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Interaction of two structurally-distinct sequence types with the clathrin terminal domain β-propeller

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Running title: endocytic protein interactions with clathrin

Abbreviations

amphII,	amphiphysin II
GST,	glutathione *S*-transferase
HC,	heavy chain
LC,	light chain
PAGE,	polyacrylamide gel electrophoresis
WT,	wild type

Summary

The amino-terminal domain of the clathrin heavy chain, which folds into a seven-bladed β-propeller, binds directly to several endocytic proteins via short sequences based on the consensus residues LLDLD. In addition to a single LLDLD-based, type-I clathrin-binding sequence, both amphiphysin and epsin each contain a second, distinct sequence that is also capable of binding to clathrin directly. Here, we have analyzed these sequences, which we term type-II sequences, and show that the ^{257}LMDLA sequence in rat epsin 1 appears to be a weak clathrin-binding variant of the sequence ^{417}PWDLW originally found in human amphiphysin II. The structural features of the type-II sequence required for association with clathrin are distinct from the LLDLD-based sequence. In the central segment of amphiphysin, the type-I and type-II sequences cooperate to effect optimal clathrin binding and the formation of sedimentable assemblies. Together, the data provide evidence for two interaction surfaces upon certain endocytic accessory proteins that could cooperate with other coat components to enhance clathrin-bud formation at the cell surface.

INTRODUCTION

The most characteristic feature of assembled clathrin is the polygonal appearance of the coat. This largely reflects the geometric arrangement of the clathrin molecule. During biosynthesis, three ~190-kDa clathrin heavy chains trimerize via the carboxyl-terminal end to form a characteristic triskelion. A ~25-kDa light chain also binds to each heavy chain, near to the site of trimerization, the central vertex. Each heavy chain projects out radially from the vertex, the three legs of the trimer splayed approximately 120° apart. The triskelion leg is a relatively rigid structure, composed of tandemly-stacked repeats of α zigzags (1,2). Lateral packing of the adjacent helix pairs generates an extended linear rod, interrupted by a kink roughly halfway along the length (3). Clathrin trimers also display an inherent sidedness (4). When viewed from above, the kink redirects the distal segment of each leg clockwise and down, positioning the globular ~350-residue amino-terminal portion, the terminal domain, of each heavy chain below the plane of both the vertex and the proximal portion of each leg (5).

As clathrin trimers begin to associate to form a coat, packing occurs by the antiparallel apposition of the proximal portion of one triskelion leg with the proximal segment of an adjacent leg. The distal segments of two other assembling trimers pack below the antiparallel proximal-leg pair (6). This leads to the generation of the hexagonal and pentagonal facets that typify the clathrin coat. Truncated trimers termed clathrin hubs, composed of roughly the carboxyl-terminal third of the heavy chain, can also assemble into pseudolattices at reduced pH in the presence of calcium (7). These assemblies do not display the characteristic curvature seen in clathrin coats however, and do not form closed spherical structures (7). As heavy chain–heavy chain leg interactions are relatively weak at physiological pH, these observations point to the amino-terminal region of the heavy chain governing clathrin-coat assembly on membranes. Indeed, when the truncated hubs are mixed with a preparation of the first 1074 residues of the heavy chain, comprising the terminal domain and the distal leg, assembly of spherical coats becomes evident (8). The three-fold symmetry of intact clathrin results in the positioning of three terminal domains,

each from a separate trimer, under each hub in the polymerized state (6). It appears that it is the contact between proximal leg segments and the distal leg domains that emanate from sub-vertex terminal domains, termed a counter-hub, that orient trimers to productively form polygons (8). The clustering of terminal domains into trimers (counter-hubs) on the membrane is likely then to be an important aspect of clathrin assembly.

At the cell surface, the terminal domain is thought to be tethered to the bud site by direct interaction with the β2 subunit of the AP-2 complex. Specifically, the short sequence ^{632}LLNLD, found within the flexible hinge that separates the globular β2-subunit appendage from the adaptor core, interacts in an extended conformation with a shallow cleft between blades 1 and 2 of the 7-bladed terminal domain propeller (9). This interaction is weak however (10), and by itself will not multimerize adjacent terminal domains. Purified AP-2 has a strong propensity to aggregate (11,12), a property which has been suggested to facilitate clathrin crosslinking (3,8,13).

Several endocytic accessory proteins also interact with the clathrin terminal domain β-propeller directly. These include AP180 (14-16), amphiphysin (16) and epsin (15,17). Intriguingly, each of these proteins also binds directly to the appendage domain of the AP-2 α subunit (18,19), raising the possibility that these clathrin-binding sequences could work in conjunction with the AP-2 β2-subunit sequence to improve clustering of clathrin at the bud site. In fact, there is already some experimental evidence to support this idea. Complexing AP-2 with AP180 leads to enhanced clathrin assembly activity (20), and we have previously shown that AP-2 recruitment to the central DPW region (residues 249–401) of epsin 1 augments clathrin binding mediated by the epsin sequence ^{257}LMDLADV (15). Here, we have further characterized this epsin sequence, and a related clathrin-binding sequence from amphiphysin, PWDLW (21). We show that juxtaposing multiple clathrin-binding sites does increase the apparent affinity for soluble clathrin trimers. A principal role for clathrin-binding sequences within various endocytic accessory proteins indeed appears to be to improve the

efficiency of clathrin-coat polymerization at bud sites on the cell surface.

EXPERIMENTAL PROCEDURES

Construct preparation —The generation of the glutathione *S*-transferase (GST)-ETLLDLDF and GST-LMDLADV fusions has been described (15). GST-TLPWDLWTTS, the distal amphiphysin I sequence, GST-SIPWDLWEPT, the distal amphiphysin II sequence, GST-ASDYQRLNLK, a TGN38 internalization sequence and GST-SYKYSKVNKE, an internalization sequence from the cation-independent mannose 6-phosphate receptor were all prepared analogously by ligation of complementary oligonucleotides, after annealing and digestion, into *Eco*RI / *Xho*I cleaved pGEX-4T-1. Alterations to these sequences were generated using QuikChange mutagenesis (Stratagene). A construct of residues 1-579 of the bovine clathrin heavy chain fused to GST was provided by Jim Keen. A smaller segment of the terminal domain, corresponding to the seven-bladed β propeller and the first α zigzag (residues 1-363) (9) was generated by using QuikChange to convert the codon for residue 364 to a TAA stop codon. Constructs of the insert domain of human amphiphysin II (residues 329-444) in pGEX-2TK, and the same sequence harboring a deletion of residues 390-397 (Δ390-397 / deletion 1; (21)) were kindly provided by Peter McPherson. The intact insert domain construct was used to prepare the Δ400-411 deletion using QuikChange mutagenesis. All of the constructs and mutations were verified by automated dideoxynucleotide sequencing.

Protein purification—GST and the various GST-fusion proteins were produced in *E. 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 hours at room temperature with constant shaking, the bacteria were recovered by centrifugation at 15,000 × g_{max} 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 23,700 × g_{max} at 4°C for 15 min. After extensive washing in PBS, GST fusions were eluted with 10 mM Tris-HCl, pH 8.0, 10 mM glutathione, 5

mM DTT on ice and dialyzed into PBS, 1 mM DTT before use in binding assays. The terminal domain was cleaved from the GST with thrombin (Amersham Pharmacia Biotech) while still immobilized on glutathione Sepharose. Digestion was as recommended by the manufacturer, followed by addition of the irreversible thrombin inhibitor PPACK (Calbiochem) to a final concentration of 25 μM. The terminal domain was then chromatograpically purified at 4°C on a Sephacryl S-100 column (1.6 × 60 cm) at 0.5 ml / min and peak fractions containing the terminal domain concentrated to 1 mg / ml using a Centricon 10 device.

The mouse α_C-subunit appendage (residues 701-938) cloned into pGEX-2T was kindly provided by Richard Anderson and the DPW domain of rat epsin 1 (residues 229-407), cloned into pGEX-4T-1, was kindly provided by Pietro DeCamilli. The GST-fusion proteins were purified and thrombin cleaved as described previously (15). The rat β2-subunit appendage + hinge (residues 592-951) cloned into pRSETc was kindly provided by Tom Kirchhausen and purified on NTA agarose as described (13).

Rat brain cytosol was prepared from either fresh or frozen rat brains (PelFreez) exactly as described previously (22). Before use, the cytosol was adjusted to 25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, 2 mM EDTA, 2 mM EGTA and 1 mM DTT (assay buffer) and centrifuged at 245,000 × g_{max} (TLA-100.3 rotor) at 4°C for 20 min to remove insoluble material. For the preparation of purified cytosolic clathrin, soluble trimers were collected preparatively with GST- ETLLDLDF bound to glutathione Sepharose and then eluted batch wise with 1.0 M Tris-HCl, pH 7.0, 1 mM DTT. Eluted clathrin was dialyzed into assay buffer and, after addition of carrier BSA to 0.1 mg / ml, centrifuged at 15,000 × g_{max} at 4°C for 20 min prior to use in binding assays. The cytosol recovered after incubation with the GST-ETLLDLDF Sepharose was used as clathrin-depleted cytosol.

Binding assays—The association of clathrin, adaptors and other endocytic

accessory proteins with the various GST-fusion proteins was performed in assay buffer in a final volume of 300 µl. Routinely, GST and the GST-fusion proteins were first each immobilized on 20 µl packed glutathione Sepharose by incubation at 4°C for 2 hours with continuous mixing. The immobilized proteins were then washed and resuspended to 50 µl in assay buffer. Rat brain cytosol or purified clathrin was added and the tubes incubated at 4°C for 60 min with continuous gentle mixing. The Sepharose beads were then recovered by centrifugation at 10,000 × g_{max} at 4°C for 1 min and 40 µl aliquots of each supernatant removed and adjusted to 100 µl with SDS-sample buffer. After washing the Sepharose pellets 4 times each with ~1.5 ml ice-cold PBS by centrifugation, the supernatants were aspirated and each pellet resuspended to a volume of 80 µl in SDS-sample buffer. Unless otherwise indicated, 10 µl aliquots, equivalent to ~1/80 of each supernatant and 1/8 of each pellet, were loaded on the gels.

For assays examining recruitment of the terminal domain (residues 1–363), the α_C-subunit appendage (residues 701-938) or the β2-subunit appendage + hinge (residues 592-951), the purified preparation was first centrifuged at 10,000 × g_{max} at 4°C for 10 min to remove insoluble material. The binding assays were in assay buffer with the proteins added to the final concentrations indicated in the figure legends with 0.1 mg/ml BSA added as a carrier. For the clathrin assembly assays, reactions were also prepared in assay buffer in a final volume of 100 µl. All protein samples were centrifuged at 245,000 × g_{max} at 4°C for 20 min prior to use. Tubes containing 350 µg/ml (~0.5 mM) purified cytosolic clathrin or 125 µg/ml (~2.5 mM) GST-amphII insert or GST-amphII (Δ390-397) were prepared on ice and then incubated at ~15°C for 30 min. After centrifugation at 100,000 × g_{max} (TLA-100.3 rotor) at 4°C for 15 min, 50 µl aliquots of each supernatant was removed and adjusted to 100 µl with SDS-sample buffer. Each pellet was resuspended to a volume of 40 µl in SDS-sample buffer and 10 µl aliquots, equivalent to ~1/20 of each supernatant and 1/4 of each pellet, were loaded on the gels.

Electrophoresis and immunoblotting—Samples were resolved on polyacrylamide

gels prepared with an altered acrylamide:bis-acrylamide (30:0.4) ratio stock solution. The decreased crosslinking 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 7.8, 150 mM NaCl, 0.1% Tween 20 and then portions incubated with primary antibodies as indicated in the individual figure legends. After incubation with HRP-conjugated anti-mouse or anti-rabbit IgG, immunoreactive bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech).

Antibodies—The anti-α-subunit mAb 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 clathrin heavy chain mAb TD.1, that recognizes the clathrin terminal domain, was kindly provided by Frances Brodsky and the antibody specific for the clathrin light chain neuronal-specific insert, mAb Cl57.3, was from Reinhard Jahn. The preparation of the affinity-purified anti-AP-1 γ-subunit antibody, AE/1 (23), anti-μ1-subunit antibody, RY/1 (23), the anti-AP-3 δ-subunit antibody, KQ/1 (24) and the epsin 1 antibodies (15) have been described. The pan-arrestin mAb F4C1 was kindly provided by Larry Donoso and the monoclonal antibodies specific for amphiphysin and AP180 were purchased from Transduction Laboratories.

RESULTS

Two distinct classes of clathrin-binding sequence—Amphiphysin (16,21) and epsin (15) each contain two discrete clathrin-binding sequences. In both proteins, one of these conforms very closely to the type-I clathrin-binding consensus L(L / I)(D / E / N)(L / F)(D / E) (25), which has also been termed the clathrin box (9). Like the type-I sequences, the second clathrin-binding site mapped in both amphiphysin (PWDLW) (16,21) and epsin 1 (LMDLADV) (15,17) also contains alternating hydrophobic and acidic residues, but the spacing and chemical nature of the side chains is different. The proximal, type-I sequence from rat amphiphysin I, ^{349}ETLLDLDF, when immobilized at high density as a GST-fusion protein, associates with clathrin very efficiently (15), and binding to soluble clathrin trimers present in brain cytosol is near quantitative (Fig. 1, lane d). No clathrin associates with GST immobilized under similar conditions (lane b). The distal clathrin-binding sequence from rat amphiphysin I, ^{379}TLPWDLWTTS, alone also binds avidly to soluble clathrin (lane f), as does the analogous sequence from rat amphiphysin II, ^{415}SIPWDLWEPT (lane h).

In addition to clathrin, a group of ~100-kDa polypeptides unexpectedly associate with the GST-PWDLW-based fusion proteins following the incubation with cytosol (Fig. 1A, lane f and h). Subunit-specific antibodies identify these proteins as the α, β1, β2 (Fig. 1B) and γ subunits (Fig. 2) of the AP-1 and AP-2 adaptor complexes. The respective μ subunits, μ1 and μ2, are also present (Fig. 1B), confirming that the adaptors bind as functional heterotetrameric complexes. The recovery of the adaptors occurs exclusively with the PWDLW-type sequences and not with the GST-ETLLDLDF (lane d), which is present at equivalent density on the Sepharose beads and which binds similar amounts of soluble clathrin. Interestingly, neither the AP-3 adaptor complex nor several other clathrin-binding endocytic-accessory proteins, including AP180, amphiphysin (Fig. 1C, lane f and h) and arrestin (Fig. 2) are recovered together with the bound clathrin. A trace amount of epsin 1 (<2% of input) associates selectively with the ^{379}TLPWDLWTTS sequence derived from amphiphysin I (Fig. 1C, lane f) but not with the related sequence from amphiphysin II (lane h).

Clathrin binding to the PWDLW sequence does not require adaptors—The hydrophobic nature of the PWDLW-type sequence prompted us to first consider the possibility that both AP-1 and AP-2 are able to recognize the sequence WDLW as an atypical variant of the YXX motif that binds directly to the μ subunit of the adaptor heterotetramer (26-28) and then recruit clathrin secondarily. Several lines of evidence argue against this idea. First, titration of the amphiphysin II-derived GST-^{415}SIPWDLWEPT fusion protein on a fixed amount of glutathione Sepharose shows that the binding profiles of clathrin and adaptors differ (Fig. 2A). While both protein complexes exhibit relatively steep binding curves, a substantial amount of cytosolic clathrin translocates onto the Sepharose under conditions where much of the AP-1 and AP-2 adaptor pools still remain soluble (lane g-j). Second, authentic tyrosine-based sorting signals do not bind to soluble adaptors efficiently in this type of assay. Direct comparison of the ability of two well characterized μ-subunit-binding sequences, ^{347}ASD**YQRL**NLK found in rat TGN38 (26) and ^{2359}SYK**YSKV**NKE from the bovine cation-independent mannose 6-phosphate receptor (29), to recruit soluble adaptors reveals that, when equivalently appended to GST, these sequences are extremely poor compared to the PWDLW sequence (data not shown). Finally, in other experiments, we have found that near quantitative immunodepletion of AP-2 from cytosol (30) has no effect on clathrin binding (not shown) and, more importantly, at similar concentrations, purified cytosolic clathrin can bind to GST-PWDLW fusions (Fig. 2B, lane f) as efficiently as the clathrin present in whole cytosol (lane h), despite the total absence of adaptor complexes. Under these conditions, the purified cytosolic clathrin does not interact with immobilized GST (lane b) but does bind avidly to GST-ETLLDLDF (lane d). These experiments show clearly that soluble clathrin trimers do not require adaptors to bind to an immobilized PWDLW-based sequence. The data then, confirm independently the presence of two functionally separate clathrin-binding sequences in amphiphysin (16,21). In addition, as there is little difference in the binding of clathrin to the PWDLW sequence derived from either amphiphysin I or II (Fig. 1), Glu422 in amphiphysin II appears dispensable. Thus, the distal sequence, which we term type-II, appears to display only a single

acidic residue in the middle of the motif (see below), unlike the type-I, LLDLD-based sequences.

To determine whether the recovery of the adaptor complexes on the GST-PWDLW beads is dependent upon bound clathrin, we compared AP-1 and AP-2 binding from either whole or clathrin-depleted rat brain cytosol (Fig 3A). Decreasing soluble clathrin levels more than fivefold blunts clathrin binding to the PWDLW sequence substantially but, surprisingly, does not affect the adaptor interaction (lane d compared to lane b). This shows that AP-1 and AP-2 bind to the PWDLW sequence directly. The DPW domain of epsin 1, which contains eight repeats of the tripeptide sequence Asp-Pro-Trp, and binds directly to both the α and β-subunit appendages (31), compromises the binding of AP-1 and AP-2 to GST-PWDLW when added to the depleted cytosol without perturbing the residual clathrin interaction (lane f compared to lane d). This illustrates that clathrin and adaptor binding to PWDLW are independent and suggests that the appendage domains of the adaptor heterotetramer likely mediate the interaction between AP-1 and -2 and the PWDLW sequence. In fact, direct association of both the α_C- and β2-subunit appendage with GST-PWDLW does occur upon simple mixing (Fig. 3B). The higher apparent affinity that the α_C appendage displays for the PWDLW sequence (lane d and f compared to lane j and l), and the lower relative concentration of AP-1 in our rat brain cytosol preparations likely explains the difference in the AP-1 and AP-2 binding curves (Fig.2A).

A type-II-like sequence in epsin 1—Alone, the proximal clathrin-binding sequence in rat epsin 1, ^{257}LMDLADV (15,17), binds to clathrin only poorly in pull-down type assays (Fig. 1, lane j). We have speculated previously (15) that this sequence might be related to the PWDLWE sequence in amphiphysin II and accordingly, mutation of this sequence to L**W**DL**W**DV results in a major increase in the clathrin-binding capacity (lane l). This fusion protein now behaves like the distal amphiphysin type-II-sequence fusions (lane f and h). Notably, the replacement of the Met and Ala residues with Trp again results in the recovery of adaptor complexes, along with the clathrin, with the pelleted Sepharose beads (lane l).

Structural features of the type-II sequence—The increased clathrin binding we observe with the GST-SSLWDLWDV substitution points to either or both Trp residues being critical elements of a type-II clathrin-binding sequence. To address the relative role of each of these residues within a type-II motif, we mutated each separately in the context of the amphiphysin II-based GST-SIPWDLWE fusion. Introduction of an Ala at either position ($W_1 \rightarrow A$ or $W_2 \rightarrow A$) completely abolishes clathrin-binding capacity (Fig. 4A, lane c and g compared to lane b). This substantiates the essential role that both these aromatic residues play in binding to clathrin. Replacement of the Trp residues with Phe has different effects. Phe is well tolerated at the distal position ($W_2 \rightarrow F$; lane h), but substitution of the first Trp in the PWDLW sequence with Phe ($W_1 \rightarrow F$) causes a substantial (~5 fold) decrease in clathrin binding (lane d). The impaired capacity of the $W_1 \rightarrow F$ mutant does not appear to reflect that hydrogen bonding is vital for a productive association with clathrin because replacement of the proximal Trp residue with either Tyr ($W_1 \rightarrow Y$) or His ($W_1 \rightarrow H$) ablates clathrin binding almost to the same extent as an Ala substitution (lane e and f).

We also tested the ability of different aliphatic side chains to substitute for the proximal Trp residue (Fig. 4B). Valine ($W_1 \rightarrow V$; lane h) is totally ineffective. Longer aliphatic residues ($W_1 \rightarrow M$, lane f; $W_1 \rightarrow L$, lane j; and $W_1 \rightarrow I$, lane l) do facilitate some clathrin binding, but to a level at least 20-fold lower than the wild-type sequence (lane d). Leucine (lane j) and methionine (lane f) appear to facilitate somewhat better clathrin recruitment than isoleucine (lane l). The weak binding afforded by the aliphatic residues might be because they were introduced in the context of the amphiphysin type-II sequence, where the preceding Pro is a relatively weak hydrophobic partner. Nevertheless, in sum these experiments suggest that the poor affinity the rat epsin 1 ^{257}LMDLADV sequence displays for clathrin in pull-down assay is likely due to the combined presence of aliphatic rather than aromatic side chains at position -1 and +2 relative to the Asp residue at the center of the sequence. The central portion of rat epsin 2, with the sequence ^{283}LLDLM, also binds clathrin relatively poorly (15,17).

The central acidic residue—The role of the acidic residue was similarly analyzed by mutagenesis of the Asp residue in the amphiphysin II-derived GST-SIPWDLWEPT construct (Fig. 5). Glu (D→E, lane h) substitutes efficiently for the wild-type Asp (lane d). Ala (D→A, lane f) is also tolerated, with clathrin binding reduced only about 50%. Charge reversal (D→K, lane j) however, is more detrimental, reducing clathrin recruitment about 5 fold. For comparison, in the type-I clathrin-box sequence, the comparable Glu residue is reported to be dispensable. In fact, individually changing either acidic residue in ^{373}LIEFE, the type-I sequence in bovine β-arrestin 2 (arrestin 3), to Lys results in only a ~30% decrease in clathrin binding (32). On conversion of the sequence to LI**K**F**K**, clathrin association is reduced roughly 60% (32). The crystal structure of the LIEFE peptide bound to the terminal domain (9) reveals that the side chain of the central Glu is fully solvent exposed and does not appear to interact directly with clathrin. Our data demonstrate clearly then that the chemical properties of the type-II sequence necessary for efficient clathrin binding are distinct from those of the type-I sequences (9,25,32).

Synergism between type-I and type-II clathrin-binding sequences—In rat epsin 1, we have shown that the sequence ^{257}LMDLADV can cooperate with a distal type-I sequence, ^{480}LVDLD, to improve clathrin binding (15). To determine whether cooperativity between type-I and type-II clathrin-binding sequences is a general phenomenon, we assessed the effect of mixing the two sequences, each fused to GST, on binding to recombinant clathrin terminal domain. Each sequence alone (Fig. 6, lane d and f) interacts with the monomeric terminal domain segment in the pull-down type assay, demonstrating clearly that the type-II sequence, like the type-I sequences, binds directly to the amino-terminal portion of the clathrin heavy chain. A weak synergistic effect on terminal-domain binding occurs on mixing the type-I and type-II sequences (Fig. 6, lane h and j). While these effects appear cooperative because recruitment of clathrin onto the mixed sequences is more than additive (lane h and j compared to lane d and f), the magnitude of the synergy is small. This might reflect steric constraints imposed by the short

linkers in our GST fusions and / or the positioning of putative clathrin-binding sites on the terminal-domain propeller surface.

To probe cooperativity between the proximal and distal sequences of amphiphysin in a more physiological setting, we examined the central portion (residues 329-444) of human amphiphysin II. This region of amphiphysin II, which is subject to alternative splicing (21,33,34), has been termed the insert domain (21). Fused to GST, this segment, which contains both the ^{390}LLDLD and ^{416}PWDLW sequences, binds efficiently to soluble clathrin trimers (16,21) (Fig. 7, lane f-i). Cooperativity between the two sequences was probed by simply deleting 12 residues (Δ400-411) of the sequence between the proximal type-I and distal type-II sequence. Even though both clathrin-binding sequences remain intact, alteration of their relative spacing diminishes the apparent affinity for clathrin. On titration, the clathrin-binding curve of the Δ400-411-fusion protein (lane f-i) is clearly different than the intact insert domain (lane b-e). The effect of reducing the spacing between the type-I and-II sequences is most pronounced at lower concentrations of the immobilized GST fusions (lane b and c compared to lane f and g). At high concentrations, the Δ400-411-fusion protein associates with more AP-2 (lane h and i) than the intact insert domain (lane d and e), and also begins to bind to AP-1, suggesting that at this density clathrin binding is similar to that of the individual PWDLW sequence. Our interpretation of this data is that two distinct sequences, the type I and type II, are able to noncompetitively engage clathrin.

Polymerization of clathrin by adjacent type I and type II sequences—To test the biological significance of adjacently arranged clathrin-binding sequences, we assessed the ability of the amphiphysin II-insert domain GST fusion to assemble soluble clathrin. When added at a 5-fold molar excess over purified cytosolic clathrin, roughly 70-80% of the total clathrin sediments together with a fraction of the GST-insert domain (Fig. 8, lane f). At pH 7.2, this bulk recovery of clathrin in the pellet fraction is dependent upon the combined presence of clathrin and the GST-fusion protein. Alone, only about 20% of the clathrin pool (lane b) and a

trace of the GST-amphiphysin II insert (lane d) sediments under similar conditions. Deletion of the type-I ^{390}LLDLD sequence (Δ390-397) from this construct substantially diminishes the efficiency with which soluble clathrin is assembled into a sedimentable form (lane j). As GST is a dimer in solution (35), these results argue that the presence of two discrete but adjacent clathrin-binding sequences improves the apparent affinity for clathrin and enhances assembly activity. Amphiphysin I and amphiphysin II are known to form both homo- and heterodimers (34,36) adding biological relevance to our observations.

DISCUSSION

We have characterized a second type of clathrin-binding motif, the type-II sequence, and provide evidence that this sequence, like the type I, associates directly with the terminal domain of the clathrin heavy chain. A repetitive motif, based on the core triplet sequence Asp-Leu-Leu / Phe (DLL / F), has also been shown to bind the clathrin terminal domain recently (37). An important question that arises then is whether the type-I and type-II (and possibly other) clathrin-binding sequences display simple quasi-equivalence in their ability to engage the shallow hydrophobic groove between propeller-blades 1 and 2 of the terminal domain, the region known to interact with the type-I, LLDLD-based sequence (9). The different chemical properties of the type-II sequence argue that the sequences are not structurally equivalent. In particular, the critical importance of the proximal Trp in the PWDLW sequence and the inability of aliphatic hydrophobic residues to efficiently substitute for this side chain distinguishes this sequence from the type-I clathrin box. None of the 12 DLL / F-type repeats in mouse AP180 (37) contains a Trp immediately preceding the DLL sequence. Several of the repeats do have a Leu, Ile, or Val residue at this position however, and two have the sequences ^{327}PVDIF and ^{636}VIDLF. Unfortunately, there are no data available on the relative affinities of these sequences for clathrin compared to the PWDLW. Moreover, although there is a correlation between the number of DLL / F repeats in AP180 and clathrin assembly activity (37), it is not known yet whether only some or all of these putative sites bind the terminal domain propeller and whether this must occur simultaneously. The hinge region of the AP-1 γ subunit also binds clathrin directly (38). The sequence LLDLL, repeated twice within the flexible hinge segment that separates the globular β1 appendage from the adaptor core, is involved in clathrin binding directly (38). Like PWDLW and LMDLA, these additional clathrin-binding sequences lack the hallmark clathrin box terminal acidic side chain that interacts electrostatically with terminal domain propeller residues Arg64 and Lys96 (9). These chemical differences might all be accommodated by the known binding site on the terminal domain β-propeller, or could possibly dictate association with a

different surface(s) of the terminal domain β-propeller, as already suggested by others (9,37). A definitive answer to whether these distinct sequences engage discrete binding surfaces on the terminal domain may come from further co-crystallization studies.

We can conclude from our experiments, however, that appropriately spaced type-I and type-II sequences in amphiphysin do improve the apparent affinity for clathrin in pull-down type assays and facilitate clathrin assembly. An even more marked synergistic effect of the type-I and type-II sequences on clathrin recruitment has also recently been observed by others. Deletion constructs within the region of amphiphysin I that contains both the LLDLD and PWDLW sequences, and mutagenesis of these sequences, also revealed that both are required for high-affinity association with clathrin (16). Superficially, this result appears discordant with our observation that either sequence alone can bind clathrin from cytosol near quantitatively (Fig. 1). The most likely explanation for the observed difference is the higher density at which we immobilize the GST-fusion proteins on Sepharose for our assays. At low density, when the clathrin-binding peptides are likely too distantly spaced to simultaneously engage a single clathrin trimer, the apparent affinity could increased in the GST-amphiphysin insert domain by having tandemly apposed type-I and -II sequences that might associate with a single terminal domain in a bivalent, intraleg fashion. Alternatively, if only a single binding site exists upon the terminal domain propeller, then efficient recruitment of clathrin by lower levels of immobilized amphiphysin insert domain might be due to the clustering of two terminal domains from different trimers, allowing stabilizing interactions involving the legs to occur (Fig. 8). By contrast, at high density, the isolated peptide sequences are arrayed closely enough on the Sepharose to permit interleg cooperativity by engaging two or three of the terminal domains of a single clathrin trimer at once. As we have suggested before (15), even if dissociation of the clathrin–peptide interaction is relatively rapid, the probability of all three terminal domains detaching simultaneously would be low. The substantially reduced interaction we see between the expressed monomeric clathrin terminal-domain fragment (although added at a much higher

concentration than clathrin present in cytosol) and the type-I or type-II sequences (Fig. 5) further supports this interpretation.

When incubated with whole cytosol, an immobilized PWDLW type-II sequence associates with AP-1 and AP-2 adaptors as well. The interaction of clathrin or adaptors with this sequence does not require the presence of the other and, since the GST-fusion protein is in considerable excess in our experiments, the interactions occur independently. This is borne out by the different binding curves clathrin and adaptors exhibit (Fig. 2A). We demonstrate that the binding of AP-1 and AP-2 probably occurs via the appendage domains because the interaction is strongly inhibited by a segment of epsin known to engage the α and β appendages, and these isolated appendages bind directly to the GST-PWDLW protein *in vitro*. The extensive binding of the purified α_C appendage fortifies the assumption that the DPW repeats within epsin represent the appendage-binding ligand (18,19,31,39).

A common feature of several endocytic accessory proteins, including epsin, AP180 and amphiphysin, is a central region containing adjacent AP-2- and clathrin-binding motifs (40). What is the physiological relevance of these coat-binding sequences? Presumably, via the carboxyl-terminal region, these accessory proteins bring additional functionality to the coat (41-44) as, for example, amphiphysin recruits dynamin and synaptojanin via the carboxyl-terminal SH3 domain (45-47). Do the binding motifs within these proteins simply serve to target them appropriately to a coated pit? Here, we show that the two dissimilar clathrin-binding sequences present in amphiphysin, and in epsin (15), can function together *in vitro*. We also show that, unlike arrestin with only a single type-I sequence (48), amphiphysin can generate pelletable clathrin assemblies within 30 min, even at neutral pH. AP180 and epsin both have epsin N-terminal homology (ENTH) domains at the amino terminus (49) and each bind to phosphoinositides directly (10,50). Tethered to phosphatidylinositol(4,5)P_2-containing membranes, AP180 recruits clathrin robustly and assembles discernible polyhedral structures (10). We have found that liposome bound GST-

epsin (residues 1-407) also recruits soluble clathrin efficiently[1]. Although amphiphysin does not contain an amino-terminal ENTH domain, the amino-terminal (BAR domain) region of the protein is known to bind phospholipid membranes (51). One interpretation of this new information is that a physiologically relevant role for clathrin-binding sequences might be, in fact, to recruit clathrin to certain regions of the plasma membrane. Juxtaposition of an accessory protein clathrin-binding determinant(s) with the terminal domain-binding LLNLD sequence in the adaptor β2-subunit hinge would promote curved lattice assembly. This contention is supported by the fact that extensive formation of clathrin-coated buds all along liposome tubules occurs when a clathrin-coat extract (containing both AP-2 and clathrin trimers) is supplemented with amphiphysin (40). Thus, multiple clathrin interaction sites within a single membrane-bound adaptor / accessory protein complex could efficiently crosslink neighboring terminal domains, allowing constrained rotation of the clathrin trimers in the plane of the membrane to facilitate productive leg alignment, packing and rapid lattice assembly.

Accumulating evidence supports some accessory proteins playing a more direct role in facilitating clathrin assembly in the absence of adaptors. For example, the type I-like sequence ^{451}LIDL(COOH) in the *Saccharomyces cerevisiae* epsin homologues Ent1p and Ent2p (52) could explain the unexpected presence of clathrin-coated vesicles in strains engineered to lack functional forms of the yeast heterotetrameric adaptors and AP180 (53,54). In mammalian cells, overexpression of an AP-2 binding region from amphiphysin I grossly disrupts the intracellular localization of AP-2 but, surprisingly, appears to leave the normal clathrin distribution at the cell surface intact (16). These results suggest that alternate docking sites for clathrin can facilitate the formation of clathrin-coated buds and vesicles. Remarkably, it has been demonstrated very recently that although AP-2 binds to the surface of phosphoinositide-containing liposomes and lipid nanotubes directly, alone the membrane-bound adaptor binds clathrin only poorly (10). Membrane-bound AP180 binds clathrin much more robustly than AP-2 but, together, invaginated buds form (10). This most likely underscores the functional role of the AP-2 and clathrin-binding sequences

located within accessory proteins.

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Footnotes

1. S. K. Mishra, N. S. Agostinelli and L. M. Traub, unpublished observations.

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Figure Legends

Fig. 1. **Association of clathrin and adaptors with isolated sequences from amphiphysin and epsin 1**. Approximately 700 μg of either GST (lane a and b) or the indicated GST-fusion protein (lane c-l) was first incubated with 20 μl of packed glutathione Sepharose, washed 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 Sepharose beads were recovered by centrifugation. Aliquots corresponding to 1 / 80 of each supernatant (S) and 1 / 8 of each washed 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-clathrin light chain (LC) mAb Cl 57.3, or the anti-AP-2 α-subunit mAb 100 / 2, or anti-AP-2 μ2-subunit antiserum, or the anti-AP-1 μ1-subunit antibody RY / 1, or anti-AP-3 δ-subunit antibody KQ / 1, or an anti-AP180 mAb, or an anti-amphiphysin mAb, or an anti-epsin 1 antibody. The position of the molecular mass standards (in kDa) is indicated on the left and only the relevant portion of each blot is shown.

Fig, 2. **Binding of clathrin, AP-1 and AP-2 to GST-SIPWDLWEPT**. *A*, Aliquots of 20 μl packed glutathione Sepharose were incubated with 0 (lane a and b), 25 (lane c and d), 50 (lane e and f), 100 (lane g and h), 200 (lane i and j) and 400 μg (lane k and l) of purified GST-SIPWDLWEPT. After washing the immobilized 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 aliquots corresponding to 1 / 80 of each supernatant (S) and 1 / 8 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 a mixture of the anti-clathrin heavy chain (HC) mAb TD.1 and the anti-β1 / β2-subunit mAb 100 / 1, or the anti-clathrin light chain (LC) mAb Cl 57.3, or the anti-AP-2 α-subunit mAb 100 / 2, or anti-AP-2 μ2-subunit antiserum, or the anti-AP-1 γ-subunit antibody

AE/1, or the anti-AP-1 μ1-subunit antibody RY/1, or the anti-pan arrestin mAb F4C1.

B, Aliquots of 20 μl packed glutathione Sepharose were first incubated with 700 μg of purified GST (lanes a and b), or GST- ETLLDLDF (lane c and d) or GST-SIPWDLWEPT (lanes e-h). After washing the immobilized the fusion proteins, purified cytosolic clathrin (lane a- f) or rat brain cytosol (lane g and h) was added to each tube to a final concentration of ~ 0.02 mg/ml (with 0.1 mg/ml BSA) and ~ 7.5 mg/ml, respectively. After incubation at 4°C for 60 min, the Sepharose beads were then recovered by centrifugation and aliquots corresponding to 1/80 of each supernatant (S) and 1/8 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 a mixture of the anti-clathrin heavy chain (HC) mAb TD.1 and the anti-β1/β2-subunit mAb 100/1, or the anti-clathrin light chain (LC) mAb Cl 57.3. Note that no AP-1 or AP-2 is present in the purified clathrin preparation. The position of the molecular mass standards (in kDa) is indicated on the left and only the relevant portion of each blot is shown.

Fig. 3. **Direct interaction of adaptors with GST-PWDLW**. *A,* Aliquots of 20 μl packed glutathione Sepharose were incubated with 400 μg of purified GST-SIPWDLWEPT. After washing the immobilized fusion protein, either whole rat brain cytosol (lane a and b), clathrin-depleted cytosol (lane c and d) or clathrin-depleted cytosol with 20 μM epsin DPW domain (residues 229-401) added (lane e and f) 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 aliquots corresponding to 1/80 of each supernatant (S) and 1/8 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 a mixture of the anti-clathrin heavy chain (HC) mAb TD.11 and the anti-β1/β2-subunit mAb 100/1, or anti-AP-2 μ2-subunit antiserum, or the anti-clathrin light chain (LC) mAb Cl 57.3.. The position of the molecular mass standards (in kDa) is indicated on the left and only the relevant

portion of each blot is shown.

B, Aliquots of 20 µl packed glutathione Sepharose were incubated with 400 µg of either purified GST (lane a and b and g and h) or GST-SIPWDLWEPT (lane c-f and i-l). After washing the immobilized proteins, purified α_C appendage (residues 701-938) or β2 appendage + hinge (residues 592-951) were added to a final concentrations of ~ 0.04 mg / ml or 0.2 mg / ml (with 0.1 mg / ml BSA) as indicated and incubated at 4°C for 60 min. The Sepharose beads were then recovered by centrifugation and aliquots corresponding to 1 / 80 of each supernatant (S) and 1 / 8 of each washed pellet (P) were resolved by SDS-PAGE and either stained with Coomassie blue. The position of the molecular mass standards (in kDa) is indicated on the left and only the relevant portion of each blot is shown.

Fig. 4. **Role of the Trp residues within the PWDLW sequence in clathrin binding**. *A,* Aliquots of 20 µl packed glutathione Sepharose were incubated without (lane a) or with 700 µg of purified GST-WT (lanes b), GST-$W_1 \rightarrow$A (lane c), GST-$W_1 \rightarrow$F (lane d), GST-$W_1 \rightarrow$Y (lane e), GST-$W_1 \rightarrow$H (lane f), GST-$W_2 \rightarrow$A (lane g) or GST-$W_2 \rightarrow$F (lane h). After washing the immobilized 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 aliquots corresponding to 1 / 8 of each washed pellet 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 heavy chain (HC) mAb TD.1 and the anti-β1 / β2-subunit mAb 100 / 1, or the anti-clathrin light chain (LC) mAb Cl 57.3, or the anti-AP-2 α-subunit mAb 100 / 2, or anti-AP-2 µ2-subunit antiserum, or the anti-AP-1 γ-subunit antibody AE / 1, or the anti-AP-1 µ1-subunit antibody RY / 1, or anti-AP-3 δ-subunit antibody KQ / 1. The asterisk indicates contaminants present in some of the fusion protein preparations.
B, Aliquots of 20 µl packed glutathione Sepharose were incubated with 700 µg of

purified GST (lane a and b), GST- WT (lane c and d), GST- $W_1 \rightarrow M$ (lane e and f), $W_1 \rightarrow V$ (lane g and h), $W_1 \rightarrow L$ (lane i and j) or GST $W_1 \rightarrow I$ (lane k and l). After washing the immobilized the fusion proteins, rat brain cytosol was added to each tube to a final concentrations of ~7.5 mg / ml and incubated at 4°C for 60 min. The Sepharose beads were then recovered by centrifugation and aliquots corresponding to 1 / 80 of each supernatant (S) and 1 / 8 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 a mixture of the anti-clathrin heavy chain (HC) mAb TD.1 and the anti-β1 / β2-subunit mAb 100 / 1, or the anti-clathrin light chain (LC) mAb Cl 57.3, or the anti-AP-2 α-subunit mAb 100 / 2, or the anti-AP-1 μ1-subunit antibody RY / 1. The position of the molecular mass standards (in kDa) is indicated on the left and only the relevant portion of each blot is shown.

Fig. 5. **Role of the central Asp residue in clathrin binding**. Aliquots of 20 μl packed glutathione Sepharose were incubated with 700 μg of purified GST (lane a and b), GST- WT (lane c and d), GST-$D \rightarrow E$ (lane e and f), GST-$D \rightarrow A$ (lane g and h) or GST-$D \rightarrow K$ (lane i and j). After washing the immobilized 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 aliquots corresponding to 1 / 80 of each supernatant (S) and 1 / 8 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 a mixture of the anti-clathrin heavy chain (HC) mAb TD.1 and the anti-β1 / β2-subunit mAb 100 / 1, or the anti-clathrin light chain (LC) mAb Cl 57.3, or the anti-AP-2 α-subunit mAb 100 / 2, or the anti-AP-1 μ1-subunit antibody RY / 1. The position of the molecular mass standards (in kDa) is indicated on the left and only the relevant portion of each blot is shown.

Fig. 6. **Binding of type-I and type-II sequences to isolated clathrin terminal**

domain. Aliquots of 20 µl packed glutathione Sepharose were first incubated with either purified GST (lanes a and b), GST-ETLLDLDF (lane c, d and g-j) or GST-SIPWDLWEPT (lane e-j) as indicated. After washing the immobilized fusion proteins, purified clathrin terminal domain (residues 1-363) was added to a final concentration of ~ 0.1 mg / ml (with 0.1 mg / ml BSA) and incubated at 4°C for 60 min. The Sepharose beads were then recovered by centrifugation and aliquots corresponding to 1 / 80 of each supernatant (S) and 1 / 8 of each washed pellet (P) were resolved by SDS-PAGE and either stained with Coomassie blue (left panel) or transferred to nitrocellulose. The blot was probed with the anti-clathrin heavy chain (HC) mAb TD.1. The position of the molecular mass standards (in kDa) is indicated on the left and only the relevant portion of each blot is shown.

Fig. 7. **Cooperativity of type-I and type-II sequences in the amphiphysin II insert domain**. Aliquots of 20 µl packed glutathione Sepharose were first incubated with 0–200 µg of either purified GST-amphII (lanes a-e) or 25–200 mg GST-amphII Δ400-411 (lanes f-i). After washing the immobilized 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 recovered by centrifugation and aliquots corresponding to 1 / 80 of each supernatant (S) and 1 / 8 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 a mixture of the anti-clathrin heavy chain (HC) mAb TD.1 and the anti-β1 / β2-subunit mAb 100 / 1, or the anti-clathrin light chain (LC) mAb Cl 57.3, or the anti-AP-2 α-subunit mAb 100 / 2, or the anti-AP-1 γ-subunit antibody AE / 1. The position of the molecular mass standards (in kDa) is indicated on the left and only the relevant portion of each blot is shown.

Fig. 8. **Clathrin assembly**. Tubes containing ~350 µg / ml purified cytosolic clathrin, 125 µg / ml GST-amphII or 125 µg / ml GST-amphIIΔ390-397 were prepared on ice as indicated. After incubation at ~15°C for 30 min, the samples were centrifuged and aliquots corresponding to 1 / 20 of each supernatant (S) and

1/4 of each resuspended pellet (P) were resolved by SDS-PAGE and stained with Coomassie blue.

Figure 1

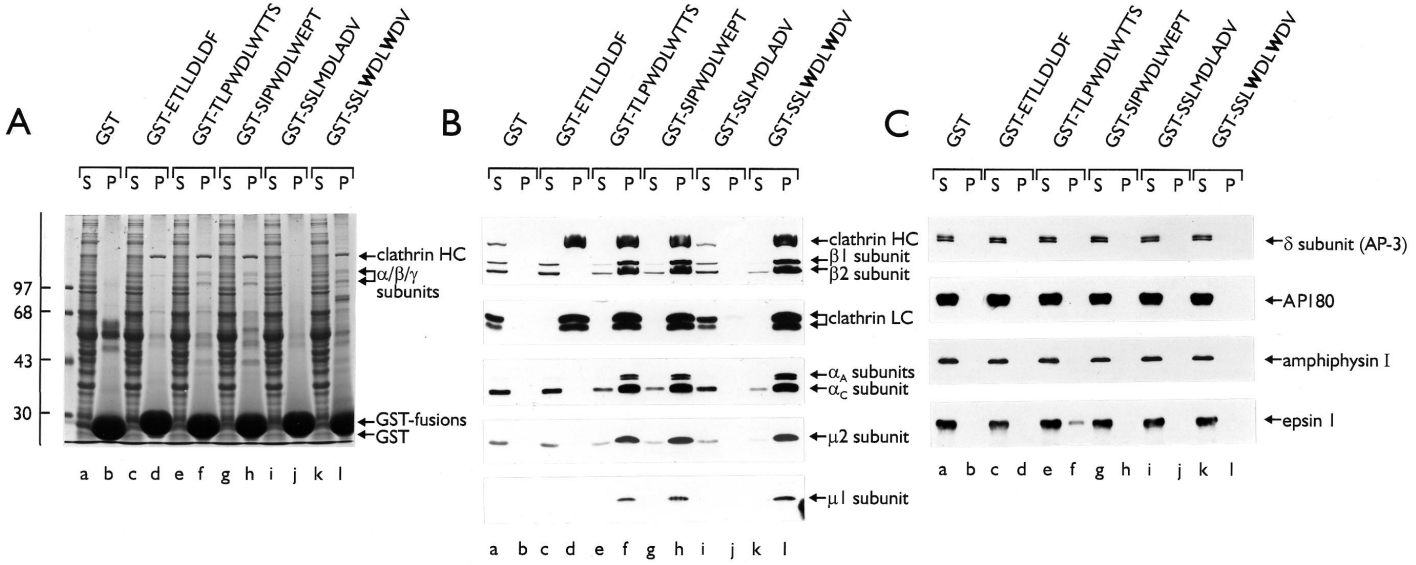

A
B
C
GST
GST-ETLLDLDF
GST-TLPWDLWTTS
GST-SIPWDLWEPT
GST-SSLMDLADV
GST-SSLWDLWDV
S P
97
68
43
30
clathrin HC
α/β/γ subunits
GST-fusions
GST
clathrin HC
β1 subunit
β2 subunit
clathrin LC
αA subunits
αC subunit
μ2 subunit
μ1 subunit
δ subunit (AP-3)
AP180
amphiphysin I
epsin 1
a b c d e f g h i j k l

Figure 2A

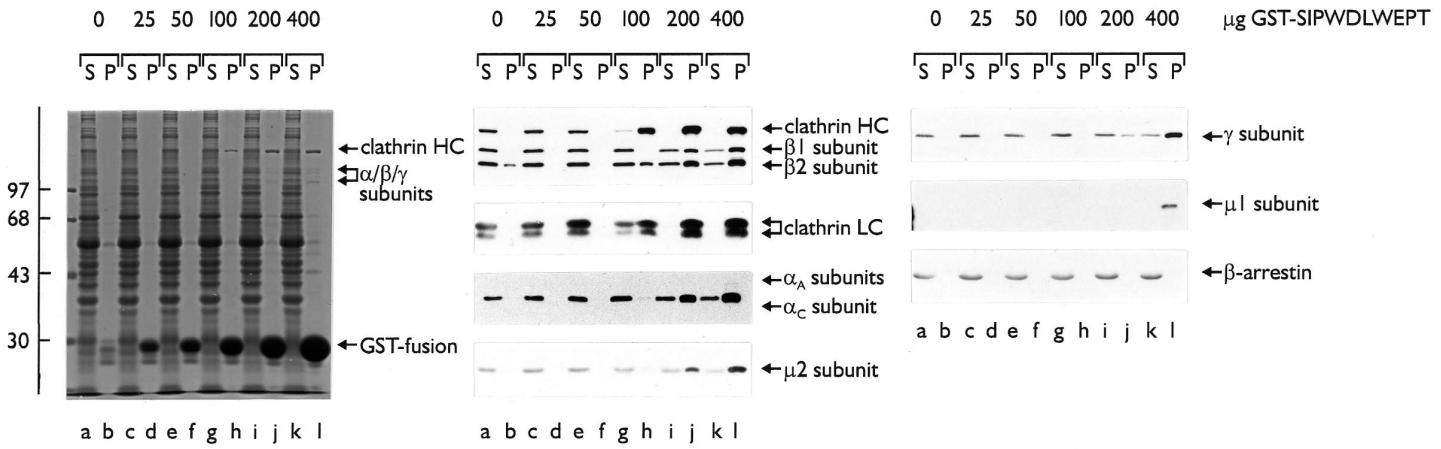

0 25 50 100 200 400
S P S P S P S P S P S P
97
68
43
30
clathrin HC
α/β/γ subunits
GST-fusion
a b c d e f g h i j k l
clathrin HC
β1 subunit
β2 subunit
clathrin LC
αA subunits
αC subunit
μ2 subunit
μg GST-SIPWDLWEPT
γ subunit
μ1 subunit
β-arrestin

Figure 2B

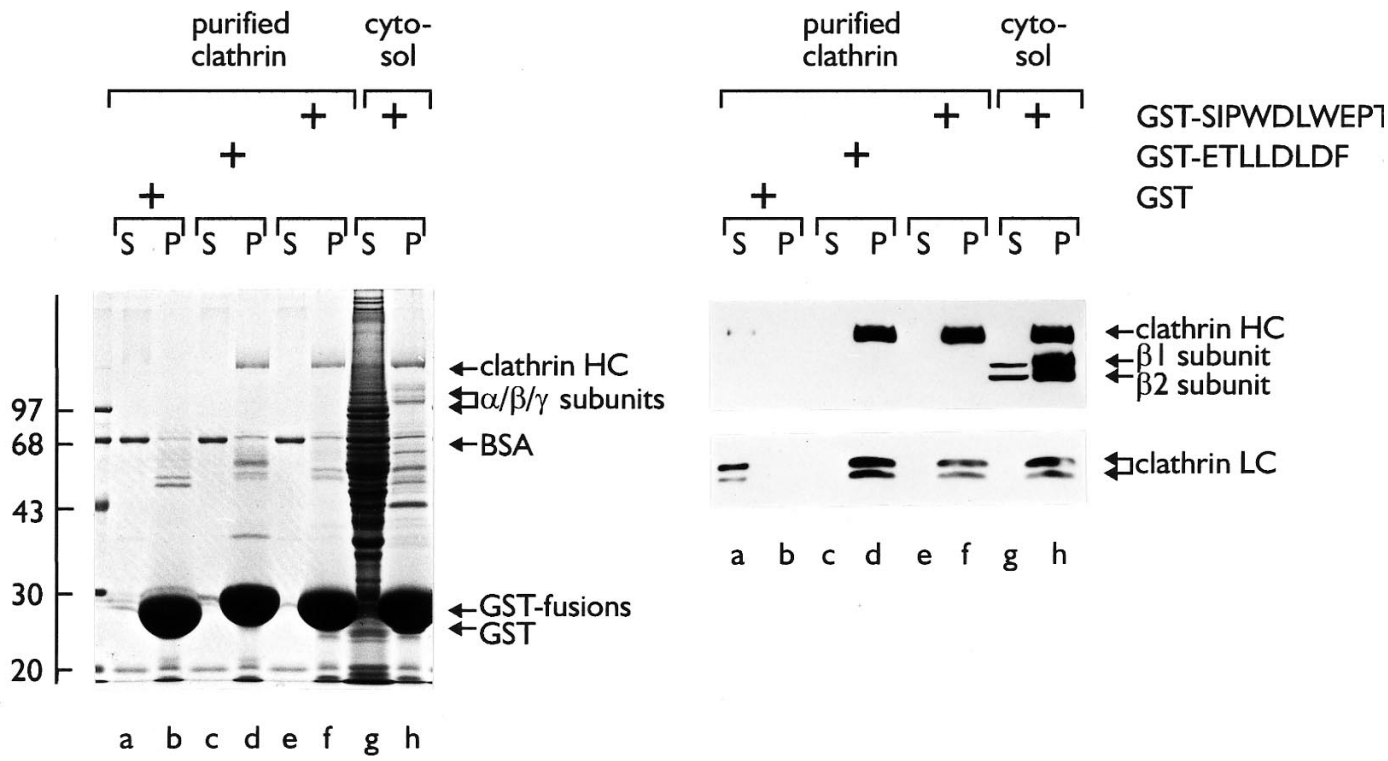

purified clathrin
cyto-sol
+
+
+
S P S P S P S P
97
68
43
30
20
←clathrin HC
⇆α/β/γ subunits
←BSA
←GST-fusions
←GST
a b c d e f g h
purified clathrin
cyto-sol
+
+
+
S P S P S P S P
GST-SIPWDLWEPT
GST-ETLLDLDF
GST
←clathrin HC
←β1 subunit
←β2 subunit
⇆clathrin LC
a b c d e f g h

Figure 3A

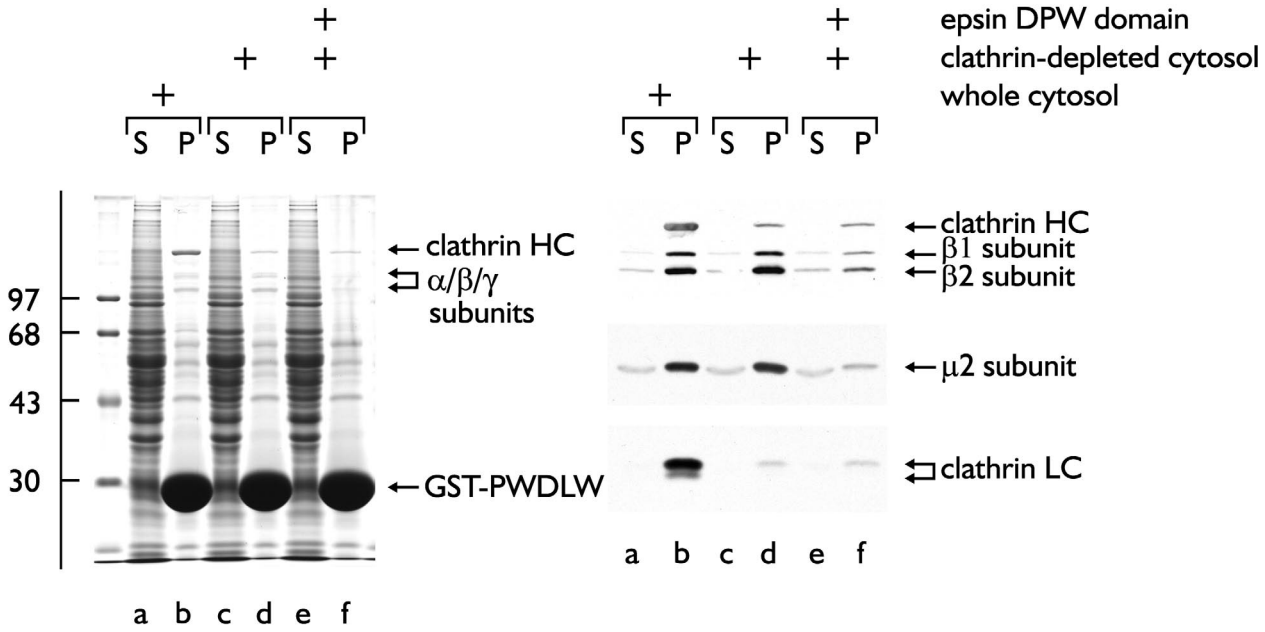

epsin DPW domain
clathrin-depleted cytosol
whole cytosol
S P S P S P
S P S P S P
97
68
43
30
clathrin HC
α/β/γ subunits
GST-PWDLW
clathrin HC
β1 subunit
β2 subunit
μ2 subunit
clathrin LC
a b c d e f
a b c d e f

Figure 3B

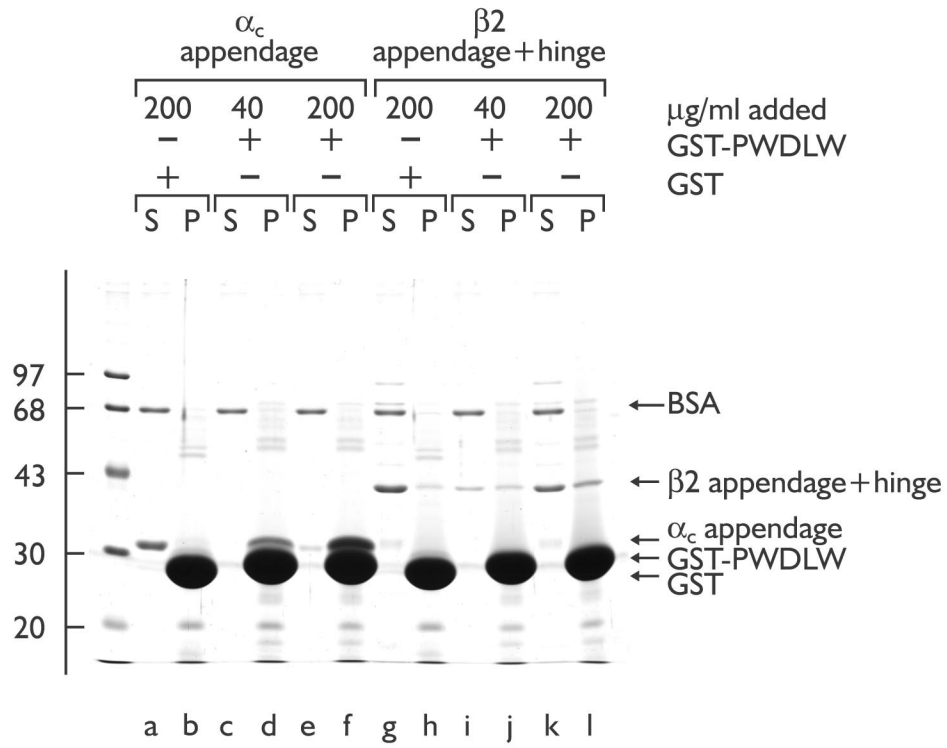
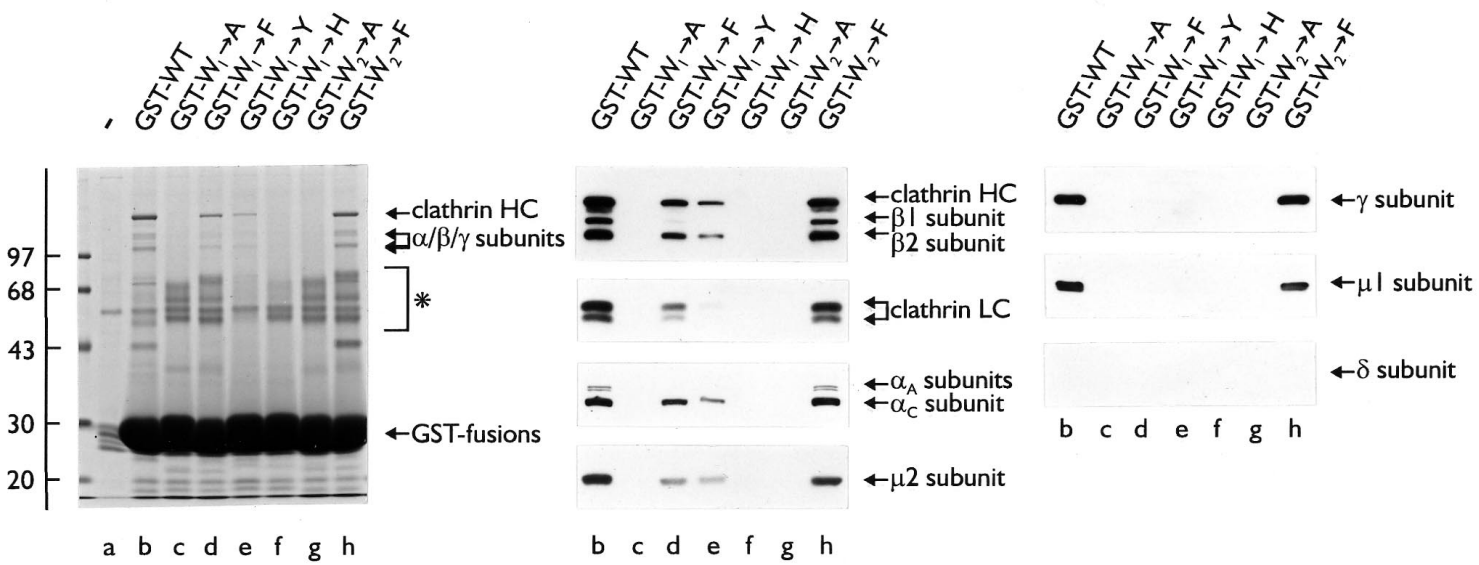

αc appendage
β2 appendage+hinge
200 40 200 200 40 200
µg/ml added
− + + − + +
GST-PWDLW
+ − − + − −
GST
S P S P S P S P S P S P
97
68
43
30
20
←BSA
← β2 appendage+hinge
← αc appendage
← GST-PWDLW
← GST
a b c d e f g h i j k l

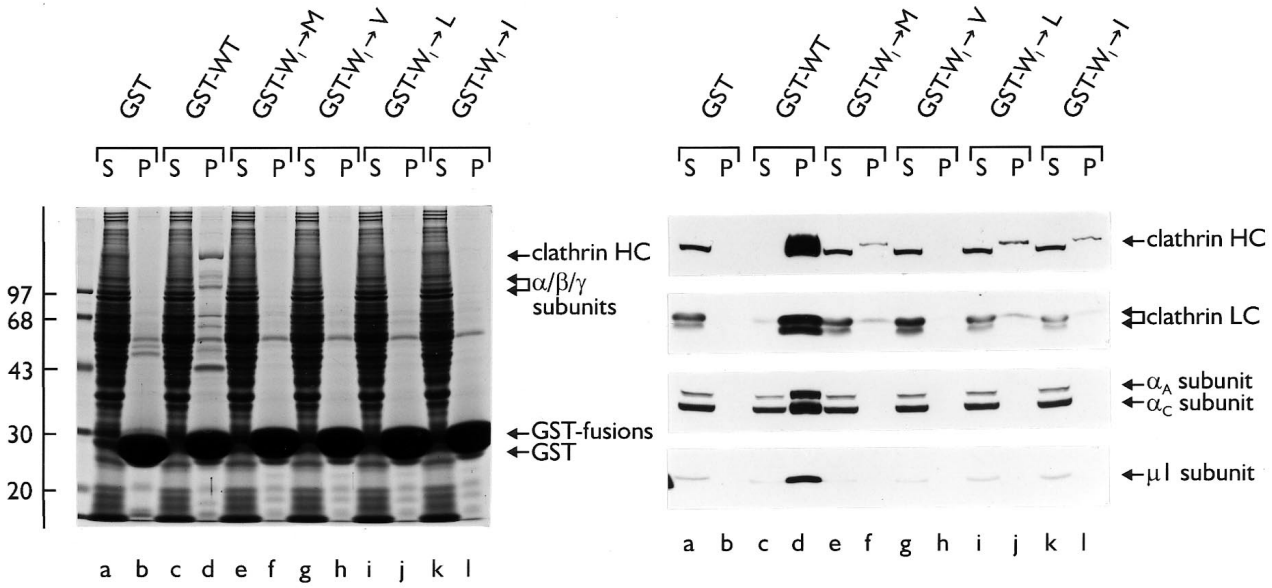
Figure 4A



GST-WT	SIPWDLWEPT
GST-W_1→A	SIP**A**DLWEPT
GST-W_1→F	SIP**F**DLWEPT
GST-W_1→Y	SIP**Y**DLWEPT
GST-W_1→H	SIP**H**DLWEPT

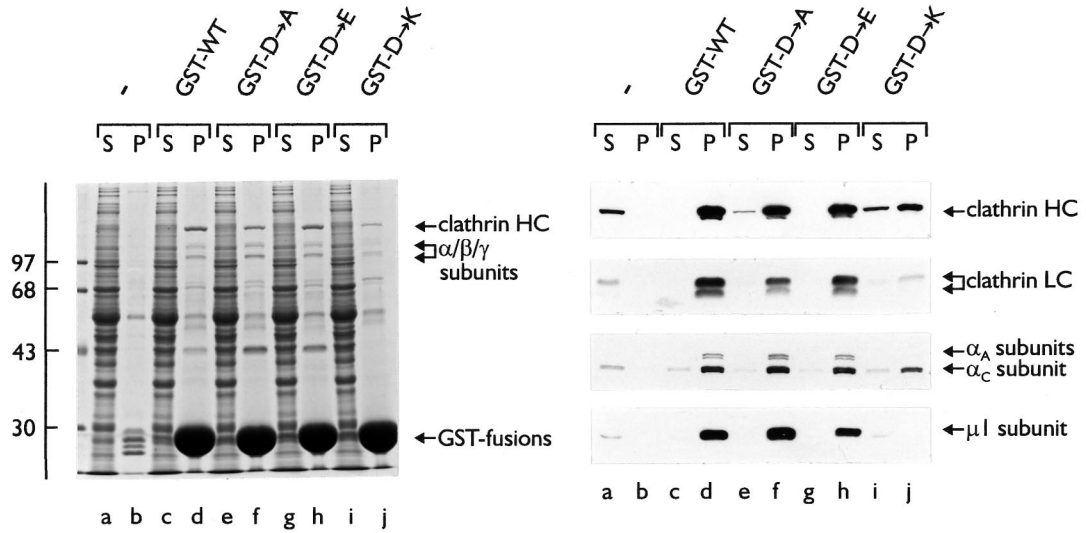
GST-WT	SIPWDLWEPT
GST-W_2→A	SIPWDL**A**EPT
GST-W_2→F	SIPWDL**F**EPT

Figure 4B



GST-WT	SIPWDLWEPT
GST-W$_I$→M	SIP**M**DLWEPT
GST-W$_I$→V	SIP**V**DLWEPT
GST-W$_I$→L	SIP**L**DLWEPT
GST-W$_I$→I	SIP**I**DLWEPT

Figure 5



GST-WT	SIPWDLWEPT
GST-D→A	SIPW**A**LWEPT
GST-D→E	SIPW**E**LWEPT
GST-D→K	SIPW**K**LWEPT

Figure 6

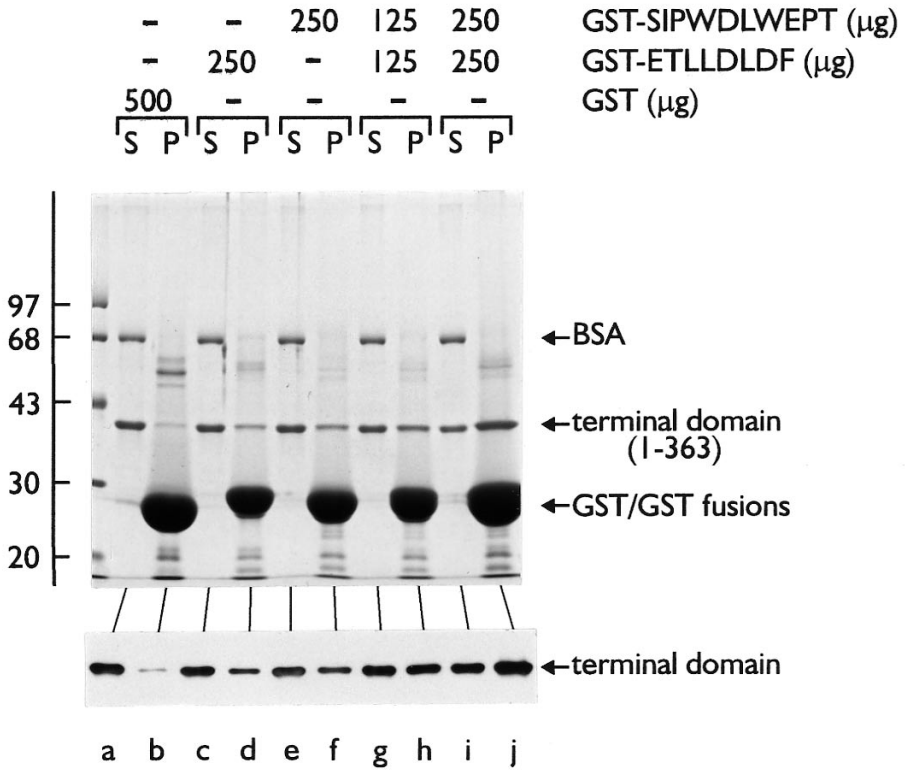
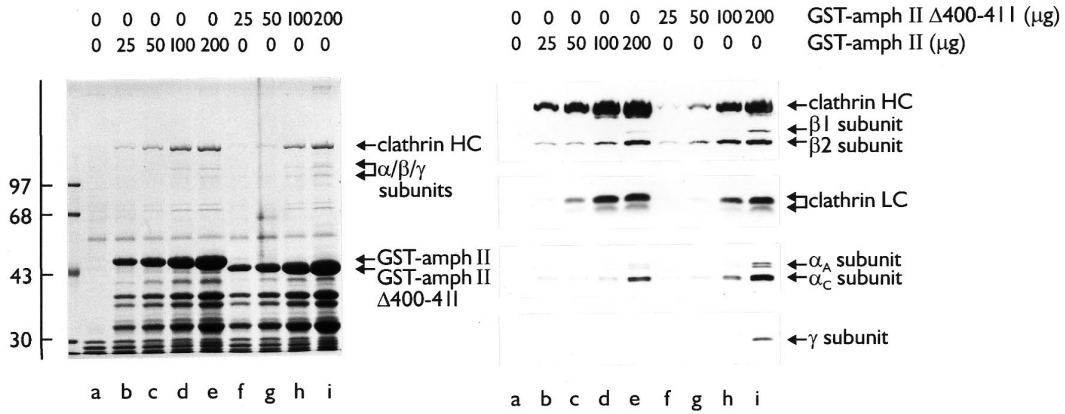

- - 250 125 250 GST-SIPWDLWEPT (μg)
- 250 - 125 250 GST-ETLLDLDF (μg)
500 - - - - GST (μg)
S P S P S P S P S P
97
68
43
30
20
←BSA
←terminal domain (1-363)
←GST/GST fusions
←terminal domain
a b c d e f g h i j

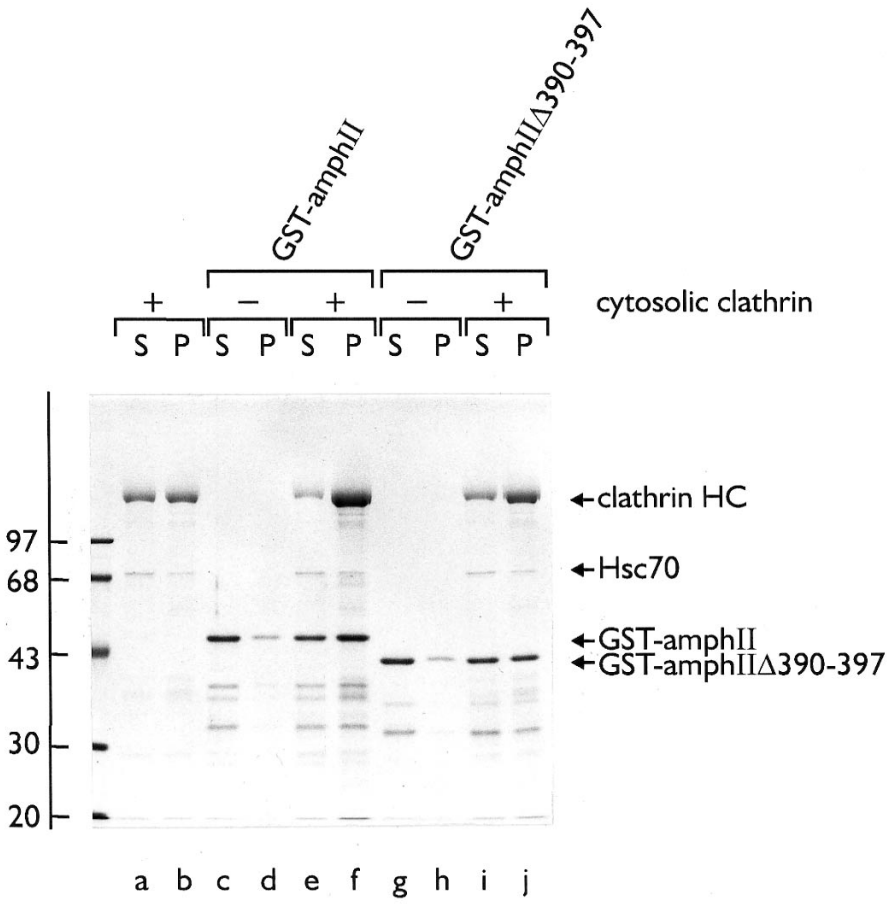
Figure 7



GST-amph II . . .[388]AS**LLDLD**FDPLPPVTSPVKAPTPSGQSI**PWDLW**EPT. . .

GST-amph II Δ400-411 . . .[388]AS**LLDLD**FDPLP GQSI**PWDLW**EPT. . .

Figure 8


GST-amphII
GST-amphIIΔ390-397
+ − + − +
cytosolic clathrin
S P S P S P S P S P
97
68
43
30
20
clathrin HC
Hsc70
GST-amphII
GST-amphIIΔ390-397
a b c d e f g h i j