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γ Subunit of the AP-1 Adaptor Complex Binds Clathrin: Implications for Cooperative Binding in Coated Vesicle Assembly

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The heterotetrameric AP-1 adaptor complex is involved in the assembly of clathrin-coated vesicles originating from the *trans*-Golgi network (TGN). The β1 subunit of AP-1 is known to contain a consensus clathrin binding sequence, LLNLD (the so-called clathrin box motif), in its hinge segment through which the β chain interacts with the N-terminal domains of clathrin trimers. Here, we report that the hinge region of the γ subunit of human and mouse AP-1 contains two copies of a new variant, LLDLL, of the clathrin box motif that also bind to the terminal domain of the clathrin heavy chain. High-affinity binding of the γ hinge to clathrin trimers requires both LLDLL sequences to be present and the spacing between them to be maintained. We also identify an independent clathrin-binding site within the appendage domain of the γ subunit that interacts with a region of clathrin other than the N-terminal domain. Clathrin polymerization is promoted by glutathione S-transferase (GST)-γ hinge, but not by GST-γ appendage. However, the hinge and appendage domains of γ function in a cooperative manner to recruit and polymerize clathrin, suggesting that clathrin lattice assembly at the TGN involves multivalent binding of clathrin by the γ and β1 subunits of AP-1.

INTRODUCTION

The transit of proteins and lipids from the *trans*-Golgi network (TGN) and the plasma membrane to endosomes within eucaryotic cells occurs via the budding and fusion of clathrin-coated vesicles (reviewed in Kirchhausen, 1999, 2000). At the TGN, this process is mediated by the heterotetrameric AP-1 adaptor complex, which consists of two large subunits, γ and β1; a medium subunit, μ1; and a small σ1 subunit. An analogous adaptor complex, AP-2 (α, β2, μ2, σ2), participates at the plasma membrane in the process of receptor-mediated endocytosis (Hirst and Robinson, 1998). At both the TGN and the plasma membrane, the first stage in the vesiculation process involves the recruitment of the respective adaptor proteins to the site of coated pit formation. After this step, cytosolic clathrin associates indirectly with the membrane by binding to the adaptor proteins, which in turn are associated with the cytoplasmic domains of transmembrane receptors. Polymerization of the soluble clathrin together with the concentrated adaptors, associated receptors, and their bound ligands eventually results in a coated transport vesicle budding off the membrane surface (Pearse and Robinson, 1990).

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A direct interaction between clathrin and the AP-1 and AP-2 complexes has been shown to occur through a clathrin binding sequence in the hinge of the β chains of the adaptor proteins interacting with a groove in the side of the clathrin N-terminal β-propeller domain (Shih *et al.*, 1995; ter Haar *et al.*, 2000). Although this β1/β2 hinge sequence was initially identified as a conserved motif for clathrin binding in the β3 chain of the AP-3 adaptor complex (Dell'Angelica *et al.*, 1998), similar sequences are now recognized to occur in a variety of other proteins known to interact with clathrin such as β-arrestin, AP-180, and amphiphysin (Kirchhausen, 2000). Presently termed a clathrin box motif, an alignment of the various sequences defined the consensus motif to consist of acidic and bulky hydrophobic residues that conform to the canonical sequence L (L, I) (D, E, N) (L, F) (D, E) (Dell'Angelica *et al.*, 1998; Kirchhausen, 2000). A single such motif, LLNLD, present within the β chains of AP-1 and AP-2 is capable of driving clathrin coat formation in vitro and was proposed to contain the primary clathrin binding site of the adaptors to stimulate lattice assembly when linked to an oligomerizing or membrane-anchored structure (Shih *et al.*, 1995). More recently, a second clathrin-binding site was demonstrated to occur within the adjacent appendage domain of the β2 subunit (Owen *et al.*, 2000). Although the β2 appendage domain by itself was incapable of promoting clathrin lattice assembly in vitro, unlike the β2 hinge region

(Shih *et al.*, 1995), a synergistic effect in clathrin binding was observed when both the β2 appendage and hinge regions were present together (Owen *et al.*, 2000). The homologous nature of the β1 subunit to β2 suggests that a similar cooperativity in clathrin binding between the β1 appendage and hinge domains is likely.

In the present study, we report the identification of a new variant of the consensus clathrin box motif that resides within the hinge region of the γ subunit of human and mouse AP-1. This sequence, LLDLL, occurs as a repeat within the γ hinge and we show both repeats as well as the spacing between the repeats to be important for binding to the clathrin N-terminal domain. Additionally, we identify an independent clathrin-binding site within the appendage domain of the γ subunit and show that this site interacts with a region of clathrin other than the N-terminal domain. Moreover, like the β2 appendage and hinge domains, we observe a substantial synergistic effect on clathrin binding and polymerization into cages when both the γ appendage and hinge are present together. The implication of these findings for the multivalent nature of clathrin-adaptor interactions is discussed.

MATERIALS AND METHODS

Antibodies

The anti-clathrin heavy chain (HC) monoclonal antibody (mAb) TD.1 was generously provided by Frances Brodsky (University of California, San Francisco). Rabbit anti-γ-synergin polyclonal antibody was a gift from Margaret Robinson (University of Cambridge, Cambridge, United Kingdom). The anti-rabaptin 5 mAb and the anti β-tubulin mAb were purchased from Transduction Laboratories (Lexington, KY).

Peptides

All peptides were synthesized at the Protein Chemistry Laboratory at Washington University in St. Louis, MO, and purified by reverse phase high-performance liquid chromatography. The amino acid sequences corresponding to the peptides used in this study are as follows: AP-1 γ hinge, NDLLDLLGGND and CDLLGDINLTGAPAAAPAPA; amphiphysin 1, KEETLLDLDFD; AP-3 δ hinge, CKQEQANNPFYIKSSPS; and AP-3 σ3, CKNINLPEIPRNINIG.

Construction of Bacterial Expression Plasmids

The various GST-γ appendage, -γ hinge, or -γ appendage + hinge constructs were made by polymerase chain reaction from the mouse γ adaptin cDNA (Robinson, 1990), and subcloned into the vector pGEX-5X-3 (Amersham Pharmacia Biotech, Piscataway, NJ) digested with *Eco*RI/*Xho*I. Mutagenesis of γ hinge or γ appendage + hinge was performed with the use of primers incorporating the desired mutations with the QuickChange system (Stratagene, La Jolla, CA). The GST-NDLLDLLG and GST-PFLLDGLS constructs were generated by annealing sense and antisense oligonucleotides and ligating the double-stranded products into *Eco*RI/*Xho*I digested pGEX-5X-3. A construct encoding residues 1–579 of the bovine clathrin heavy chain subcloned into pGEX-2T was kindly provided by James Keen (Thomas Jefferson University, Philadelphia, PA), whereas GST-ETLLDLDF was kindly provided by Linton Traub (Washington University). GST-γ2 appendage and GST-γ2 appendage + hinge were made by polymerase chain reaction from a human EST clone, GenBank accession number T49401 (Incyte Genomics, St. Louis, MO). All constructs and mutations were confirmed by dideoxynucleotide sequencing.

Protein Expression and Purification

The various GST-γ fusion proteins were expressed in the *Escherichia coli* strain BL21(RIL) (Stratagene) essentially as described (Drake *et al.*, 2000). Cells from 1L of culture were lysed into 20 ml of B-PER reagent (Pierce, Rockford, IL), sonicated briefly, and centrifuged at 27,000 × *g* at 4°C for 15 min to remove insoluble material. The clarified lysate was then mixed by tumbling at 4°C for 4 h with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) preequilibrated with 20 mM Tris-Cl, pH 7.5, containing 0.1% Triton X-100. After four washes with the 20 mM Tris/0.1%Triton X-100 buffer and a single wash with either detergent-free 50 mM Tris-Cl, pH 8.0, or phosphate-buffered saline (PBS), the GST-fusion proteins were competitively eluted with 10 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0, or in the case of GST TD 1–579, cleaved with thrombin in PBS per manufacturer's instruction (Amersham Pharmacia Biotech) to separate the clathrin terminal domain from GST. Proteins eluted with reduced glutathione were dialyzed overnight against PBS before use in pull-down experiments.

Rat liver cytosol was prepared as described (Traub *et al.*, 1993). Soluble clathrin was purified from bovine brain cytosol by incubation of cytosol with GST-NDLLDLLG followed by elution with buffer A (1 M Tris-Cl pH 7.4, 2 mM dithiothreitol [DTT], and 3 mM 3-([3-cholamidopropyl]dimethylammino)-2-hydroxy-1-propanesulfonate). The clathrin was either dialyzed against PBS for use in GST pull-down experiments or stored in buffer A for clathrin polymerization and coat assembly assays.

Binding Assays

The binding of the various GST fusion proteins with clathrin was assayed in buffer B (25 mM HEPES-KOH pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT, and 0.1% Triton X-100) in a final volume of 300 μl in 1.5 ml of presiliconized microcentrifuge tubes (Midwest Scientific, St. Louis, MO). Routinely, the GST-fusion proteins were first immobilized at room temperature on 30 μl of packed glutathione-Sepharose to concentrations of 3–6 mg/ml. The bound proteins were pelleted by centrifugation at 750 × *g* for 1 min, the beads washed once with cold buffer B, and 300 μl of rat liver cytosol or purified soluble clathrin in buffer B at a final concentration of 7.5 mg/ml or 5 μg/ml, respectively, was added to the washed beads. For binding assays with clathrin terminal domain, 50 μg of purified TD 1–579 in 300 μl of buffer B was added to each reaction. The reactions were allowed to proceed for 1 h at 4°C with tumbling, after which the samples were subjected to centrifugation at 750 × *g* for 1 min. An aliquot of the supernatant was saved, and the pellets were washed four times each with 1.5 ml of cold buffer B by centrifugation at 750 × *g*. The pellets were resuspended in SDS sample buffer and unless indicated otherwise, 1/10th of each pellet and 1/30th of each supernatant were loaded on SDS gels for assays with rat liver cytosol or purified triskelia, whereas 1/10th of each pellet and 1/100th of each supernatant were loaded for assays with the use of purified terminal domain.

GST pull-down assays in peptide inhibition studies were performed as described above except reactions were carried out in a final volume of 500 μl containing the indicated concentrations of the various peptides. In this case, 1/10th of each pellet and 1/50th of each supernatant were loaded on SDS gels. Clathrin binding curves were generated by densitometric analysis of the pellet fractions of Coomassie blue-stained gels with the use of a Molecular Dynamics personal laser densitometer (Sunnyvale, CA) and the Image Quant software.

Clathrin Coat Assembly

Clathrin coats were reconstituted essentially as described (Gallusser and Kirchhausen, 1993). Briefly, purified soluble clathrin in buffer A (1.5–2 μM) was mixed with an eightfold molar excess of the various GST fusion proteins in buffer B. The final concentration of clathrin in

Figure 1. Sequence comparison of the hinge of mouse γ (mγ), human γ (hγ), human γ2 (hγ2), and human α (hα) adaptins, and GST-γ adaptin fusion constructs. (A) Schematic of γ adaptin showing the trunk (T), the hinge (H), and the appendage (A) also called the ear. An alignment of part of the hinge region shows that only γ and γ2 adaptin but not α adaptin possess a variant of the consensus clathrin binding sequence (underlined). (B) Construction of the various GST-γ adaptin fusion proteins is described under MATERIALS AND METHODS.

the assays was 0.75 μM. The reactions (300 μl) were dialyzed overnight at 4°C against coat assembly buffer (100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, 2 mM DTT, and 2 mM EDTA) with the use of Pierce Slide-A-Lyzer 0.5-ml dialysis cassettes. Samples were recovered and centrifuged at 12,000 × *g* for 5 min at 4°C to remove aggregated material, after which coats were separated from nonassembled protein by ultracentrifugation in a TLA-100 rotor at 60,000 rpm for 10 min at 4°C. The pellets were resuspended either in 1× sample buffer for SDS-PAGE analysis or in coat assembly buffer for electron microscopy. The percentage of clathrin in the pellet and supernatant fractions was quantified by densitometry of Coomassie blue-stained gels as described above.

Electron Microscopy

Assembled clathrin coats were diluted into coat assembly buffer and placed for 1 min onto 3- × 3-mm square glass coverslips premoistened with KHMgE (70 mM KCl, 30 mM HEPES, pH 6.5, 5 mM MgCl, 3 mM EGTA). The coverslips were then plunged into 10 ml of 2% glutaraldehyde in KHMgE, fixed for 15 min at room temperature, and washed with four exchanges of double distilled H_2O. Freeze-drying and subsequent sample preparations were performed as described (Heuser, 1989). Replicas on 75-mesh Formvar-coated electron microscopy grids were viewed with the use of a JEOL 200CX electron microscope operating at 100 kV and imaged at 50,000 magnification.

Electrophoresis and Immunoblotting

Proteins were resolved on 8% SDS-polyacrylamide gels and either transferred to nitrocellulose or stained with Coomassie brilliant blue for direct visualization. Blots were blocked with Tris-buffered saline/Tween (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk for 1 h at room temperature. Different portions of the blots were then probed with primary antibodies as indicated in the individual figure legends, followed by horseradish peroxidase-conjugated anti-mouse IgG, and the immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

RESULTS

γ Subunit of AP-1 Binds to Soluble Clathrin Trimers

The canonical clathrin box motif first noted by Dell'Angelica *et al.* (1998) is now known to be present in a variety of proteins involved in clathrin-mediated endocytosis, as well as the β subunits of the adaptor proteins AP-1, AP-2, and AP-3. Analysis of the amino acid sequence of the γ subunit of human and mouse AP-1 revealed the presence of two copies of a variant, LLDLL, of the consensus clathrin binding sequence within the hinge region of the γ chain (Figure 1A). To determine whether the γ subunit of mouse AP-1 is a clathrin binding partner of the AP-1 complex, we constructed and expressed various GST-γ fusions (Figure 1B), and assayed them for clathrin binding with the use of rat liver cytosol as the source of clathrin. Because GST-LLDLD with a perfectly conserved clathrin box motif was shown to display strong clathrin binding in pull-down experiments (Drake *et al.*, 2000), it served as a positive control in our assays, whereas GST or GST-α appendage served as a negative control. Both GST-γ 595–702 (hinge with 2 LLDLL repeats) and GST-γ 703–822 (appendage) displayed significant clathrin binding capacity (Figure 2, A and B), suggesting the presence of independent clathrin-binding sites within the γ hinge and appendage domains. When both the appendage and the hinge were present (GST-γ 595–822), a marked enhancement in clathrin recruitment was observed (compare GST-LLDLD and GST-γ 595–822, Figure 2A).

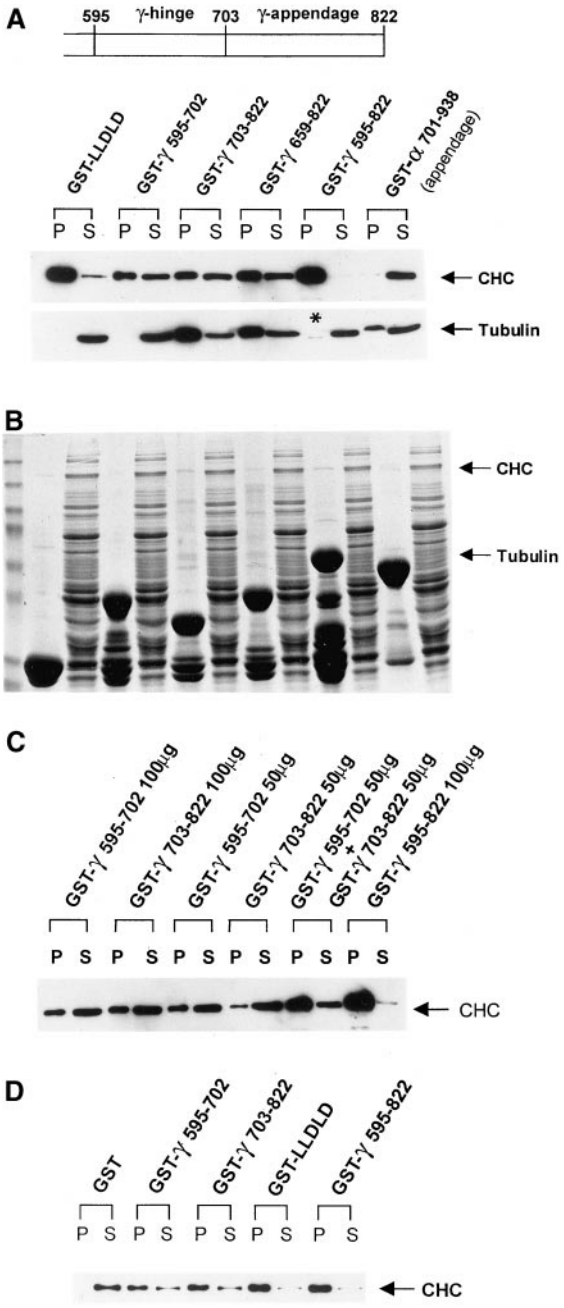

Figure 2. GST-γ hinge and GST-γ appendage bind clathrin independently and cooperatively. (A) Immunoblot of GST pull-down assay with 200 μg of immobilized fusion protein, which had been incubated with rat liver cytosol at a final concentration of 7.5 mg/ml. Portions of the blot were probed with the anti-clathrin HC mAb TD.1 or an anti-tubulin mAb. (B) Coomassie blue-stained gel of the same samples as in A indicates the approximately equivalent loading of the different fusion proteins. In this case, each pellet lane corresponds to 1/20th of the total pellet fraction, whereas 1/60th of each supernatant was loaded. (C) Immunoblot of GST pull-down assay with the use of 50 or 100 μg of immobilized fusion proteins, individually or in combination, with rat liver cytosol. (D) Immunoblot of GST pull-down assay with the use of purified soluble clathrin triskelia isolated from bovine brain cytosol.

GST-γ 659–822, which includes part of the hinge but lacks the two LLDLL repeats, does not show this synergistic effect, suggesting that the LLDLL sequences within the γ hinge mediate clathrin binding. GST-α appendage as described previously fails to bind any appreciable amount of clathrin (Shih *et al.*, 1995; Wang *et al.*, 1995; Owen *et al.*, 2000). When the γ hinge and appendage domains on separate fusion proteins were mixed and immobilized on glutathione-Sepharose before reacting with rat liver cytosol, the cooperative nature of the γ hinge and appendage domains in interacting with clathrin was mostly restored (Figure 2C). That these interactions between the various GST-γ fusions and clathrin are direct is demonstrated in the binding assays with purified cytosolic clathrin (Figure 2D). The similar binding ability of the different γ fusions with rat liver cytosol or with purified clathrin precludes the possibility of other cytosolic proteins mediating the binding.

In addition to clathrin, two prominent bands in the 50–60-kDa range were seen with GST-γ 703–822 and GST-γ 659–822 but not GST-γ 595–702 or GST-LLDLD in the Coomassie blue-stained gels (Figure 2B). GST pull-down experiments with bovine brain cytosol suggested that these two bands may correspond to the two isoforms of tubulin, a major component of brain cytosol. Immunoblotting with an anti-tubulin antibody confirmed that the γ appendage domain but not the γ hinge region interacted with tubulin (Figure 2A). GST-γ 595–822 with an intact appendage is expected to bind tubulin as well but the apparent lack of a signal on the immunoblot (Figure 2A, *) is due to the fusion protein comigrating with tubulin on 8% SDS gels (Figure 2B). Although this tubulin binding may be nonspecific, it should be noted that two groups have reported that α- and β-tubulin are stoichiometric components of clathrin-coated vesicles isolated from brain and liver tissue (Kelly *et al.*, 1983; Pfeffer *et al.*, 1983). There is also recent evidence that AP-1 is a motor adaptor protein for directional movement along microtubules (Nakagawa *et al.*, 2000). The ability of the γ appendage to bind tubulin could potentially have a role in this process.

γ Hinge But Not γ Appendage Binds Clathrin Terminal Domain

The consensus clathrin binding motifs of β-arrestin 2 and β3 of the AP-3 adaptor complex were recently shown to bind to a groove between blades 1 and 2 in the side of the clathrin N-terminal β-propeller domain (ter Haar *et al.*, 2000). Thus, it was of interest to determine whether the γ hinge LLDLL sequence also bound to the clathrin terminal domain, especially because the γ hinge sequence lacked an important acidic residue in the fifth position to fit into the polar pocket of the binding site in the clathrin groove. As shown in Figure 3, GST-γ 595–702 (hinge) but not GST-γ 703–822 (appendage) bound purified TD 1–579 as did GST-LLDLD. Neither GST alone nor GST α appendage bound any TD 1–579.

Both γ Hinge LLDLL Sequences with Correct Spacing Are Required for Clathrin Binding

Because the hinge regions of the β1, β2, and β3 chains contain only a single clathrin box motif, the occurrence of two LLDLL sequences within γ hinge raised the possibility of redundancy within this region. Alternately, both sites

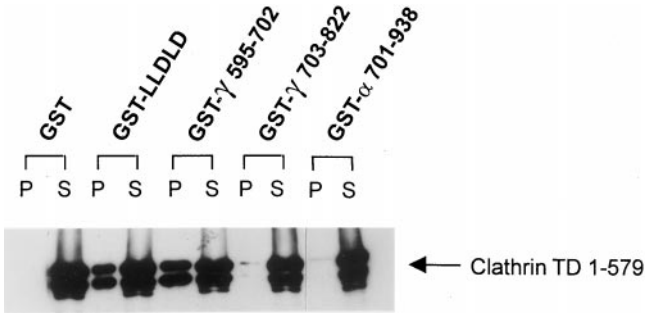

Figure 3. GST-γ hinge but not GST-γ appendage binds clathrin terminal domain. Clathrin TD 1–579 was expressed as described under MATERIALS AND METHODS and separated from GST after cleavage with the protease thrombin. Purified TD (50 μg) was incubated with 200 μg of the immobilized GST fusion proteins as indicated. Blots were probed with the anti-clathrin HC mAB TD.1. Only the GST-γ 595–702 (hinge) contains the LLDLL sequence and binds terminal domain.

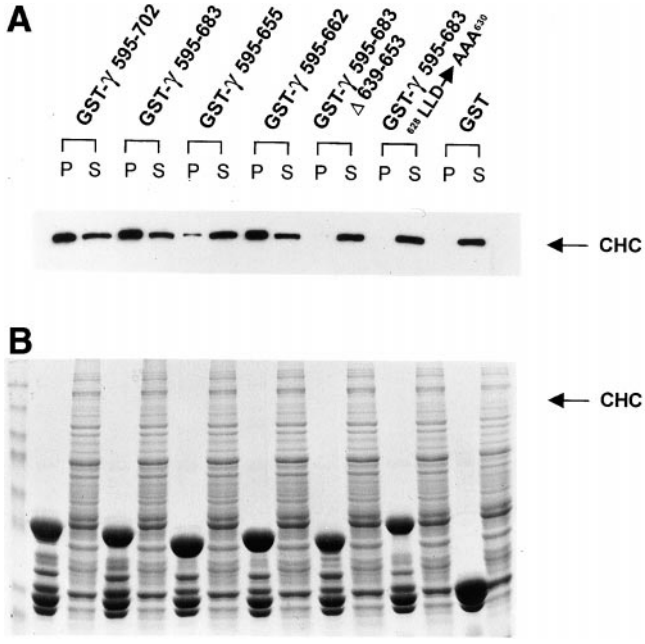

Figure 4. Both γ hinge LLDLL sequences with the correct spacing are required for clathrin binding. GST-γ 595–655 lacks the second ^{656}LLDLL^{660} sequence, whereas GST-γ 595–683 ^{628}LLD → AAA^{630} has the first LLDLL sequence mutated. (A) Immunoblot of the various GST-γ hinge fusion proteins incubated with rat liver cytosol, probed with the TD.1 mAb. (B) Coomassie blue-stained gel of the same samples indicated in A.

may be important for clathrin binding, as was shown to hold true for amphiphysin 1 (Slepnev *et al.*, 2000). We therefore tested the requirement for the presence of two clathrin-binding sequences within the γ hinge by constructing a series of hinge variants and determining their ability to bind clathrin in pull-down assays. Both GST-γ 595–702 and γ 595–683 with two intact LLDLL motifs bound clathrin efficiently, but GST-γ 595–655 in which the second LLDLL was deleted displayed a dramatic decrease in binding capacity (Figure 4, A and B). Replacement of only seven amino acids (residues 656–662) encompassing the second LLDLL sequence fully restored clathrin binding in GST-γ 595–662. Furthermore, an internal deletion of residues 639–653 or mutations of the first LLDLL sequence all but abolished clathrin binding. These results indicate that not only are both LLDLL sequences critical for the function of the γ hinge in interacting with clathrin but also that a correct spacing between the two sequences is necessary. An alternate explanation is that all of the clathrin binding activity resides in the proximal LLDLL sequence, and the various deletions somehow prevent this LLDLL motif from interacting with the clathrin terminal domain, either by affecting its conformation or its accessibility. Although we cannot exclude this possibility without mutating the distal LLDLL motif, we believe this to be unlikely.

We next asked whether the internal deletion between the two LLDLL sequences within the γ hinge negates the cooperativity observed in GST γ-595–822. As shown in Figure 5, GST-γ 595–822 Δ 639–653 displayed reduced clathrin binding, at the level observed with the γ appendage domain alone, consistent with the previous finding. Furthermore, a construct, GST-γ 653–822, with only the second LLDLL sequence present also failed to show cooperativity in clathrin binding, which similarly was reduced to the level of the γ appendage domain by itself (Doray and Kornfeld, unpublished observation). These data indicate that both γ hinge LLDLL repeats are necessary for the γ hinge and appendage domains to interact in a synergistic manner to bind clathrin.

An examination of the human γ2 sequence by alignment reveals significant identity between the γ2 and γ appendage domains (49% identity) (Takatsu *et al.*, 1998). The γ2 hinge region, however, is more dissimilar in primary structure and length to the γ hinge, except for the presence of an LLDLL and an LLDLP sequence within the γ2 hinge that occur at the same spacing observed between the 2 LLDLL sequences in γ adaptin (Figure 1A). This prompted us to investigate whether human γ2 adaptin also bound clathrin. In pull-down assays, GST-γ2 666–785 (appendage) behaved in a

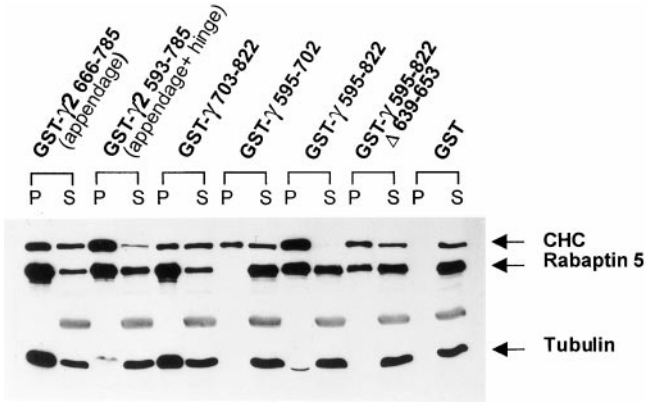

Figure 5. γ 2 Adaptin binds the same subset of proteins as does γ adaptin. Immunoblots of the pull-downs of GST-γ2 and GST-γ fusion proteins. Portions of the blot were probed with the anti-clathrin TD.1 mAB, an anti-tubulin mAb, or an anti-rabaptin 5 mAb.

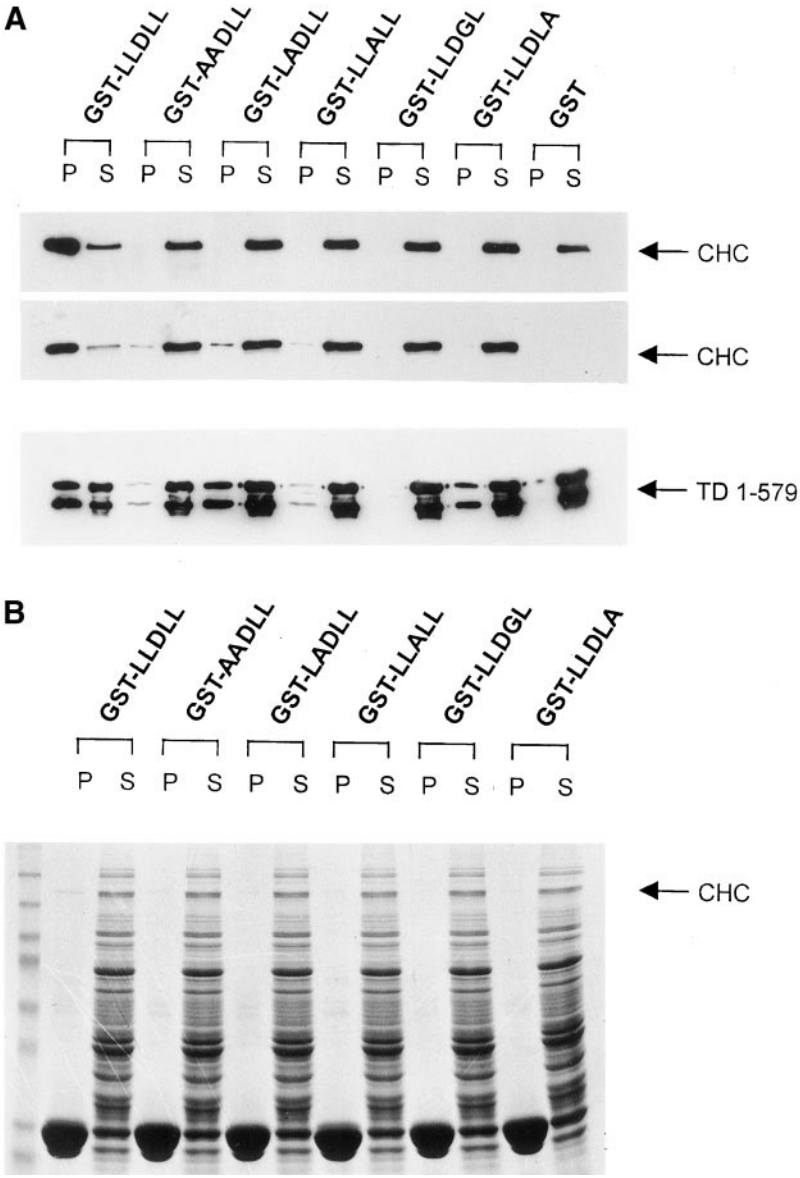

Figure 6. Each residue within the γ hinge LLDLL sequence is important for binding clathrin trimers. (A) Binding assays performed with rat liver cytosol in the presence of 0.1% Triton X-100 (top) and in the absence of detergent (middle), or with purified clathrin TD 1–579 (bottom). (B) Coomassie blue-stained gel of the same samples indicated in A incubated with rat liver cytosol in the presence of 0.1% Triton X-100.

similar manner to GST-γ 703–822 not only in terms of clathrin recruitment but in binding tubulin and rabaptin 5 (Figure 5), and also γ synergin (Doray and Kornfeld, unpublished observation). Moreover, GST-γ2 593–785 (appendage + hinge) also cooperatively bound clathrin like the γ appendage + hinge fusion, although a somewhat less pronounced effect was noted, which may be attributed to the second motif having a proline instead of a leucine residue in the fifth position (Figure 5). Nonetheless, these findings are consistent with γ2-adaptin having a role in clathrin-mediated protein trafficking (Lewin *et al.*, 1998).

Mutagenesis of γ Hinge LLDLL Sequence

To delineate the critical residues of the γ hinge pentapeptide sequence involved in clathrin binding, a series of alanine or glycine mutants was constructed, expressed, and tested for their ability to recruit clathrin in pull-down assays with the use of rat liver cytosol. Mutation of any residue to alanine or glycine within this variant γ hinge clathrin box motif completely abolished clathrin binding under the standard assay conditions (buffer A) used throughout this study (Figure 6A, top, and B). When the detergent Triton X-100 was omitted from both the binding and wash steps, trace amounts of clathrin were detected in the pellet fraction of all the mutants with the exception of LLDGL after incubation with cytosol (Figure 6A, middle). Similar results were obtained when purified terminal domain was used in the binding assays in place of cytosolic clathrin (Figure 6A, bottom). However, in this case mutation of the second leucine had only a small effect on terminal domain binding and mutation of the last leucine resulted in impaired, but not absent,

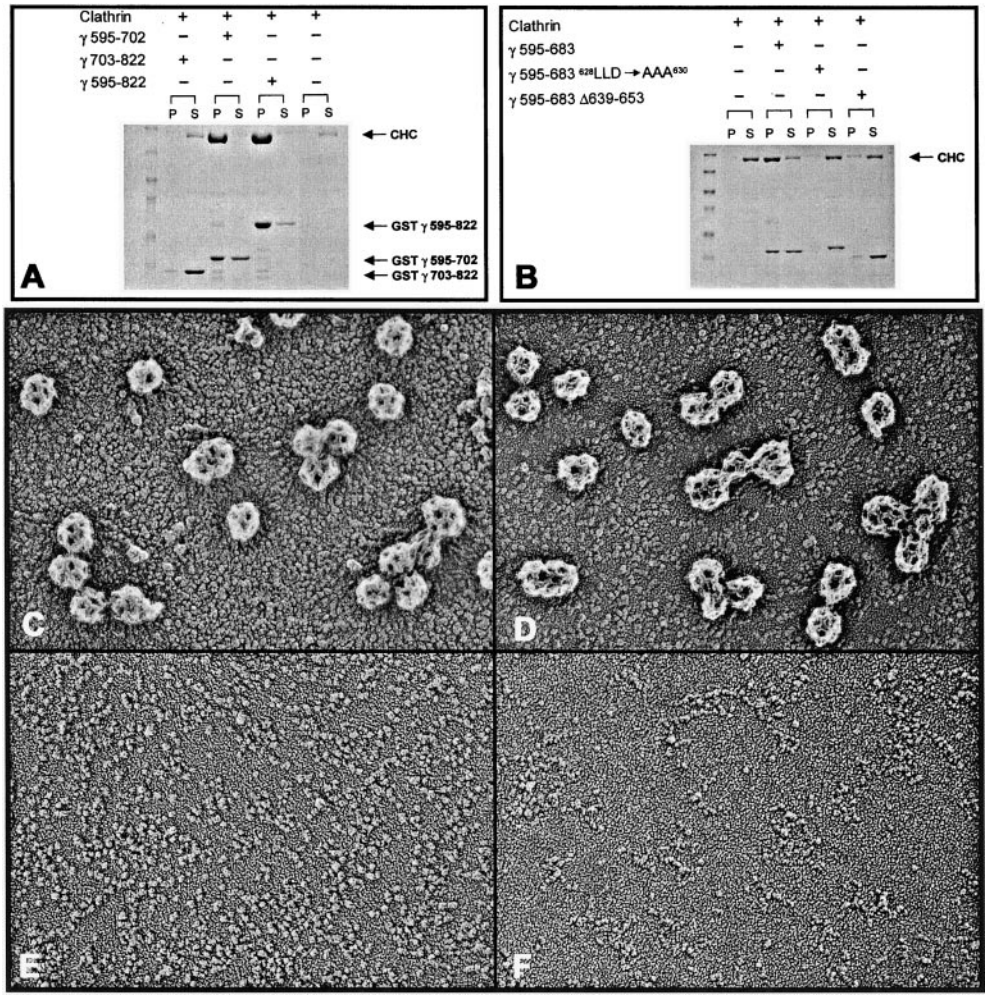

Figure 7. γ Hinge/appendage facilitates the polymerization and assembly of clathrin lattices. The polymerization assays were carried out as described under MATERIALS AND METHODS. (A) Coomassie blue-stained gel of the polymerization of cytosolic clathrin in the presence of GST γ appendage, GST γ hinge, and GST γ appendage + hinge. By densitometric analysis, 3% of the clathrin was in the high-speed pellet for GST γ appendage, 50% for GST γ hinge, 90% for GST γ appendage + hinge, and 9% for clathrin alone. The values represent the average of two independent experiments with the use of two different preparations of clathrin. (B) Coomassie blue-stained gel of the polymerization of cytosolic clathrin in the presence of GST γ 595–683, GST γ 595–683Δ639–653, and GST γ 595–683 ^{628}LLD → AAA^{630}. (C–F) Electron microscopy images obtained from high-speed pellets of assembled clathrin of the samples indicated in A. (C) GST γ appendage + hinge and clathrin; (D) GST γ hinge and clathrin; (E) GST γ appendage and clathrin; (F) clathrin alone.

binding. It should be noted that the conditions of the two assays differed in that an ~100-fold molar excess of terminal domain was present compared with the concentration of clathrin with the use of rat liver cytosol. Such a high concentration of terminal domain is necessary to compensate for the weak affinity of the monomeric interaction that occurs between the terminal domain and the GST-peptide fusion protein. We suspect that the difference in the binding profiles obtained with the two assays is a consequence of the condition of the assays. Taken together, these data suggest that substitution of alanine or glycine residues within the γ hinge LLDLL sequence is not favorable for its interaction with the clathrin N-terminal domain, underscoring the specificity of the pentapeptide-terminal domain interaction. It should be noted that because the first leucine residue was not individually mutated, we cannot be certain of its role at this time.

γ Hinge/Appendage Drives Clathrin Lattice Assembly

Because the GST-γ appendage, GST-γ hinge, and the GST-γ appendage + hinge fusion proteins when immobilized on glutathione-Sepharose beads were able to bind clathrin from cytosol, we wanted to determine whether these proteins could also facilitate the polymerization of soluble clathrin into cages. With the use of an in vitro coat assembly assay (Gallusser and Kirchhausen, 1993), we show that GST-γ hinge is sufficient to polymerize cytosolic clathrin into a sedimentable state (Figure 7A). In contrast, GST-γ appendage only produced background levels of clathrin in the pellet fraction. However, GST-γ appendage + hinge was more effective than the hinge alone in polymerizing clathrin, consistent with the results from the pull-down experiments (Figure 7A). Deletion of the residues between the two LLDLL sequences of the γ hinge severely impaired the ability of the hinge to drive lattice formation, whereas mutation of the first LLDLL to AAALL reduced clathrin in the pellet fraction to background levels (Figure 7B). To determine whether the clathrin recovered in the pellets was in fact incorporated into cages, the samples were subjected to electron microscopy. The polymerized clathrin associated with either the GST-γ appendage + hinge or GST-γ hinge was assembled into discrete cages as seen in Figure 7, C and D, respectively. Neither GST-γ appendage nor clathrin by itself did so under the prevalent assay conditions (Figure 7, E and F).

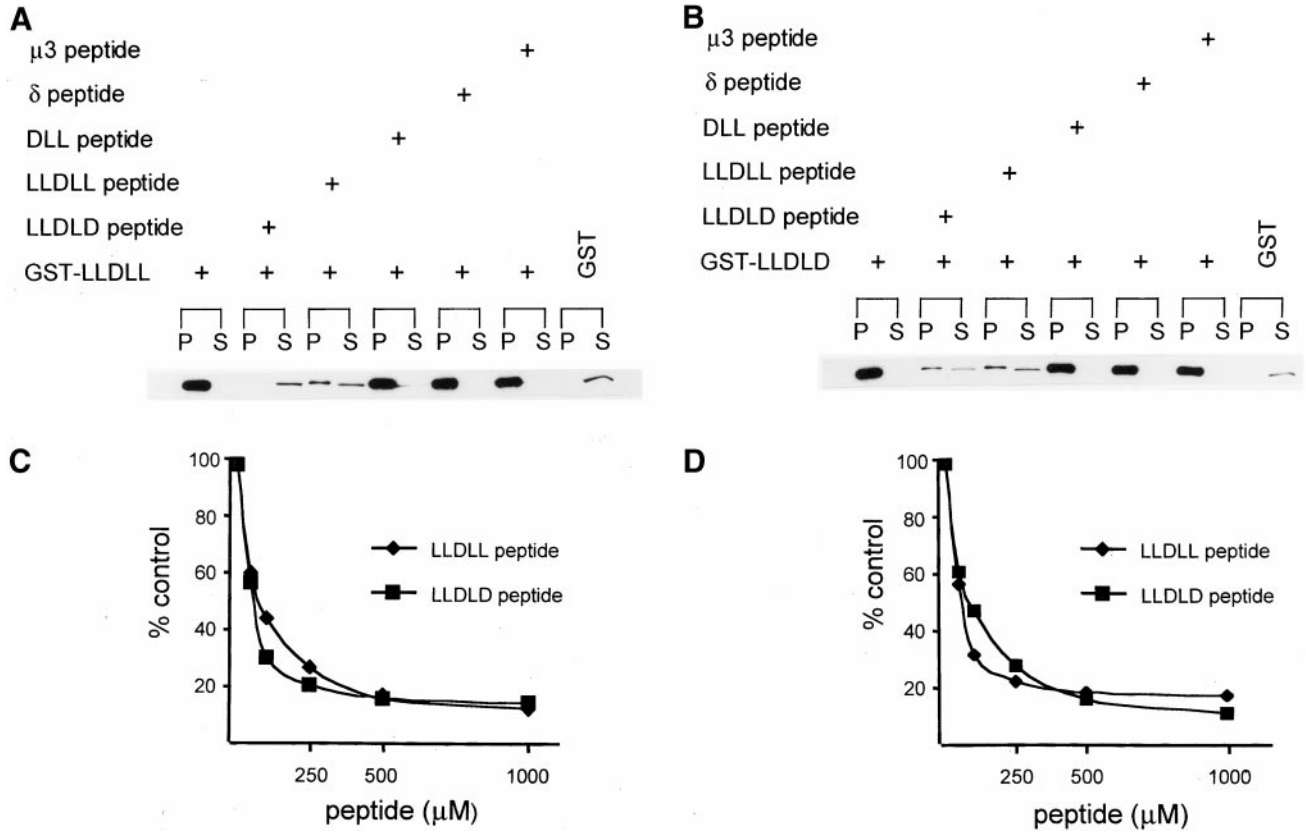

Figure 8. Both LLDLL and LLDLD peptides inhibit GST-LLDLL and GST-LLDLD. Inhibition assays were performed as described under MATERIALS AND METHODS. (A–B) Concentration of each free peptide was 1 mM. (A) GST-LLDLL immobilized on glutathione-Sepharose 4B. (B) Immobilized GST-LLDLD. (C and D) Free peptide concentrations varied from 50 μM to 1 mM. Curves were generated from densitometric analysis of the pellet fractions of the pull-down assays at different peptide concentrations. (C) GST-LLDLL immobilized on glutathione-Sepharose 4B. (D) Immobilized GST-LLDLD.

Peptide Inhibition of GST-LLDLL and -LLDLD Pull-Down of Clathrin

To address the issue of whether the LLDLL motif binds to the same groove of the clathrin terminal domain as the LLDLD motif, peptides derived from γ hinge and amphiphysin 1 incorporating their respective clathrin binding sequences were synthesized. As control peptides in these assays, we used a peptide partially overlapping the distal clathrin binding site (Figure 1A) of γ hinge and containing the sequence DLL, or peptides derived from the hinge segment of the δ subunit of AP-3 or the σ3 subunit. As shown in Figures 8, A and B, the ability of GST-LLDLL and GST-LLDLD to bind clathrin is strongly inhibited by either the LLDLL or the LLDLD peptides at 1 mM. Neither the DLL nor the δ hinge or the σ3 peptides displayed any inhibitory effect at the same concentration, indicating the inhibition to be specific to the peptide sequences in question. As shown in Figure 8, C and D, the LLDLL and the LLDLD peptides are equally effective in inhibiting clathrin binding to either GST-LLDLL or GST-LLDLD.

DISCUSSION

A number of studies have shown that the TGN-associated AP-1 and plasma membrane-associated AP-2 adaptor complexes interact directly with clathrin and induce the assembly of clathrin-AP coats in vitro (Ahle and Ungewickell, 1989; Gallusser and Kirchhausen, 1993; Shih *et al.*, 1995). One important mechanism of the clathrin-adaptor interaction involves the binding of a short peptide motif, the clathrin box sequence, present in the β chains of adaptor proteins to the terminal domain of clathrin (Dell'Angelica *et al.*, 1998; ter Haar *et al.*, 2000). In addition, over the past several years evidence has been obtained that the α appendage of AP-2 also has a role in coated vesicle assembly (reviewed in Owen and Luzio, 2000). A number of proteins involved in endocytosis, including amphiphysin, epsin, Eps 15, AP-180, and auxilin have been shown to associate with the α appendage, and most of these interact directly with clathrin (Ahle and Ungewickell, 1990; Morris *et al.*, 1993; Ramjaun and McMahon, 1998; Drake *et al.*, 2000). This is in contrast to the α appendage or the α appendage + hinge, which displays no clathrin binding ability (Shih *et al.*, 1995; Wang *et al.*, 1995;

Owen *et al.*, 2000). These data suggest that clathrin lattice assembly with AP-2 involves bivalent binding of clathrin with adaptor, directly via the β2 subunit and indirectly via the α subunit-associated proteins. In contrast to these findings with the α appendage, the γ appendage is only known to associate with γ-synergin, GAK, or auxilin 2, and rabaptin 5 (Page *et al.*, 1999; Hirst *et al.*, 2000; Umeda *et al.*, 2000). γ-Synergin, an EH domain-containing protein, has been proposed to function as an adaptor adaptor in linking the AP-1 complex to other proteins at the TGN (Page *et al.*, 1999). However, it has not been shown to interact with clathrin. GAK or auxilin 2 does bind clathrin but it is believed to act as a cofactor for the hsc 70-mediated clathrin coat dissociation rather than participating in clathrin coat assembly (Umeda *et al.*, 2000). The significance of rabaptin 5 binding to the γ appendage is unknown. Thus, we are unaware of any prior evidence that the γ appendage + hinge participates in clathrin lattice assembly.

The results of our study establish that the γ subunit of mouse AP-1 has two independent clathrin-binding sites, one located within the hinge and the other in the appendage. The γ hinge clathrin binding site comprises two LLDLL sequences with a similar spacing to the LLDLD and PWDLW clathrin binding motifs of amphiphysin 1 (Slepnev *et al.*, 2000). In addition to the γ hinge of human and mouse AP-1, the LLDLL sequence is also present in human and mouse γ2 proteins, as well as yeast β1 (LLELL) and β2 adaptins. Also, the hinge region between the GAT domain and the γ adaptin homologous appendage domain of human Vear (GGA2) has an LLDLL sequence. We have shown that the Vear hinge interacts with clathrin and that both this LLDLL motif and the LIDLE sequence that is also present within the Vear hinge are required for clathrin binding (Zhu *et al.*).

The LLDLL sequence is significantly different from the canonical clathrin box sequence in that it lacks an acidic residue at the fifth position. The potential importance of this residue in binding to the clathrin terminal domain was revealed in the crystal structures of the clathrin heavy chain residues 1–363 cocrystallized with the β-arrestin 2 LIEFE and AP-3 LLDLD peptides (ter Haar *et al.*, 2000). These structures showed that the canonical clathrin box sequence binds to a groove between blades 1 and 2 of the seven-bladed β-propeller module with the terminal acidic residues engaging in electrostatic interactions with lysine 64 and arginine 96 of the clathrin terminal domain. That the free carboxyl group of the final glutamate or aspartate is essential for clathrin binding was further demonstrated with the yeast Ent1p protein whose clathrin binding motif, LIDL, forms the acidic C terminus of the polypeptide chain. Thus, the fusion protein GST-RGYTLIDL bound clathrin, whereas GST-RGYTLIDLAAAAA with five additional alanine residues did not (Wendland *et al.*, 1999). The clathrin-binding motifs of the γ hinge not only lack an acidic residue at the fifth position but also in the sixth position, as occurs with the epsin proximal clathrin binding sequence (Rosenthal *et al.*, 1999). Still, GST-NDLLDLLG derived from the γ hinge recruited clathrin triskelia from cytosol as efficiently as GST-ETLLDLDF from amphiphysin 1. Furthermore, the γ hinge binding occurs with the clathrin terminal domain, similar to the LLDLD peptide. Our findings from the peptide inhibition studies suggest that both these sequences may engage the same site(s) on the clathrin terminal domain. This is rather surprising from the perspective of the crystallographic data, which clearly show the terminal acidic residue to be critical. An alternate explanation for our results is that in fact the two peptides bind to different sites on the terminal domain but upon peptide binding to one site the terminal domain undergoes a conformational change so as to preclude binding to the other site. Hence, the only way to categorically determine the precise binding site of the LLDLL motif would be to analyze a cocrystal of this sequence with the clathrin terminal domain.

One of the striking findings was that the GST-γ hinge facilitated the polymerization of soluble clathrin into cages, whereas GST-γ appendage failed to do so. This process required that both LLDLL sequences be present and that the spacing between them be maintained. There are several possible ways in which the GST-γ hinge could serve to promote the lateral association of clathrin legs to enhance the polymerization of soluble clathrin. One potential mechanism is that the two LLDLL motifs in the γ hinge bind to two terminal domains of a single clathrin triskelion to induce a conformational change that facilitates interaction with a second trimer, ultimately leading to enhanced polymerization. Alternately, the two LLDLL motifs in the γ hinge could cross-link terminal domains from two different triskelions, thereby stabilizing the interactions. In both of these models, reducing the distance between the LLDLL sequences or mutating one of the sequences would be predicted to preclude a simultaneous binding of the γ hinge to two terminal domains. At this point, we are unable to distinguish between these two models. Because the GST is a dimer, another possibility is that the two LLDLL sequences in the γ hinge bind simultaneously to different grooves within a single terminal domain β-propeller. In this case, the two γ hinges of the GST dimer would also bind to different terminal domains. This model would require that each terminal domain have two or more peptide binding sites for the LLDLL motif. There is evidence suggesting that the two clathrin binding motifs of amphiphysin 1 may perform an analogous cross-linking role in clathrin lattice assembly at the cell surface by way of aggregating the terminal domains by one of the described mechanisms (Traub, personal communication). Moreover, it was shown that mutation of either the LLDLD or the PWDLW sequence of amphiphysin 1 severely impaired clathrin binding (Slepnev *et al.*, 2000), again reflecting the poor affinity of a single motif for the clathrin terminal domain and the necessity for a bipartite clathrin binding site in both γ hinge and amphiphysin 1.

In a study published by Anderson and colleagues identifying the α appendage domain of AP-2 as a high-affinity binding site for dynamin, it was noted that GST-γ appendage (704–822) bound clathrin from bovine brain cytosol in GST pull-down experiments (Wang *et al.*, 1995). Because intact AP-1 and AP-2 were also observed in the immunoblots of the pull-downs, the investigators suggested that the GST-γ appendage fusion protein interacted with AP-1 and AP-2, which in turn bound clathrin, presumably through their β chain hinge regions. In their study, the hinge region of the γ subunit was not tested for clathrin binding. We show that the γ appendage domain is capable of binding soluble cytosolic triskelia directly but displays no affinity for the clathrin N-terminal 1–579 amino acids, which include the terminal domain β-propeller and part of the α helical

zigzag linker (ter Haar *et al.*, 1998). This indicates that it binds to a more proximal site in the heavy chain. In this regard, Brodsky and colleagues recently showed that the minimum requirement for the β2 appendage + hinge domain to reconstitute complete clathrin basket formation is the presence of the clathrin heavy chain N-terminal domain and distal leg extending to residue 1074 (Greene *et al.*, 2000). A clathrin heavy chain fragment from residues 1–545 when combined with the β2 appendage + hinge domain produced no baskets. Further, our results demonstrate a strong synergistic effect on clathrin binding and polymerization into cages when both the appendage domain and the hinge region of the γ subunit are present at the same time, supporting the idea of the γ chain interacting simultaneously with the clathrin terminal domain and distal leg in bivalent manner. In the study by Owen *et el.* (2000), the β2 appendage and hinge domains exhibited a similar cooperativity in clathrin binding and polymerization, which led the authors to suggest that the bipartite nature of the β2 appendage + hinge interaction could serve to orient domains of clathrin triskelia correctly in order to drive clathrin cage formation in vivo. Our data impose upon this model yet another level of multivalency in the clathrin assembly process that occurs at the TGN. The end result is an effective cross-linking of the clathrin trimers through the hinge segments as well as the appendage domains of both the β1 and the γ subunits of AP-1, which could then efficiently drive the formation of a coated vesicle.

While this manuscript was in preparation, Morgan *et al.* (2000) reported that a motif containing the sequence DLL, which exists in multiple copies in many clathrin adaptor proteins, serves as a clathrin assembly motif. These investigators showed that peptides with this sequence had a low affinity for clathrin and that promotion of efficient clathrin polymerization required peptides with multiple copies of the DLL motif. The authors suggest that the large number of clathrin binding motifs in the adaptor proteins may allow multiple interactions with the grooves between the blades of the clathrin terminal domain, thereby facilitating clathrin assembly by cross-linking the terminal domains of adjacent triskelia. Based on our mutagenesis analysis of the LLDLL sequence (Figure 6) and our inhibition studies with the different peptides (Figure 8), it appears that LLDLL binds clathrin with a considerably higher affinity than DLL. This is also suggested by the finding that the α appendage + hinge fails to bind clathrin even although it has two DLL motifs (Shih *et al.*, 1995). The relationship between the LLDLL and the DLL motifs will require additional studies.

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