assay cytosolic β-arrestin 1 and 2, detected with both a pan-arrestin mAb and a polyclonal antibody,[3] interact with the GST-TD very poorly (*lane d* compared with *lane b*). The large excess of GST-TD in these experiments rules out competition for binding sites on the propeller blades of the terminal domain.

Because the GST-TD experiments were performed with whole cytosol, it was important to exclude the possibility that epsin only binds to the clathrin-terminal domain indirectly. To do this, we examined the association of these protein domains directly. To obviate the formation of GST-TD-GST-DPW heterodimers, the terminal domain was cleaved off the fusion protein and coupled to CNBr-activated Sepharose 4B. Immobilized in this way, the terminal domain still retains the ability to bind to AP180, AP-2, amphiphysin, and epsin present in brain cytosol (data not shown). When mixed with GST-DPW, the TD-Sepharose retains a fraction of the fusion protein (Fig. 5*B*, *lane d*), whereas the control Sepharose beads do not (*lane b*). These experiments demonstrate that the interaction between epsin 1 and the clathrin-terminal domain is direct. If thrombin-cleaved epsin 1 DPW domain is added to the TD-Sepharose, this fragment also interacts with the terminal domain and some is recovered on the beads (*lane h*). However, given the molar excess of the DPW domain added, the association of the monomeric form with the immobilized terminal domain appears substantially less efficient than the binding of dimeric GST-DPW fusion protein.

We also tested the ability of the GST-DPW fusion to bind to preassembled clathrin cages. When mixed together with the clathrin, again a small fraction of the GST-DPW fusion binds to the cages and is recovered in the pellet (Fig. 6, *lane f*). Incubations in the absence of the clathrin cages show that epsin associated with the pellet is not simply due to sedimentation of

[2] Circular dichroism analysis of the DPW domain of epsin 1 yields a spectrum indicative of a random coil structure, suggesting this region might be largely disordered. D. Levine, D. H. Fremont, and L. M. Traub, unpublished observations.

[3] L. M. Traub, unpublished observations.

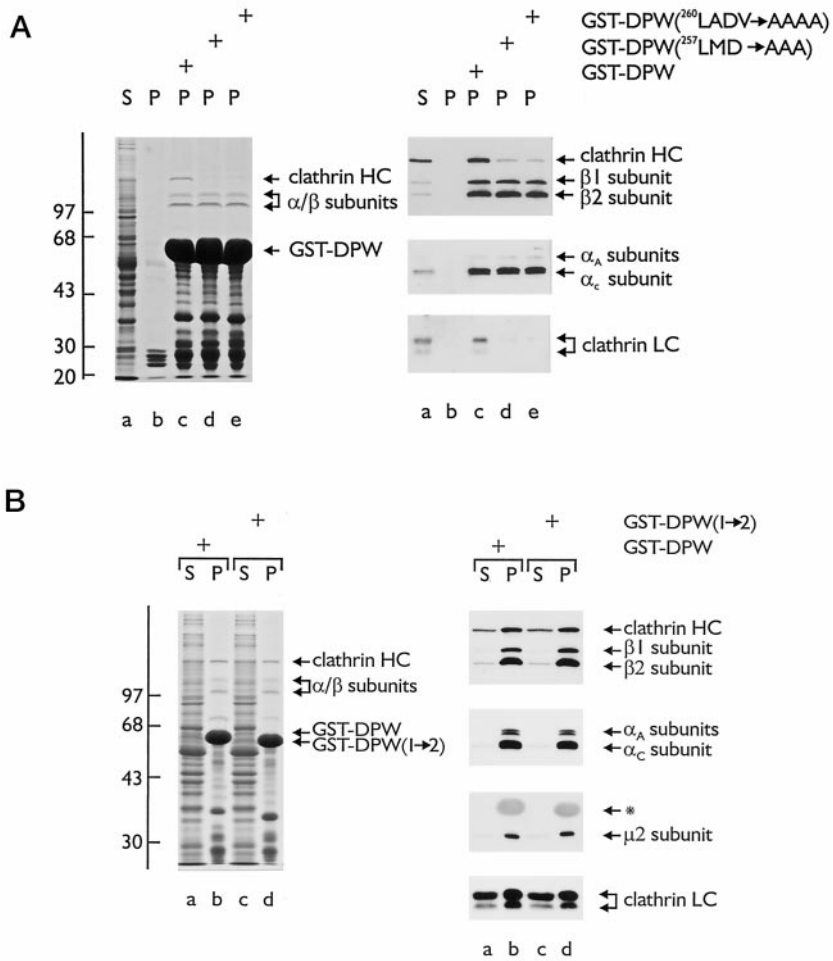

FIG. 4. **Identification of the clathrin-binding sequence in epsin 1.** *A*, aliquots of 50 μl packed glutathione-Sepharose were mixed alone (*lanes a* and *b*) or with ~350 μg of GST-DPW (*lane c*), ~350 μg of GST-DPW (^{257}LMD → AAA) (*lane d*), or ~350 μg of GST-DPW (^{260}LADV → AAAA) (*lane e*). After immobilizing the fusion proteins, rat brain cytosol was added to each tube to a final concentration of ~7.5 mg/ml and incubated at 4 °C for 60 min. The Sepharose beads were then recovered by centrifugation and washed. Aliquots corresponding to 1/200 of each supernatant (*S*) and 1/15 of each pellet (*P*) were resolved by SDS-PAGE and either stained with Coomassie Blue (*left panel*) or transferred to nitrocellulose. Portions of the blots were probed with a mixture of the anti-clathrin HC mAb TD.1 and the anti-β1/β2 subunit mAb 100/1, the anti-AP-2 α subunit mAb 100/2, and the anti-clathrin LC mAb Cl 57.3. The positions of the molecular mass standards (in kDa) are indicated on the *left*, and only the relevant portion of each blot is shown. *B*, aliquots of 50 μl of packed glutathione Sepharose containing ~200 μg of prebound GST-DPW (*lanes a* and *b*) or GST-DPW (1 → 2) (*lanes c* and *d*) were each incubated with brain cytosol and then aliquots corresponding to 1/200 of each supernatant (*S*) and 1/15 of each pellet (*P*) were resolved by SDS-PAGE and either stained with Coomassie Blue (*left panel*) or transferred to nitrocellulose. Portions of the blots were probed with a mixture of the anti-clathrin HC mAb TD.1 and the anti-β1/β2 subunit mAb 100/1, the anti-AP-2 α subunit mAb 100/2, anti-AP-2 μ2 subunit antiserum, and the anti-clathrin LC mAb Cl 57.3. The positions of the molecular mass standards (in kDa) are indicated on the *left*, and only the relevant portion of each blot is shown. The *asterisk* indicates weak cross-reactivity of the GST-DPW fusion proteins with the rabbit-polyclonal serum used. Notice that substituting the epsin 2 sequence for the endogenous epsin 1 sequence in GST-DPW results in a minor alteration in mobility of the protein on the gel and different degradation products.

aggregated protein (Fig. 6, *lane d*). The GST-DPW (^{257}LMD → AAA) and GST-DPW (^{260}LADV → AAAA) mutants both show a diminished capacity to associate with the clathrin cages (Fig. 6, *lanes j* and *n*), in agreement with the reduced affinity these mutants display for soluble clathrin trimers (Fig. 3).

Two Distinct Clathrin-binding Sites in Epsin 1—Epsin binds to AP-2 via the DPW repeats within the DPW domain (9, 31, 32). When a series of GST-DPW truncations is tested for adaptor binding, the association of AP-2 diminishes as the number of DPW triplets in the GST-DPW fusion is reduced (Fig. 7*B*). The reduction in AP-2 binding is paralleled by a loss of clathrin association in these constructs. In the GST-DPW(2 → STOP) construct, with only a single DPW motif present (Fig. 7*A*), binding of both AP-2 and clathrin is minimal (*lane l*), and the amount of the two protein complexes found in the supernatant fraction (*lane k*) does not differ much from the control (*lane a*). The inhibitory effect on clathrin binding was unexpected because, in all cases, the ^{257}LMDLADV sequence remains intact (Fig. 7*A*). One interpretation of these results, consistent with the weak binding of the DPW domain to clathrin (Fig. 5*B*), is that the relative affinity of the LMDLADV sequence alone for the clathrin-terminal domain is low, and stable binding requires at least two binding sites to engage a single terminal domain or a clathrin trimer. A second clathrin-binding sequence, ^{632}LLNLD, is located on the β2 subunit of the AP-2 adaptor complex and would be in close proximity because the AP-2 adaptor binds to the GST-DPW fusion via the adjacent appendage.

Intriguingly, epsin 1 and epsin 2 each contain an additional linear sequence, located in the carboxyl segment, that has previously been recognized as a potential clathrin-binding motif (10) (Table I). These distal sequences actually conform more closely to a general consensus sequence (37) for clathrin binding (L(L/I)(D/E/N)(L/F)(D/E)) than the clathrin-binding site we have mapped to the DPW domain in that there is only a single aliphatic hydrophobic residue between the first and second

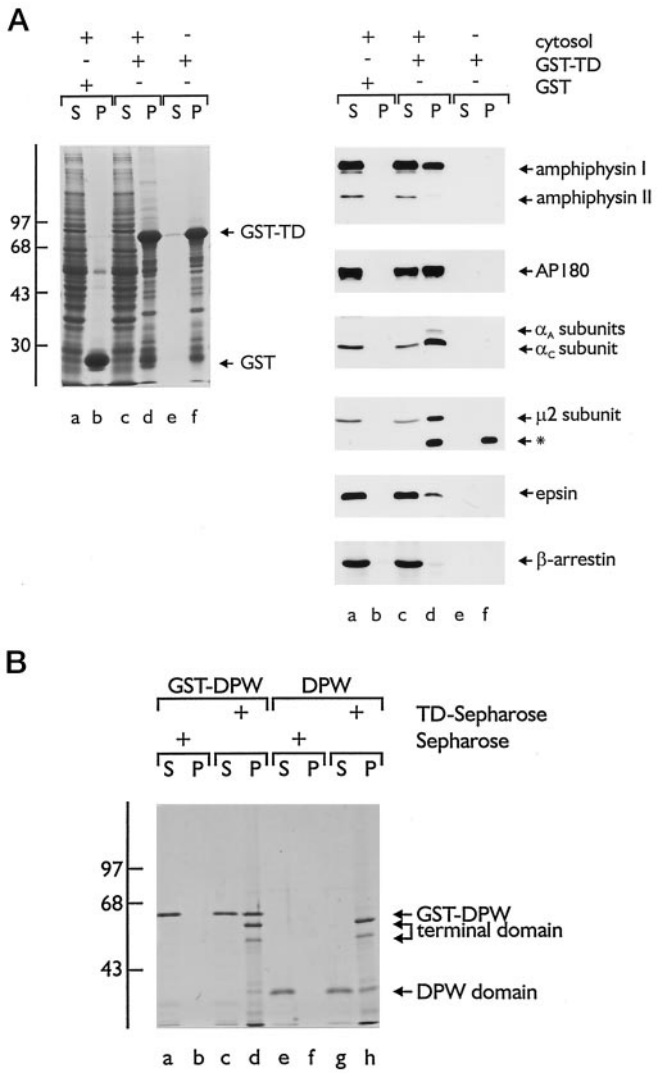

FIG. 5. **Association of cytosolic coat-associated proteins with the clathrin-terminal domain.** *A*, approximately 200 μg of either GST (*lanes a* and *b*) or GST-TD (*lanes c–f*) was first immobilized on 50 μl of packed glutathione Sepharose and then mixed either with buffer (*lanes e* and *f*) or with rat brain cytosol to give a final concentration of ~7.5 mg/ml (*lanes a–d*). After incubation at 4 °C for 60 min, the Sepharose beads were recovered by centrifugation. Aliquots corresponding to 1/200 of each supernatant (*S*) and 1/15 of each washed pellet (*P*) were resolved by SDS-PAGE and either stained with Coomassie Blue (*left panel*) or transferred to nitrocellulose. Portions of the blots were probed with an anti-amphiphysin mAb that recognizes both amphiphysin I and II, an anti-AP180 mAb, the anti-AP-2 α subunit mAb 100/2, anti-AP-2 μ2 subunit antiserum, affinity purified anti-epsin 1 antibodies, or the pan-arrestin mAb F4C1. The positions of the molecular mass standards (in kDa) are indicated on the *left*, and only the relevant portion of each blot is shown. The *asterisk* indicates a cross-reactive protein that contaminates the GST-TD preparation (*lane f*). *B*, aliquots of 50 μl of either Sepharose 4B or TD-Sepharose 4B were mixed with 150 μg/ml GST-DPW (*lanes a–d*) or with 150 μg/ml thrombin-cleaved DPW domain (*lanes e–h*) as indicated. After incubation at 4 °C for 60 min, the Sepharose beads were recovered by centrifugation. Aliquots corresponding to 1/200 of each supernatant (*S*) and 1/10 of each washed pellet (*P*) were resolved by SDS-PAGE and stained with Coomassie Blue. The positions of the molecular mass standards (in kDa) are indicated on the *left*.

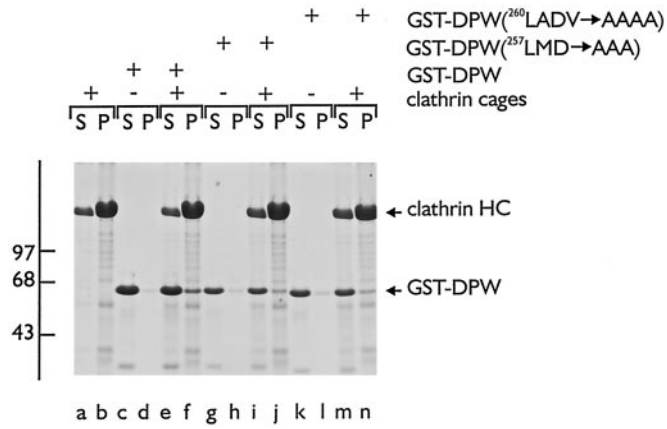

FIG. 6. **Interaction between GST-DPW and preassembled clathrin cages.** Samples of assembled clathrin cages (500 nM trimers) (*lanes a*, *b*, *e*, *f*, *i*, *j*, *m*, and *n*) and ~1.25 μM GST-DPW (*lanes c–f*), GST-DPW (^{257}LMD → AAA) (*lanes g–j*) and GST-DPW (^{260}LADV → AAAA) (*lanes k–n*) were prepared on ice in buffer A, pH 6.8, as indicated. After incubation at room temperature for 20 min, the clathrin cages were recovered by centrifugation, and equal aliquots of the resultant supernatant (*S*) and pellet (*P*) fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The positions of the molecular mass standards (in kDa) are indicated on the *left*.

acidic residue (Table I). Unfortunately, our initial attempts to determine whether the distal sequence also conveys clathrin binding were hampered by extensive degradation of either the DPW and NPF domains of epsin 1 or the carboxyl-terminal NPF domain alone fused to GST.[4] We also found that transient transfection of Myc epitope-tagged full-length epsin 1 inhibits the endocytic uptake of transferrin,[3] making it infeasible to probe the role of the clathrin-binding sequences by transfection. Instead, we compared the ability of these two short linear sequences to bind to soluble clathrin trimers when fused directly to GST. As positive controls, we have used the proximal ^{349}ETLLDLDF clathrin-binding sequence derived from amphiphysin I (38) and the sequence ^{447}RGYTLIDL from the *S. cerevisiae* epsin homologue Ent1p (YBL161w) (27). When mixed with brain cytosol, the amphiphysin-derived sequence binds to clathrin very efficiently (Fig. 8, *lane d*), as reported previously (38), leaving negligible amounts of clathrin in the supernatant (*lane c*). The yeast sequence behaves similarly (*lanes i* and *j*), but the structurally related distal epsin 1 sequence, AALVDLDS, recruits substantially less soluble clathrin (*lane h*). The proximal epsin 1 sequence, SSLMDLADV, alone binds only trace amounts of cytosolic clathrin, although this is above the background of the GST alone (*lane b*). The inability of the ^{257}LMDLADV sequence to bind to clathrin efficiently is in full agreement with the minimal association of clathrin with the GST-DPW (2 → STOP) construct (Fig. 7). When the two epsin 1 clathrin-binding sequences, each fused to GST, are mixed together, immobilized on glutathione Sepharose, and then incubated with brain cytosol, a significant increase in the amount of clathrin that binds to the beads relative to each sequence alone is seen (Fig. 8*B*, *lane f* compared with *lanes b* and *d*). Because the concentration of the proximal and distal sequences on the mixed beads (*lane f*) is only about half that of the unmixed beads (*lanes b* and *d*), the increase in clathrin binding seen on mixing must be due to cooperative association of clathrin trimers with the two epsin-derived sequences. Clathrin binding to the two epsin sequences together is still weaker than the binding to the amphiphysin sequence, in general agreement with the relative affinities of these proteins for the GST-TD (Fig. 5*A*).

DISCUSSION

Our study reveals that rat epsin 1, like amphiphysin, arrestin, and AP180, contains discrete binding determinants for both AP-2 adaptors and clathrin triskelia. These results extend the demonstration that intersectin-binding protein 2 (Ibp2; epsin 2) binds to clathrin (42) by showing that two clathrin-

[4] M. A. Downs and L. M. Traub, unpublished observations.

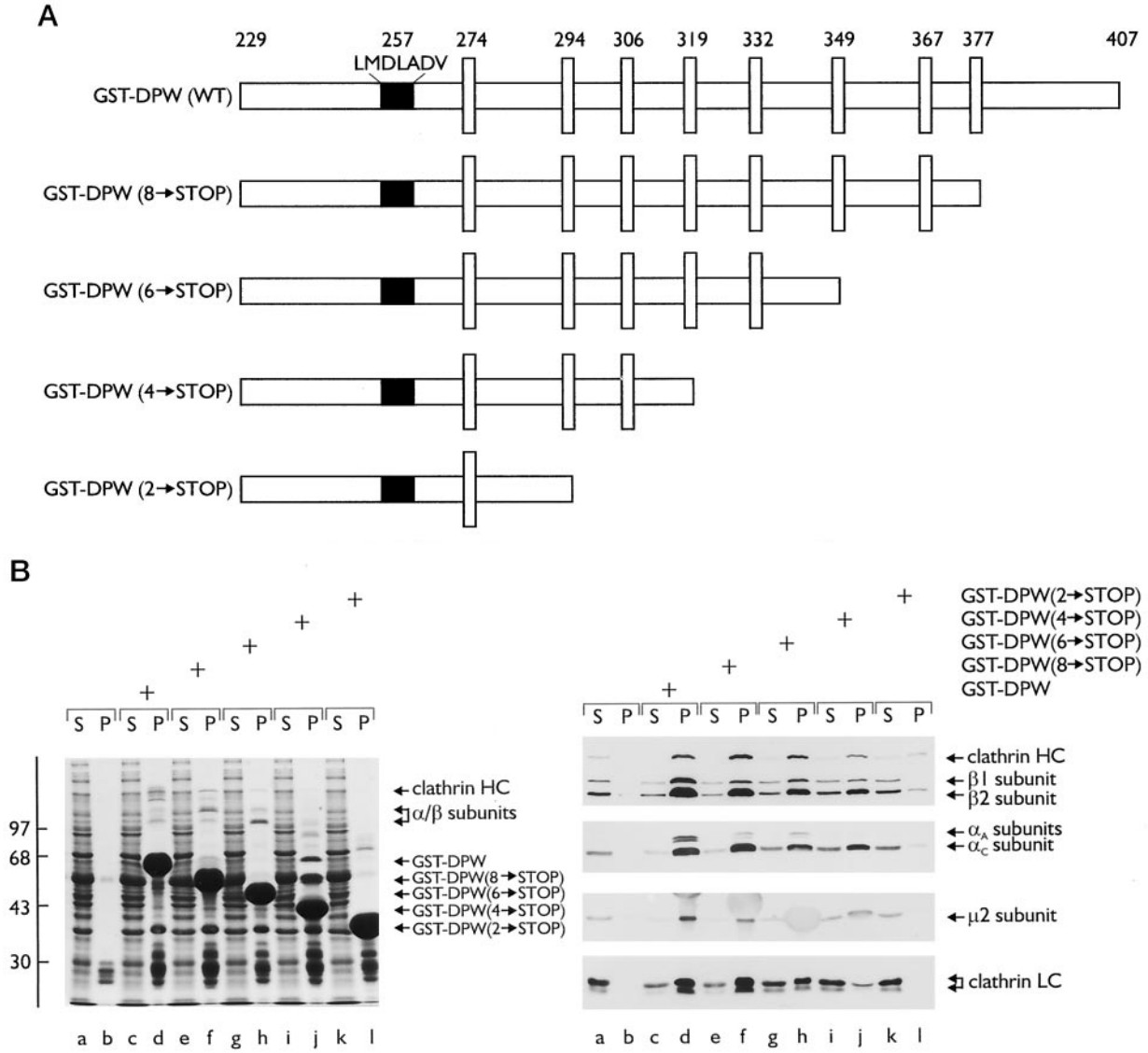

FIG. 7. **Association of clathrin and AP-2 with GST-DPW truncation mutants.** *A*, schematic illustration of the GST-DPW fusion proteins used. Each DPW triplet is demarcated by a *wide vertical box*, and the residue position of the aspartic acid of each triplet is indicated. The position of the clathrin-binding sequence LMDLADV is also shown. *B*, approximately 300 μg of GST-DPW (*lanes c* and *d*), GST-DPW (8 → STOP) (*lanes e* and *f*), GST-DPW (6 → STOP) (*lanes g* and *h*), GST-DPW (4 → STOP) (*lanes i* and *j*), or GST-DPW (2 → STOP) (*lanes k* and *l*) immobilized on ~50 μl of packed glutathione Sepharose or 50 μl of packed glutathione Sepharose alone (*lanes a* and *b*) was each mixed with rat brain cytosol to give a final concentration of ~7.5 mg/ml. After incubation at 4 °C for 60 min, the Sepharose beads were recovered by centrifugation. Aliquots corresponding to 1/200 of the supernatant (*S*) and 1/15 of each pellet (*P*) were resolved by SDS-PAGE and either stained with Coomassie Blue (*left panel*) or transferred to nitrocellulose. Portions of the blots (*right panel)* were probed with a mixture of the anti-clathrin HC mAb TD.1 and the anti-β1/β2 subunit mAb 100/1, the anti-AP-2 α subunit mAb 100/2, anti-AP-2 μ2 subunit antiserum, or the anti-clathrin LC mAb Cl57.3. The positions of the molecular mass standards (in kDa) are indicated on the *left*, and only the relevant portion of each blot is shown. Note that the ~180–68-kDa bands that decrease in size with the decrease in the number of DPW repeats (*lanes d*, *f*, *h*, *j*, and *l*) and obscure the clathrin and adaptor bands (*lanes d*, *f*, and *h*) are contaminants derived from the fusion protein preparations. The μ2 signal in *lane h* of the blot is obscured by the co-migrating GST fusion protein.

binding regions in epsin are involved. Both the sequences that we have delineated are similar to the leucine-aspartic/glutamic acid-based sequences found in several proteins known to play a role in the clathrin-coat assembly process and endocytosis (27, 37–39) (Table I), but we believe that each sequence within epsin 1 binds to clathrin at a different site. The association between clathrin and epsin is also likely to be physiologically relevant, and we believe that the localization of overexpressed epsin 1 to clathrin-coat structures at the TGN in Chinese hamster ovary cells (9) is evidence of this. We show that compared with AP-2, the DPW domain of epsin associates only poorly with AP-1 and minor amounts of endogenous epsin are found associated with the TGN at steady-state. The interaction between AP-2 and epsin requires the independently folded carboxyl-appendage domain of the α subunit (9). The crystal structure of the α subunit appendage shows that a binding site for the DPW triplet is created by a surface-exposed hydrophobic pocket located on the extreme carboxyl platform domain of the appendage (31, 32). The sequence of the analogous chain of AP-1, the γ subunit, displays little homology to the α subunit over the carboxyl-terminal portion (43), and based on the alignments (43) and our α-appendage crystal structure, it is unlikely that the γ subunit appendage is able to fold into a structure that contains both of the subdomains that comprise the functional α subunit appendage. Instead, we suspect that when overexpressed, epsin might begin to associate with assembled clathrin on the TGN via the clathrin-binding sites rather than interacting directly with the AP-1 adaptor complex alone.

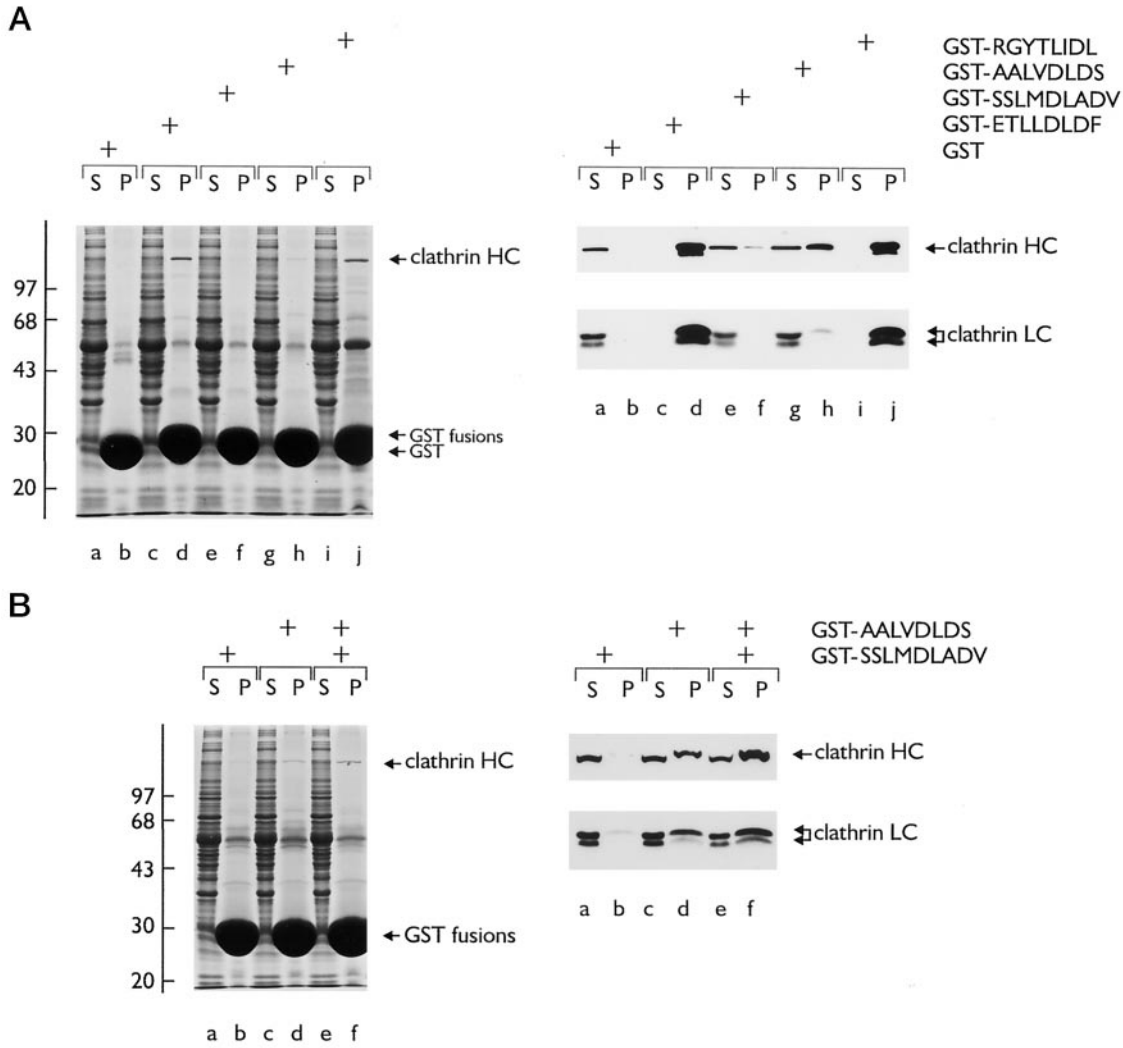

FIG. 8. **Binding of soluble clathrin to clathrin-binding sequences fused to GST.** A, approximately 350 μg of either GST (*lanes a* and *b*) or GST-ETLLDLDF (*lanes c* and *d*), GST-SSLMDLADV (*lanes e* and *f*), GST-AALVDLDS (*lanes g* and *h*), or GST-RGYTLIDL (*lanes i* and *j*) was first immobilized on ~50 μl of packed glutathione-Sepharose and then each mixed with rat brain cytosol to give a final concentration of ~7.5 mg/ml. After incubation at 4 °C for 60 min, the Sepharose beads were recovered by centrifugation. Aliquots corresponding to 1/200 of each supernatant (*S*) and 1/12 of each washed pellet (*P*) were resolved by SDS-PAGE and either stained with Coomassie Blue (*left panel*) or transferred to nitrocellulose. Portions of the blots (*right panels*) were probed with the anti-clathrin HC mAb TD.1 and the anti-clathrin LC mAb Cl57.3. The positions of the molecular mass standards (in kDa) are indicated on the *left*, and only the relevant portion of each blot is shown. *B*, approximately 350 μg of either GST-SSLMDLADV (*lanes a* and *b*) or GST-AALVDLDS (*lanes c* and *d*), or a 1:1 (w/w) mixture of GST-SSLMDLADV and GST-AALVDLDS (*lanes e* and *f*) was first immobilized on ~50 μl of packed glutathione Sepharose and then mixed with rat brain cytosol to give a final concentration of ~7.5 mg/ml. After incubation at 4 °C for 60 min, the samples were analyzed as in *A*.

A functional clathrin-binding sequence is also located at the carboxyl terminus of two *S. cerevisiae* epsin homologues, Ent1p and Ent2p (27). These yeast epsin-like proteins lack the central array of DPW triplets seen in higher organisms, and it is unclear at present whether they also bind to adaptor complexes directly as well. The clathrin-binding sequences delineated here in rat epsin 1 are absolutely conserved in the *Xenopus laevis* epsin homologue MP90 (42, 44). An epsin homologue in *Drosophila melanogaster* displays good conservation over both clathrin-binding sequences (Table I), which are separated by a region of the polypeptide encompassing six DPW triplets. In a *Caenorhabditis elegans* epsin, at least one of the sequences is sufficiently conserved to be likely able to associate with the clathrin-terminal domain (Table I). What the epsin family members do have in common then is a ~160-amino acid epsin N-terminal homology (ENTH) domain and the ability to bind to clathrin (45). These might constitute essential portions of the molecule, because deletion constructs encoding essentially these two elements, the epsin N-terminal homology (ENTH) domain and the clathrin-binding sequence of either Ent1p or Ent2p, can each rescue an *ENT1 ENT2* null yeast strain (27). The evolutionary conservation of clathrin-binding sites within the epsins again argues that this is an important aspect of epsin function.

Structural Requirements for Interaction with the Terminal Domain—Our results allow us to conclude that in the LLDLD-type clathrin-binding sequence, which we term the type I sequence, the second leucine residue is critical for high affinity binding. Substitution at this position with valine, as seen in the distal epsin 1 site, causes a major decrease in the ability to associate with free clathrin trimers. This suggests that the second or perhaps both the first and second leucine residue(s) (37, 39) interact with the clathrin-terminal domain in a hydrophobic pocket created at the outer edges of propeller blades 1 and 2 (41) and that valine is not of sufficient length to bind tightly at this site. Our *S. cerevisiae* Ent1p data show that isoleucine is able to replace leucine at the second position, although the yeast sequence is an unusual variation of the type

I consensus. Isoleucine is a rare choice at the second position of the known type I sequences (Table I). The critical importance of the first two leucine residues is reiterated by the demonstration that a synthetic proteosome inhibitor derivative, Leu-Leu-Leu(aldehyde), when immobilized on beads, is also able to capture clathrin trimers from cytosol (46). Because this interaction requires the clathrin heavy chain and not the light chains (46), we presume that the compound mimics part of the type I sequence and also binds directly to the terminal domain propeller. The final aspartic acid of the type I sequence can be replaced by the free carboxyl group of the preceding leucine because the yeast Ent1p sequence ^{451}LIDL(COOH) binds clathrin as well as the proximal ^{351}LLDLD sequence from amphiphysin I. The requirement for a free carboxylate is shown by the failure of the Ent1p sequence to bind clathrin when five alanines are appended to the end of the sequence (27).

A Second Binding Site on the Terminal Domain—Compared with the majority of characterized clathrin-binding motifs (type I sequences), the central regions of epsin 1 and 2 that we have shown also bind to clathrin are unusual in two respects. First, the aspartic acid residues within the motif have two intervening nonpolar residues instead of one, and second, the methionine present in both sequences is a rare choice of a hydrophobic amino acid (Table I). Once the precise molecular contacts between the type I clathrin-binding motif and the clathrin-terminal domain are established by crystallography, it should be possible to determine whether this difference in the epsin 1 and 2 sequences will preclude binding to the site between blades 1 and 2 of the clathrin-terminal domain. Our data compel us to conclude, however, that the proximal epsin sequence must bind elsewhere on the terminal domain surface. Otherwise, it is difficult to explain why this sequence exhibits a cooperative effect on clathrin binding together with two different type I sequences, the ^{632}LLNLD from the AP-2 β2 subunit and ^{480}LVDLD from the carboxyl segment of epsin 1. There is other experimental evidence for the existence of a second binding site on the terminal domain propeller. The central segment of amphiphysin II has two independent clathrin-binding sequences (38). The proximal one is a typical type I sequence (Table I), but the second, which seems to bind clathrin equally well (38), is dissimilar (^{416}IPWDLWEP) but reminiscent of the proximal epsin 1 ^{257}LMDLADV sequence as again there are two intervening hydrophobic residues between two acidic amino acids. The internalization signal within the cytosolic domain of the LDL receptor, ^{804}NPVY, is also reported to interact with the clathrin-terminal domain, apparently while adopting a β-tight turn conformation (47). Additional work will be needed to identify and characterize a second binding site on the terminal domain propeller, but it is worth mentioning that the β subunit of heterotrimeric G proteins, which also folds into a seven-bladed β propeller (48), associates with the G protein α subunit and effector molecules (adenylate cyclase and phospholipase C) by utilizing different surfaces of the toroidal structure (49).

Amphiphysin I and II (38) and β-arrestin 1 and 2 (8) reportedly interact with clathrin with nanomolar affinities. We have made no attempt to determine binding constants in this study. This is because we have shown that the binding we observe in our assays is heavily dependent on the way they are performed. The epsin DPW domain will recruit free clathrin trimers if immobilized on Sepharose beads at high density. Binding of soluble GST-DPW to immobilized terminal domain or to assembled clathrin cages is considerably weaker, and binding of monomeric epsin DPW is relatively poor. These results illustrate that the nature of the binding assay affects the extent of the interaction between epsin and clathrin observed. Because cytosolic clathrin is a three-legged structure, at high densities of GST-DPW, all three terminal domains of a trimer could associate with the immobilized epsin DPW domain and the associated AP-2. Even if the off rate of these interactions is reasonably high, the probability of all three terminal domains detaching at once would be low. This likely explains why binding of clathrin trimers to the central portion of epsin is best seen when cytosol is mixed with GST-DPW Sepharose. Even when the proximal and distal clathrin-binding sites are combined, however, the relative affinity of epsin 1 for clathrin is weaker than that of amphiphysin. Epsin 1 contains eight DPW repeats within the central segment of the protein and rat epsin 2 has three, as well as several redundant DX(W/F) sequences. Amphiphysin, on the other hand, has only a single DPF triplet and, consequently, has a lower affinity for AP-2 than epsin (32). The *S. cerevisiae* Ent1p and Ent2p lack DPW/F repeats entirely. It is possible that the different relative affinities that these proteins display for clathrin and AP-2 might affect how, when, and where they are incorporated into the assembling lattice.

AP180 was identified on the basis of clathrin binding and polymerization properties (30). Several discrete regions of the AP180 molecule bind to clathrin (50–52), but only the 58-kDa carboxyl segment has cage assembly activity (52). Although the precise molecular basis for the interaction between AP180 and clathrin remains to be determined, it is known that AP180 requires an intact terminal domain on the clathrin trimer for productive association with assembled cages (53). Type I-like clathrin-binding sequences are discernible in both AP180 and CALM, the ubiquitous non-neuronal form of the protein (54, 55) (Table I). These sequences are found in the amino-terminal portion of each protein, regions of both AP180 and CALM that act in concert with the respective carboxyl-terminal region to elicit maximal clathrin-binding activity (52, 55). Further mutagenesis and structural studies should determine whether the type I-like sequences in AP180 are involved in clathrin binding. Nevertheless, because several endocytic proteins do display multiple clathrin-binding sequences within a single molecule (Table I), one obvious role for the two different sites could be to ensure a specific orientation of the molecule within the assembling lattice relative to the terminal domain. Thus, the clathrin-binding sites defined here, in addition to the AP-2 and eps15/intersectin/POB1-binding sites on epsin, might orient epsin appropriately within the lattice. Unlike AP-2 and clathrin, neither epsin nor amphiphysin are enriched in preparations of clathrin-coated vesicles (9, 38),[3] which indicates that these proteins must largely exit the latticework before the final detachment event. A major challenge for the future is to understand how these proteins, with multiple binding sites for lattice components, are able to associate reversibly with the lattice over the short time frame of the budding reaction.

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