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A Clathrin-binding Site in the Hinge of the β2 Chain of Mammalian AP-2 Complexes*

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The assembly of cytosolic clathrin into the cytoplasmic face of coated pits and coated vesicles appears to be driven by the clathrin-associated protein (AP) complexes. We have previously shown that one of the large chains of the AP complexes, the β chain, is sufficient to drive coat assembly *in vitro*. This chain consists of two domains, the amino-terminal trunk and the carboxyl-terminal ear, linked by a "hinge." We report here that presence of the hinge in recombinant β trunk or in recombinant β ear fragments is essential for driving *in vitro* assembly of clathrin into coats. We have also used a binding assay to map the clathrin-binding site by nested deletion of hinge sequences to a 50-residue region in the center of the hinge. This sequence is conserved in all known β sequences from multicellular organisms. The interaction of a single β hinge with a clathrin triskelion is weak, and we propose that recruitment of cytosolic clathrin to a forming coated pit involves simultaneous contacts between the legs of single clathrin trimers and the β hinges of two or three membrane-bound AP complexes. Uncoating is likely to require interruption of these contacts.

Clathrin-coated pits and coated vesicles carry the endocytic and regulated exocytic vesicular traffic in eukaryotic cells. The assembly of the clathrin lattice on the cytosolic side of the membrane initiates the formation of a coated pit, and a section of the membrane is captured, becoming a coated vesicle (1). The membrane that forms the coated vesicle selectively retains specific membrane protein receptors, which are then targeted to the appropriate organelle (for recent reviews see Refs. 2–4).

Specific protein complexes isolated from coated vesicles are known to drive clathrin coat formation at physiological ionic strength (5–8) and are likely to direct the assembly of clathrin *in vivo*. The best studied of these are the AP-1 and AP-2 complexes, related heterotetrameric structures (9, 10) that preferentially localize at the trans-Golgi network and at the plasma membrane, respectively (11, 12). Coated pits at the Golgi and the plasma membrane selectively retain different subsets of membrane receptors, and the APs are believed to mediate receptor selection. This view is supported by a number of observations, including the direct association of receptor tails with APs *in vitro* (13–18) and *in vivo* (19–22). For this reason, these complexes have come to be called adaptors.

AP-1 and AP-2 complexes each contain two large subunits (γ and β1 or αa/αc and β2, respectively), one medium subunit (μ1 or μ2), and one small subunit (σ1 or σ2) (9, 10). The large subunits have a proline/glycine/alanine-rich region of about 100 amino acids that is sensitive to proteases (9, 23–25). This portion, referred to variously as the "hinge" or "linker," contains the majority of the differences between β1 and β2 or between αa and αc. When AP-2 complexes are examined by electron microscopy, they appear as a brick-like core with two smaller lobes attached (26). After proteolysis, the brick remains, but the two small lobes (referred to as "ears," "heads," or "appendages" and now known to be the carboxyl-terminal domain of the α/γ and β1/β2 subunits) are lost (9, 25, 26). It is therefore believed that the hinge connects the amino-terminal domain or "trunk" of a large chain (~600 amino acids) to the corresponding ear (~250 amino acids).

We have recently shown that the β1 and β2 subunits are the clathrin assembly components of the AP complexes (8). Recombinant β subunits can by themselves drive clathrin coat formation, and β chains compete with whole APs for the same binding site. Which parts of the β chain are crucial for coat formation? We show here using recombinant proteins that a β2 fragment containing the trunk joined to its hinge or a β2 fragment containing the hinge and the ear can drive clathrin assembly. Presence of the hinge is essential because comparable β2 fragments lacking it fail to drive coat formation. The hinge between the trunk and the ear is usually dismissed as a potential clathrin-binding element, because this section is the most divergent between β1 and β2, and it is generally assumed that the clathrin-binding site (or sites) is likely to have a conserved sequence. There are, however, two regions of similarity between the β1 and β2 in the center of the hinge, and we show that these conserved segments are indeed essential for inducing clathrin lattices. Our observations lead to the proposal that the β hinge contains the primary clathrin-binding site and that stimulation of lattice assembly occurs when this site is linked to an oligomerizing or membrane-anchored structure.

MATERIALS AND METHODS

Construction of Bacterial Expression Vectors—The αc hinge/ear and the β2 hinge/ear fragments listed in Fig. 1*A* were created from PCR products derived from previously cloned rat brain αc and β2 cDNA (25, 27) by ligation into the blunted *Nde*I site of the bacterial expression vector pRSETc. Six histidine residues were added to the amino terminus to facilitate purification by affinity chromatography on Ni-NTA-agarose[1] beads (Qiagen). The constructs were checked by DNA sequencing. The β2 trunk/hinge fragment was created by ligation of the

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[1] The abbreviations used are: Ni-NTA, nickel nitriloacetate; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholinepropanesulfonic acid.

appropriate polymerase chain reaction product from the rat brain β2 cDNA into the *Nde*I and *Pvu*II sites of the expression vector pRSETc. The expression vectors for intact β2 and for β2 trunk have been previously described (8).

Overexpression of Recombinant Proteins in Escherichia coli—*E. coli* BL21(DE3) were transformed with the expression vectors and grown overnight in LB (ear/hinge fragments) or M9A (all others) medium containing 100 μg/ml ampicillin. One-liter cultures were inoculated with 10 ml of overnight culture and grown at 37 °C until the cell density reached an A_{600} of ~0.5. Cultures expressing the hinge/ear fragments were cooled to room temperature in an ice water bath, and expression was induced with a final concentration of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. Cultures were grown for an additional 5–8 h at room temperature. Cultures expressing the intact β2, β2 trunk/hinge, or β2 trunk were kept at 37 °C until the cell density reached A_{600} = ~0.7, and expression was induced for 2–4 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside (8). The cells were then harvested by centrifugation at 7,000 rpm (JA-10, Beckman) for 5 min at 4 °C.

Purification of Recombinant Proteins—Pellets from 1–4-liter cultures expressing αc or β2 hinge/ear fragments were resuspended in 30–60 ml of sonication buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 0.2% Triton X-100, and 10 mM β-mercaptoethanol) and sonicated 5 × 30 s with alternate 30-s cooling intervals by incubation in an ice water bath. The lysates were spun at 17,000 rpm (JA-17 rotor, Beckman) for 30 min at 4 °C, and the supernatants mixed with 0.5 ml of Ni-NTA-agarose beads (QIAGEN) for 2–3 h. The resin was collected by gravity flow over an open-ended column (diameter, 2.5 cm) and washed five times by resuspension of the beads in 10 ml of sonication buffer containing 10 mM imidazole. Histidine-tagged proteins were obtained with five sequential 1-ml elution steps of 200 mM imidazole, 300 mM NaCl, and 40 mM Tris, pH 8.0. EDTA at 1.0 mM and phenylmethylsulfonyl fluoride at 250 μM were immediately added (phenylmethylsulfonyl fluoride was not added to β2 ear or β2-Δ83 hinge/ear eluates, which precipitate in the presence of ethanol). After overnight dialysis at 4 °C into 300 mM NaCl, 50 mM Tris, pH 8, and 1.0 mM EDTA, the sample was spun at 85,000 rpm (TLA100.4; Beckman) for 30 min at 4 °C. The supernatants were injected into a preparative Superdex 75 (Pharmacia Biotech Inc.) HR10/50 column equilibrated with storage buffer (20 mM HEPES, pH 8.0, 300 mM NaCl, and 1 mM EDTA) and eluted at a flow rate of 0.5–1.0 ml/min. For the construct β2-Δ83 hinge/ear, a Superose 12 (Pharmacia) HR5/30 column was used instead. The recombinant proteins eluted mostly (>70%) as a single peak with an estimated overall purity of at least 90% determined by SDS-PAGE and Coomassie Blue staining and with yields ranging from 0.5 to 5 mg/l of culture. β2, β2 trunk/hinge, and β2 trunk were purified as inclusion bodies, solubilized in 6 M GuHCl, refolded, and used without further purification (8).

Purification of Bovine Brain Clathrin and AP Complexes—Bovine brain clathrin was separated from AP complexes by sizing chromatography (8, 9, 28), and AP-2 complexes were purified by MonoQ (Pharmacia) ionic exchange chromatography (8, 12).

Rat Liver Cytosol Preparation—Livers (2) from rats starved overnight or prefrozen (Pel-freez) were minced into 3 volumes of 25 mM HEPES-KOH, pH 7.4, 250 mM sucrose, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 trypsin inhibitor units/ml aprotinin, and 5 μg/ml leupeptin and homogenized with 10 passes on a Van-Potter homogenizer coupled to a drill rotating at ~120 rpm. The sample was spun at 5,500 rpm (JA17, Beckman) for 10 min at 4 °C, and the supernatant was cleared sequentially at 10,000 rpm (JA17, Beckman) for 20 min and at 38,000 rpm (Ti60, Beckman) for 60 min at 4 °C. The top lipid layer was discarded, and the supernatant (40 ml) was stored in frozen aliquots at −70 °C. Aliquots were dialyzed overnight against binding buffer (20 mM HEPES, pH 7.0, 100 mM NaCl) at 4 °C and cleared at top speed on a microfuge (Eppendorf) for 10 min at 4 °C before use.

Binding Assay—Recombinant αc and β2 hinge/ear constructs were dialyzed overnight at 4 °C against binding buffer. The hinge/ear and rat liver cytosol preparations were cleared at 85,000 rpm (TLA100.4, Beckman) for 30 min at 4 °C before mixing. A typical experiment consisted of 100 μg of recombinant protein and 3 mg of rat liver cytosol in a total volume of 0.34 ml. After incubation for 30 min in ice, 10 μl of Ni-NTA-agarose beads preequilibrated in binding buffer were added and incubated in an Eppendorf tube for 1 h at 4 °C with constant mixing by rotation. The beads were washed four times with 200 μl of binding buffer plus 0.2% Triton X-100. Bound proteins were eluted by incubation for 2 min at room temperature with 30 μl of 8 M urea and 0.1 M HEPES, pH 8, and analyzed by SDS-PAGE. Because the release of histidine-tagged recombinant proteins is minimal under this condition, it was possible to verify that in all experiments the amount of recombinant protein bound to the beads was always the same. This analysis was done using SDS-PAGE and Coomassie Blue staining of the eluted beads after boiling with 1 × Laemmli sample buffer containing 100 mM imidazole.

Assembly of Clathrin Coats and Electron Microscopy—Assembly of soluble bovine brain clathrin into coats and electron microscopy were done exactly as described previously for mixtures containing clathrin and recombinant β chains (8).

Western Blot Analysis—Samples fractionated by SDS-PAGE were electrotransferred to nitrocellulose (Boehringer Mannheim) and probed with a mouse monoclonal IgM antibody directed against clathrin heavy chain (CHC5.9, IBL Research) or with rabbit serum containing polyclonal antibodies raised against the rat recombinant αc or against the β2 hinge/ear fragments (20). The membranes were developed by enhanced chemiluminesce with a secondary goat anti-mouse antibody coupled to horseradish peroxidase (Amersham Corp.) and exposed to X-OMAT x-ray film (Kodak) for different times to insure linearity of the response.

Amino-terminal Sequencing—Approximately 10 μg of recombinant αc or β2 hinge/ear were digested with elastase followed by SDS-12.5% PAGE. The cleaved products were electrotransferred to a polyvinylidene difluoride membrane, and the ~28 kDa fragments were subjected to six cycles of automated Edman degradation (9, 25, 29).

RESULTS

αc and β2 Have Extended Hinges and Globular Ears—We expressed the complete hinge ear portion of the rat brain αc as well as a series of rat brain β2 fragments containing parts of the hinge plus the complete carboxyl-terminal ear (Figs. 1*A* and 2). We compared the elution profile of native αc and β2 hinge/ear domains prepared by tryptic digestion of bovine brain AP-2 complexes with the elution profile of the recombinant αc and β2 hinge/ears. The various hinge/ear fragments all have Stokes radii that are larger than predicted from their molecular weights, assuming a compact, globular conformation (Table I). Moreover, the native and recombinant fragments have similar Stokes radii and therefore similar shapes, excluding the possibility that the histidine tags on the recombinant fragments might account for their anomalous elution profiles.

Electron microscopy shows that the AP-2 complexes consist of a brick-like core linked to two smaller globular structures (26). The distinction between hinge and ear was made largely on the basis of sequence data, because the proline/glycine-rich region now called the hinge seemed unlikely to fold into a compact domain (25, 30). If this model of a globular domain plus extended "stalk" is correct, it can account for the observed Stokes radii of the 34- and 40-kDa αc and β2 hinge/ear fragments. Elastase treatment of native APs yields AP cores plus a set of smaller fragments (about 28 kDa) derived from the large chains (24, 26). Similar proteolytic treatment of our recombinant αc and β2 hinge/ear fragments also yields 28-kDa fragments with amino termini at Ser-688 (αc) and Ser-701 (β2). These 28-kDa fragments have Stokes radii appropriate for compact proteins (Table I). Thus it appears that the hinges of the αc and β2 chains are relatively extended structures joining the respective amino-terminal trunks with the globular ears. Given the extensive sequence identity between the related αa and αc chains (31) and between β1 and β2 chains (25, 32), it is likely that the members of each type of large chain have very similar structures.

β2 Hinge/Ear Contains a Binding Site For Clathrin—Expression of recombinant β2 hinge/ear has made it possible to test directly for proteins that bind to this portion of the AP-2 complex and for its ability to promote assembly of clathrin lattices. When β2 hinge/ear was mixed with rat liver cytosol extracts and recovered on a Ni-NTA-agarose column, significant amounts of clathrin were found to be associated with it (Fig. 3*A*). Comparing the Coomassie Blue staining pattern of Fig. 3*A*, *lane 2* (absence of β2 hinge/ear) with that of *lane 3* (presence of β2 hinge/ear), one can see that the main difference is a band with the same electrophoretic mobility of the clathrin

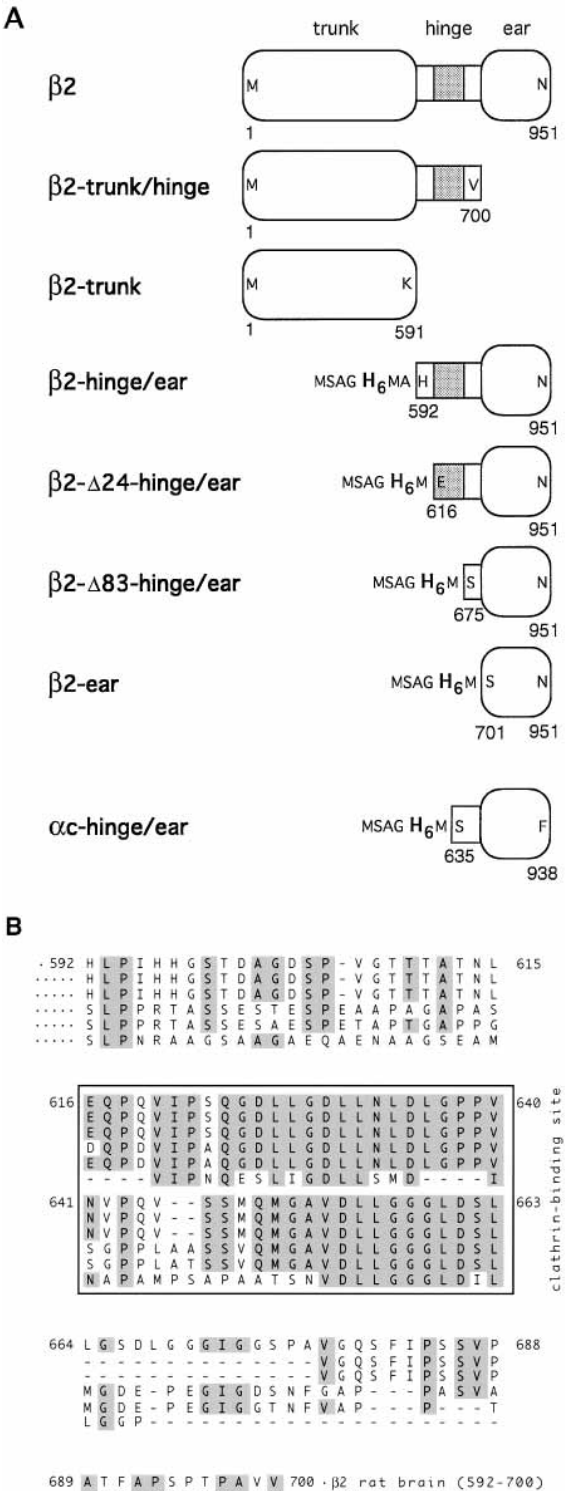

FIG. 1. **Schematic representation of recombinant molecules.** *A*, portions of rat brain αc and β2 expressed in *E. coli*. The amino acids at the beginning and the end of each fragment and the amino acids introduced at the amino-terminal end of the ear/hinge constructs are indicated. *B*, optimal sequence alignment of the hinge regions of β chains (25, 30, 33, 34) was obtained with the Clustal method (DNAstar). Portions with four or more matches are *shaded*. The proposed clathrin-binding site is *boxed*.

heavy chain (*lane 1*). Indeed, clathrin is specifically bound to the β2 hinge/ear fragment (Fig. 3*B*, *lane 6*), and over 50% of the clathrin in the cytosol preparation is extracted by this procedure even in the presence of high amounts of Tris (Fig. 4*C*; see below). This binding is specific, because clathrin is recruited from an unpurified cell extract (Fig. 3*A*, *lane 3*) and because there is no recruitment by the Ni-NTA-agarose beads alone (Fig. 3*A*, *lane 2*) nor by αc hinge/ear fragment (Fig. 3*A*, *lane 7*). Similar results were obtained using 0.5 M Tris-HCl, pH 7.4, a condition that depolymerizes clathrin coats (Fig. 4*B*, *lane 3*), and by using a number of sources of clathrin, including bovine brain cytosol and 1% Triton X-100 total extracts of A431 cells (data not shown). To define the site of clathrin binding more accurately, we tested the ability of truncated versions of β2 hinge/ear to extract clathrin from cytosol preparations. Removing 24 residues from the amino terminus of the hinge/ear (β2-Δ24 hinge/ear) had no effect on clathrin binding (Fig. 3*A*, *lane 4*), whereas fragments lacking either the amino-terminal 83 residues of the hinge (β2-Δ83 hinge/ear; Fig. 3*A*, *lane 5*, and Fig. 4*B*, *lane 4*) or the entire hinge (β2 ear; Fig. 3*A*, *lane 6*) completely failed to bind clathrin. Thus, the residues required for this interaction lie between 616 and 674. As indicated in Fig. 1*B*, the hinge segment between residues 592 and 700 is responsible for most of the sequence divergence between the mammalian β1 and β2 chains, but the central segment including Val-620 to Val-640 and Val-653 to Leu-663 is well conserved among mammalian species (25, 30, 33) and in the single β chain of Drosophila (34).

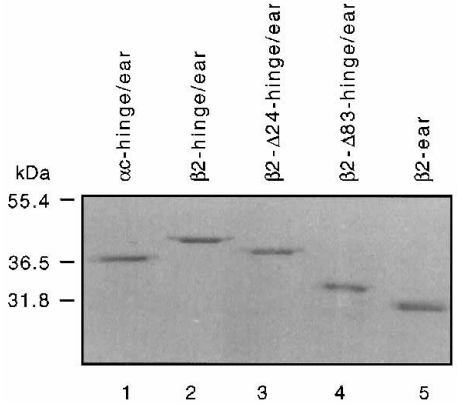

FIG. 2. **Electrophoretic analysis of recombinant rat αc and β2 hinge/ear fragments expressed in *E. coli*.** SDS-12.5% PAGE and Coomassie Blue staining of purified αc hinge/ear (*lane 1*), β2 hinge/ear (*lane 2*), β2-Δ24 hinge/ear (*lane 3*), β2-Δ83 hinge/ear (*lane 4*), and β2 ear (*lane 5*). Size markers are indicated.

The results from the binding experiment just described cannot rule out the possibility that another protein, present in cytosol or in the cell extracts and of a size similar to one of the proteins that binds nonspecifically to the-agarose beads, might in fact serve as a linker in the association of clathrin to β2 hinge/ear. We therefore examined whether in the absence of other cellular proteins, β2 hinge/ear will still bind clathrin purified from coated vesicles (Fig. 4*A*). It does even when it is added in Tris-containing buffers (Fig. 4*A*, *lane 6*). Thus, at least for this aspect of the interaction between clathrin and the AP complexes, not only is there no apparent requirement for other proteins but also there is no apparent difference between coated vesicle-derived clathrin and the majority of the clathrin trimers found in cytosol. Fig. 4*A* also shows that the β2 ear alone and the αc hinge/ear bind clathrin far more weakly than β2 hinge/ear, consistent with the absence of detectable binding of cytosolic clathrin by these fragments. It is important to note, however, that, in the moderate ionic strength of the various buffer conditions used here, the association of β2 hinge/ear with clathrin is only detectable when the hinge/ears are immobilized on Ni-NTA-agarose beads. In the absence of the beads, free β2 hinge/ears do not co-elute with clathrin trimers from a

TABLE I

Stokes radii of native and recombinant β2 fragments

The samples were injected into a Superose 12 HR5/30 column run at room temperature with storage buffer (0.5 ml/min). The elution volumes (Ve) were obtained from the peaks in the $A_{280\ nm}$ tracings. The Stokes radii were calculated from the molecular weights of the fragments assuming a globular structure or estimated from their Ve by logarithmic interpolation between the Ve values of bovine serum albumin and carbonic anhydrase (47, 48). AP-2 complexes (kept in the buffer from which they elute from the MonoQ column) were treated with trypsin to cleave the large chains and to separate their hinge/ear domains from the remaining AP cores (24, 25). Recombinant αc and β2 hinge/ear (in 30 μl storage buffer) were digested with elastase (Sigma) for 10 min at room temperature, and the cleavage was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride. The identity of the samples was established by SDS-PAGE and Western blot analysis or Coomassie Blue staining.

Sample	Molecular weight from DNA	Molecular weight from SDS-PAGE	Stokes Radius from molecular weight	Ve	Stokes radius from Ve
			nm	*ml*	*nm*
Albumin	66,000		3.6	11.8	3.6
β2-hinge/ear tryptic from AP-2		39,000	2.5	12.2	3.2
β2 hinge/ear recombinant	40,000		2.5	12.2	3.2
αc hinge/ear tryptic from AP-2		35,000	2.2	12.8	2.6
αc hinge/ear recombinant	34,000		2.2	12.8	2.6
Carbonic anhydrase	29,000		2.0	13.6	2.0
Elastase ear fragment from recombinant αc hinge/ear		28,000	2.0	13.8	1.9
β2 ear recombinant	29,000		2.0	13.8	1.9
Elastase ear fragment from recombinant β2 hinge/ear		28,000	2.0	13.8	1.9

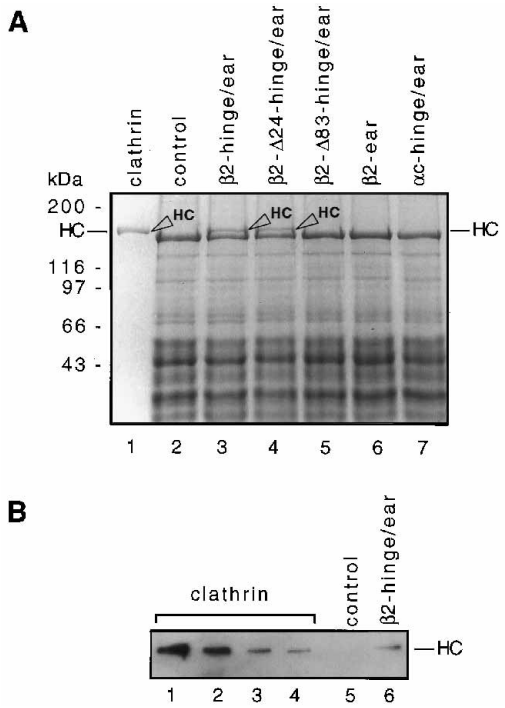

FIG. 3. **Immobilized β2 hinge/ear and β2-Δ24 hinge/ear bind clathrin from rat liver cytosol.** This binding experiment was performed by incubation of rat liver cytosol (3 mg) with recombinant ear/hinge fragments (100 μg). *A*, 75% of the samples containing proteins bound to the Ni-NTA-agarose beads were analyzed by SDS-12.5% PAGE and Coomassie Blue staining. *Lane 1*, 200 ng of purified bovine brain clathrin; *lane 2*, cytosol and beads only, negative control; *lane 3*, cytosol with beads and β2 hinge/ear; *lane 4*, β2-Δ24 hinge/ear; *lane 5*, β2-Δ83 hinge/hinge; *lane 6*, β2 ear; *lane 7*, αc hinge/ear. The *arrowheads* highlight clathrin heavy chain (*HC*). *B*, Western blot analysis using chemiluminescence detection with the monoclonal antibody CHC5.9 (against clathrin heavy chain). *Lanes 1–4* show the signals from serial 2-fold dilutions of purified bovine clathrin (200–25 ng). *Lanes 5* and *6* show the clathrin signal from 3 μl (10%) of the eluted samples described above corresponding either to cytosol and beads (*control*) or to cytosol, beads, and β2 hinge/ear, respectively.

Superose 6 column (Fig. 5). Our interpretation of this result is that the interaction between clathrin and the β2 hinge/ear is too weak to be detected in solution, but a clathrin trimer can bind simultaneously to two or three ear/hinges arrayed on the surface of a Ni-NTA-agarose bead, resulting in measurable interaction. We return to this point under "Discussion."

β2 Hinge/Ear Drives Assembly of Clathrin Lattices—Immobilized β2 hinge/ear binds clathrin specifically. Can it drive coat formation? Low concentrations of the fragment do not (9, 24), but at concentrations greater than 50 μg/ml the β2 hinge/ear can indeed promote clathrin assembly (Fig. 6). The coats formed by β2 hinge/ear (Fig. 6*B*) are more heterogeneous in size and more irregular in structure than the characteristic small barrel-type coats formed with intact β2 (Figs. 6*A* and 8*A*), β2 trunk/hinge (Fig. 8*B*), or APs (Ref. 8 and data not shown). Furthermore, most of the clathrin assemblies formed by β2 hinge/ear lack the core of excluded negative stain at the center characteristic of coats containing intact β2 (Figs. 6*A* and 8*A*) or β2 trunk/hinge (Fig. 6*B*). Incorporation of β2 hinge/ear to the newly formed clathrin assemblies was established by SDS-PAGE analysis of the high speed supernatants and the pellets from the assembly experiment (Fig. 7, *lanes 7* and *8*). Judging by the relative intensity of the Coomassie Blue-stained bands of clathrin heavy and light chains and of β2 hinge/ear in the high speed pellet (lane 8), we estimate an approximate ratio of one clathrin leg to one β2 hinge/ear fragment in the lattice. The high speed pellet in the control with β2 ear had no clathrin (Fig. 7, *lane 6*), and the pellet in the absence of clathrin had reduced ammounts of β2 hinge/ear (Fig. 7, *lane 14*). In coat assembly buffer III, a portion of β2 hinge/ear tended to self-associate and appear in the low speed pellet. In binding buffer, however, β2 hinge/ear remains soluble and does not interact with clathrin (Fig. 5), and in this buffer, β2 hinge/ear does not promote coat formation (not shown). We therefore believe that fragments of β2 hinge/ear stimulate coat assembly by simultaneously self associating and binding to clathrin, thereby driving the clathrin trimers together to form a lattice.

β2 trunk/Hinge Drives Clathrin Coat Formation—Can the hinge segment promote coat assembly when attached to structures other than the ear? To answer this question, we mixed β2 trunk/hinge with purified clathrin and looked for the formation of coats. The trunk/hinge induces coats (Fig. 8*B*) that are indistinguishable from the barrel-shaped coats made using intact β2 subunits (Fig. 8*A*) or whole AP complexes (Ref. 8 and data not shown). As negative controls for the assembly we have repeated our previous observation (8) that a recombinant protein containing only the trunk of β2 does not induce coats (Fig. 8*C*) and that clathrin alone does not form cages under the conditions used (Fig. 8*D*).

Association of clathrin with the β2 trunk/hinge was confirmed by SDS-PAGE analysis (Fig. 9) of supernatants and pellets obtained from the coat assembly experiment described above. The similar amounts of intact β2, β2 trunk/hinge, and clathrin found in the high speed pellets containing coats (Fig. 9,

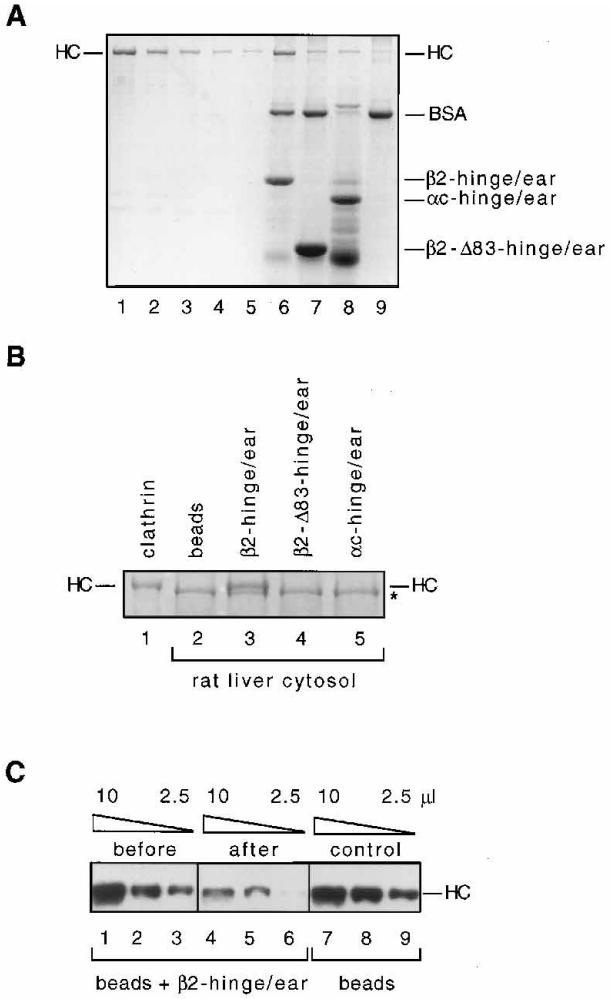

FIG. 4. **Immobilized β2 hinge/ear binds purified and cytosolic clathrin in the presence of Tris.** *A*, 100 μg of purified clathrin was mixed with 50 μg of β2 hinge/ear, β2 ear, or αc hinge/ear or with Ni-NTA-agarose beads alone, in a final volume of 0.5 ml (*lanes 6–9*). The composition of the solution was 0.1 M Tris-HCl, pH 7.4, 100 mM NaCl, and 0.5 mg bovine serum albumin (*BSA*). Bound proteins were eluted with 50 μl of 0.2 M imidazole, and ⅓ of the sample was analyzed by SDS-12.5% PAGE and Coomassie Blue staining. The light chain composition of clathrin recruited by β2 hinge/ear and of purified clathrin was the same, indicating that there was no preferential recruitment of a subpopulation of clathrin. Presence of αc hinge/ear prevents binding of bovine serum albumin to the beads. 2-fold serial dilutions of clathrin starting with 1 μg were included to calibrate the gel (*lanes 1–5*); clathrin with beads and β2 hinge/ear (*lane 6*); β2-Δ83 hinge/ear (*lane 7*); αc hinge/ear (*lane 8*); and clathrin and beads only (*lane 9*). *B*, samples of 3.7 mg of rat liver cytosol, dialyzed into 0.5 M Tris-HCl, pH 7.4, were mixed with the recombinant fragments in a total volume of 0.4 ml (*lanes 2–5*). The final buffer was 0.5 M Tris-HCl, pH 7.4, and the samples were analyzed by SDS-10% PAGE as in Fig. 3*A*. *Lane 1*, 200 ng clathrin; *lane 2*, cytosol with beads only; *lane 3*, cytosol with beads and β2 hinge/ear; *lane 4*, β2-Δ83 hinge/ear; *lane 5*, αc hinge/ear. The band below clathrin heavy chain, indicated with an *asterisk*, is a cytosolic protein that binds nonspecifically to the beads also detected in Fig. 3*A*. *C*, comparison by SDS-8% PAGE and Western blot analysis of the amount of clathrin remaining in the rat liver cytosol after its depletion by β2 hinge/ear bound to the Ni-NTA-agarose beads. The samples probed with CHC5.9 correspond to 2-fold serial dilutions starting with 10 μl of the cytosol supplemented with β2 hinge/ear before addition of the beads (*lanes 1–3*) and after binding to the beads (*lanes 4–6*) and a control of cytosol incubated with beads in the absence of β2 hinge/ear (*lanes 7–9*). The comparison of the signal intensities of *lanes 3* and *5* indicates that at least 50% of cytosolic clathrin bound to the β2 hinge/ear fragment.

lanes 4 and *8*) indicate that they have essentially the same stoichiometry of association with clathrin. Thus, the hinge is required for coat formation.

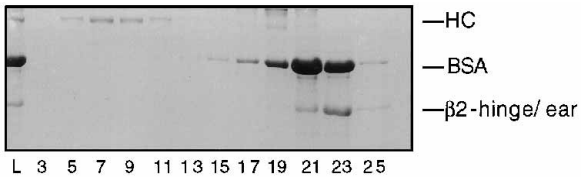

FIG. 5. **β2 hinge/ear does not bind clathrin in solution.** 100 μg of clathrin (*HC*) was incubated with 100 μg of β2 hinge/ear and with 2.5 mg bovine serum albumin (*BSA*) in a total volume of 0.5 ml of binding buffer for 60 min in ice followed by centrifugation at 85,000 rpm (TLA100.4) for 10 min. The amount of these proteins remaining in the supernatant was the same whether they were spun individually or as a part of the mixture. The supernatant was applied at 0.5 ml/min to a Superose 6 HR5/30 (Pharmacia) column equilibrated in binding buffer, and 0.1-ml fractions were collected and concentrated. 10 μl of the injected sample (*L*) and the indicated alternating fractions were analyzed by SDS-12.5% PAGE and Coomassie Blue staining.

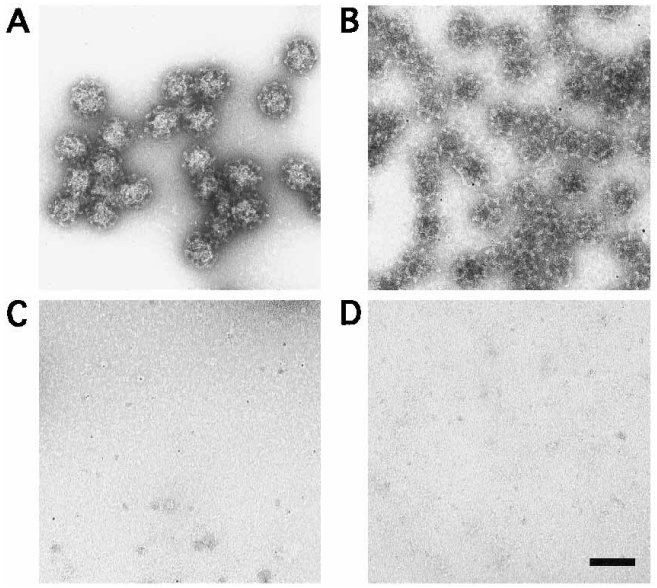

FIG. 6. **β2 hinge/ear facilitates the assembly of clathrin lattices.** Negatively stained images obtained from high speed pellets of clathrin assemblies generated by overnight dialysis at 4 °C against coat assembly buffer III (0.1 M Na-MES, pH 6.5, 2 mM EDTA, and 2 mM dithiothreitol). Aggregated material was removed by top speed centrifugation in a table-top microfuge followed by centrifugation at 60,000 rpm for 12 min (TLA100, Beckman) to separate the assembled from nonassembled material. Clathrin:β2 coats (*A*) and clathrin assemblies (*B*, *C*, and *D*) were obtained from mixtures containing clathrin (~0.5 mg/ml) together with β2 (~0.2 mg/ml) and β2 hinge/ear, β2 ear, or αc hinge/ear (~0.5 mg/ml), respectively. In contrast to the clathrin coats obtained with intact β2 (*A*), those obtained with β2 hinge/ear (*B*) are more irregular in size and shape, and their centers appear empty. This experiment was performed five times with identical results using independent preparations of clathrin and recombinant proteins. Values indicate the number of free-standing clathrin assemblies counted in three random fields. *A*, clathrin:β2, 1194, positive control; *B*, clathrin:β2 hinge/ear, 1145; *C*, clathrin:β2 ear, 68, negative control; *D*, clathrin:αc hinge/ear, 59, negative control. *Scale bar*, 100 nm.

DISCUSSION AND CONCLUSIONS

The β1 and β2 subunits of clathrin-associated AP complexes interact with clathrin (8, 23, 35) and drive coat assembly (8). This activity depends on structures contained within the carboxyl-terminal end of the protein, because neither the amino-terminal β trunk (8) nor the full AP core stimulates coat formation (24). We have therefore studied the properties of the carboxyl-terminal part of the β2 chain. Our experiments show that this 39-kDa fragment, previously defined by tryptic digestion of intact APs (25), can be subdivided into two segments, an extended hinge and a compact ear. These correspond respectively to the stalk and appendage seen in electron micrographs

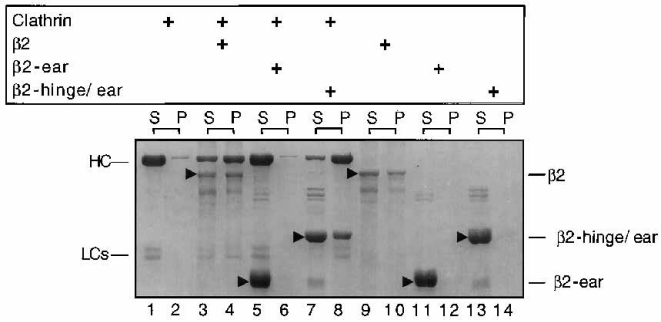

FIG. 7. **Incorporation of recombinant β2 hinge/ear into clathrin coats.** The mixtures correspond to the samples used in Fig. 6 (*lanes 3–8*). Clathrin (*lanes 1* and *2*) and the recombinant β fragments (*lanes 9–14*) were included as negative controls. The high speed supernatants (*S*) and pellets (*P*) were analyzed by SDS-12.5% PAGE and Coomassie Blue staining. *HC* and *LCs* indicate clathrin heavy chain and light chains, respectively. The recombinant proteins β2 (~100 kDa), β2 hinge/ear (~40 kDa), and β2 ear (~28 kDa) are indicated by *arrowheads*.

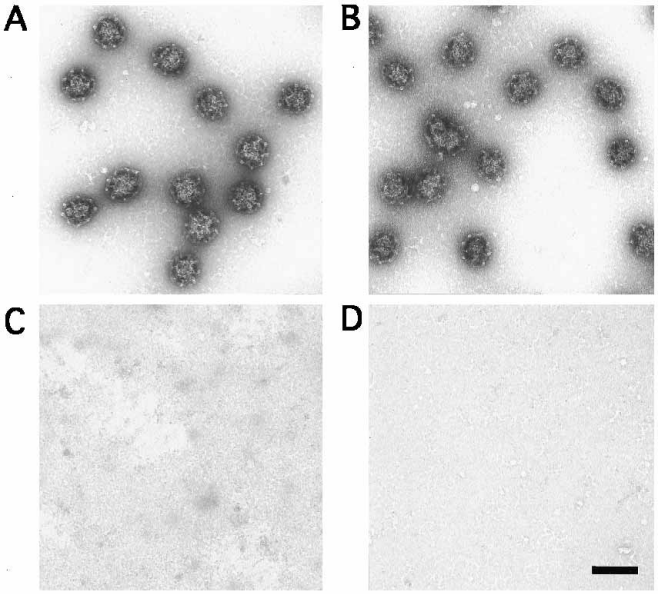

FIG. 8. **β2 trunk/hinge but not β2 trunk fragment assembles clathrin into coats.** Negatively stained high speed pellets from samples of coats formed in assembly buffer III supplemented with 1% Triton X-100 using purified bovine brain clathrin (final concentration, ~0.5 mg/ml) and refolded recombinant rat β2, β2 trunk/hinge, or β2 trunk chains (~0.2 mg/ml). The coats are mostly of the barrel-type (~70 nm in diameter) and display at their center a characteristic electron-dense material. The values indicate the number of free-standing coats found in four randomly chosen fields. *A*, clathrin:β2, 586. *B*, clathrin:β2 trunk/hinge, 721. *C*, clathrin:β2 trunk, 0. *D*, clathrin, 0. *Scale bar*, 100 nm.

of intact APs (26). The entire fragment, when immobilized on Ni-NTA-agarose, can bind clathrin; the ear alone does not. The residues required for binding lie between 616 and 674, a region that is well conserved from *Drosophila* to man, despite variations in sequences surrounding it (25, 30, 33, 34). Not only does the hinge/ear fragment bind clathrin to beads, it can also stimulate assembly of clathrin lattices. This latter property appears to require some self-association of the fragment, coupled to the direct interaction with clathrin. We have also shown that attachment of the hinge region to the β trunk confers on it a similar capacity to stimulate lattice formation. Again, association of the trunk portions is probably necessary to drive assembly.

A significant aspect of our results is the identification of the clathrin-binding site on β2 chains as part of an extended stalk structure. In three-dimensional image reconstructions of small

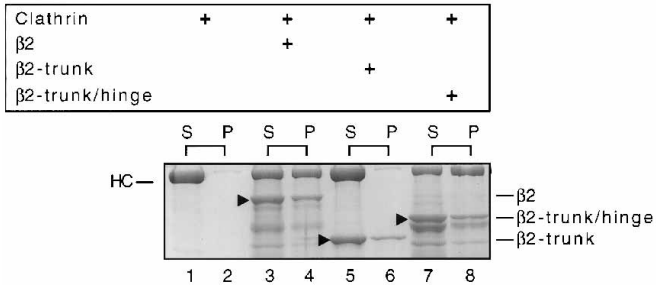

FIG. 9. **Incorporation of recombinant β2 and β2 trunk/hinge into clathrin coats.** The samples correspond to the assembly experiment described in Fig. 8. The high pellets were resuspended back into the same original volume, and aliquots of the supernatants (*S*) and pellets (*P*) were analyzed by SDS-10% PAGE and Coomassie Blue staining. *HC* indicates clathrin heavy chain. The *arrowheads* indicate the recombinant β2 (~106 kDa), β2 trunk (~67 kDa), and β2 trunk/hinge (~77 kDa) chains. Two additional and usually less abundant smaller recombinant proteins (~70–75 kDa), observed with the β2 and β2 trunk/hinge samples are partial translation products (8).

coats, elements likely to be the inward facing terminal domains of clathrin legs are seen to contact other structures, identified as APs, at smaller radii (36). It is therefore reasonable to imagine that the hinge segments project outwards toward the clathrin terminal domains and that the AP cores lie against a coated vesicle membrane without making direct contact with the framework of the lattice composed of the proximal and distal segments of the clathrin legs (the edges of the empty hexagonal and pentagonal facets). This view is supported by two additional observations. On the membrane side, AP cores as well as intact APs bind to isolated Golgi membranes in a nucleotide-dependent manner (37–39). On the clathrin side, terminal domain is required for the association of clathrin with AP complexes (40) and with β2 hinge/ear.[2]

Our results are consistent with a number of earlier observations on AP-clathrin interactions. Limited enzymatic proteolysis of APs leads to release of the hinge/ear parts of the large chains (α and γ, β1 and β2), leaving the AP cores otherwise intact (24, 25). These cores are inactive in stimulating coat assembly (24). Proteolysis of APs when they are part of a clathrin-AP coat also leads to the release of hinge ear fragments (9, 23, 25). This result has been interpreted previously to indicate that the hinge/ear domain does not interact with the clathrin lattice. However, our results suggest that there is a another explanation. The interaction of a single hinge segment with its target site on clathrin is too weak to immobilize an otherwise free peptide. For example when presented on Ni-NTA-agarose beads, the hinge can capture clathrin triskelia by virtue of two or three parallel contacts, one per leg, whereas isolated hinge ear fragments fail to interact under similar conditions. Likewise, AP complexes associated with each other under *in vitro* assembly conditions or arrayed together on the surface of a membrane can bind clathrin effectively. Thus, it is reasonable to expect that once rendered monovalent by cleavage from its trunk, the hinge/ear will dissociate from a clathrin lattice as observed. Consistent with this picture is the behavior of cleaved APs obtained by proteolysis of coated vesicles in tartrate-containing buffer (23). Under these conditions, which prevent aggregation of intact APs but do not release them from coats, both AP cores and β chain hinge ears dissociate as soon as the β chain is cleaved. Again, it appears that a monovalent interaction is not sufficient to retain the hinge ear, nor do there appear to be strong clathrin contacts to the cores. Other interactions between clathrin and AP-2 cores have been described (41), perhaps through the α chains (42), but these interactions

[2] A. Contreras and T. Kirchhausen, manuscript in preparation.

do not alone lead to the assembly of organized structures. The role suggested here for the β hinge as the principal clathrin-binding site is also consistent with recent results showing that intact AP-1 complexes bound to Golgi membranes recruit clathrin but proteolyzed AP cores do not (37).

This view differs from a conclusion that we reached earlier. We suggested, on the basis of coat stability when exposed to low concentrations of Tris before and after proteolytic treatment, that core contacts with clathrin were required for coat stability (9). Incomplete digestion of the APs in those experiments could readily account for the results, and we believe that the present work with pure recombinant chains is more definitive. Our interpretation here also differs from a conclusion, drawn by others, in which it was found that gentle treatment with elastase of isolated AP-containing plasma membranes did not affect ability of these membranes to form coats (43). It was concluded that AP cores, which are produced by digestion and remain attached to the membrane, have a direct role in coated pit formation. An alternative explanation is that a fraction of APs on the membrane remained undigested, because only the release of α but not of β hinge ear fragments was monitored in those experiments.

Coated vesicles contain arrays of binding sites at two levels, the clathrin lattice and the membrane-bound APs, linked by the interaction we report here. Isolated bovine brain coated vesicles contain many more clathrin chains than APs (about 2–3 clathrin trimers/AP), so that only some of the potential sites on the clathrin lattice are occupied. An individual AP-clathrin contact need not be a strong one, because the coat is stabilized by a number of such contacts in parallel. Disassembly of the clathrin lattice will occur if these contacts are weakened under conditions that do not strongly favor clathrin-clathrin interactions (*e.g.* normal physiological pH and ionic strength). This may be the mechanism of disassembly *in vivo*. Alternatively, coat disassembly will result from solubilization of the AP complexes, again under conditions that do not stabilize the clathrin lattice, because most clathrin trimers have no more than one AP contact, and we have shown that one contact is insufficient to anchor a triskelion. This is the mechanism of Tris-induced disassembly. Tris buffers have traditionally been used to dissociate clathrin and APs from coated vesicles and to depolymerize assembled coats and cages (28). Tris is known to disrupt clathrin-clathrin interactions and to prevent aggregation of APs. Uncoating of coated vesicles in low concentrations of Tris leaves most of the APs and about 10–30% of the clathrin attached to the membrane vesicle, about the expected ratio if two or three contacts are required per triskelion (44).[3] Uncoating in high concentrations of Tris solubilizes the APs, which fall off their clathrin-binding sites. Results reported here suggest that Tris does not, however, directly destabilize the clathrin-AP contacts. None of the earlier observations requires that it should do so. We have shown that a stable assembly requires multiple contacts between clathrin and APs arrayed on a substrate. Dissociation of APs from membranes and disruption of clathrin lattice interactions would thus be sufficient to solubilize both components.

In summary, coat assembly, or clathrin binding, appears to occur *in vitro* whenever the hinge peptide is arrayed in polyvalent form, either by association of the APs on which it is borne or by immobilization on a suitable matrix. Thus, we arrive at the following picture for assembly of the coated vesicle lattice (Fig. 10). Clusters of APs, prebound to plasma or trans-Golgi network membranes, serve as attachment points for cytosolic clathrin. This clathrin binds to the cytoplasmic face of the membrane much as it does to β2 hinge/ear segments presented in our experiments on Ni-NTA-agarose beads. The presence of several clathrin trimers in a localized region of membrane would be expected to favor lattice formation, including recruitment of additional clathrin molecules from the cytosol. These clathrins might in turn recruit further AP complexes, because they offer free sites for interaction with β chain hinges. The end product, a coated vesicle, is known to contain an excess of clathrin over APs, showing that not all legs are bound to a β chain.

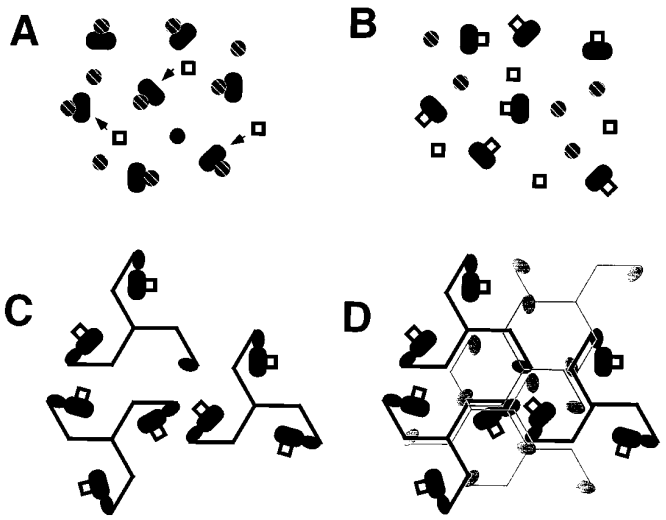

FIG. 10. **Model for the association of AP complexes and clathrin.** *A*, APs are first bound to the target membrane by association with a docking protein (*circle*). *B*, APs are then transferred to the cytoplasmic tails of membrane proteins (*square*) to be sorted in clathrin-coated pits and coated vesicles. *C*, cytosolic clathrin triskelions attach via their terminal domains to the β2 hinge of two or three APs, thereby becoming membrane-bound. *D*, additional cytosolic clathrin can now interact directly with membrane-bound clathrin and start the cooperative process that leads to the formation of the coated pit lattice.

The model just described relies on the way the β chain hinge/ear projects from the AP complex, on the ability of APs to cluster in membranes, and on the cooperativity of clathrin lattice formation. It requires, as indeed observed, that APs are bound to membranes prior to association with clathrin (19, 22). Clustering of membrane-bound APs is as suggested by immunolabeling experiments (45). The model predicts that APs containing truncated β1 and β2 chains lacking the hinge ear region will not be included in coated vesicles. Expression of such chains in Chinese hamster ovary cells indeed shows that they incorporate well into APs but poorly into clathrin-containing structures.[4]

Our model differs form an earlier proposal, which postulated the capture of two clathrin trimers by a single, divalent AP complex (6). That proposal was based on the incorrect assumption that α and β chains had similar properties and similar functions, conferring a pseudo 2-fold symmetry upon the complex, which was also believed to contain two medium and two small chains. Subsequent determination of the correct composition of the complex and of the primary structure of its components (reviewed in Ref. 3) required revision of this picture. In our model, AP complexes are monovalent, and it is their clustering in a membrane that creates a multi valent attachment site.

We suggest that induction of coat formation *in vitro* by APs,

[3] W. Shih, A. Gallusser, and T. Kirchhausen, unpublished observation.

[4] K. Clairmont, W. Boll, and T. Kirchhausen, manuscript in preparation.

β chains, or hinge-containing β2 chain fragments follows essentially the same mechanism. Like clustering in membranes, self-association of the APs leads to formation of a polyvalent nucleus for clathrin assembly. The small diameter of coats induced *in vitro* (7–9, 46) is probably related to initiation of assembly around a relatively small core of AP complexes.

The demonstration that the β2 chain hinge forms the essential bond between APs and clathrin predicts that interruption of this contact should be a key step in uncoating. We suggest that the mechanism of uncoating can now be analyzed further, in light of the findings presented here.

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A Clathrin-binding Site in the Hinge of the 2 Chain of Mammalian AP-2 Complexes

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