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Clathrin Domains Involved in Recognition by Assembly Protein AP-2*

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The domains on clathrin responsible for interaction with the plasma membrane-associated assembly protein AP-2 have been studied using a novel cage binding assay. AP-2 bound to pure clathrin cages but not to coat structures already containing AP that had been prepared by coassembly. Binding to preassembled cages also occurred in the presence of elevated Tris-HCl concentrations (≥200 mM) which block AP-2 interactions with free clathrin. AP-2 interactions with assembled cages could also be distinguished from AP-2 binding to clathrin trimers by sodium tripolyphosphate ($NaPPP_i$), which binds to the α subunit of AP-2 (Beck, K., and Keen, J. H. (1991) *J. Biol. Chem.* 266, 4442–4447). At concentrations of 1–5 mM, $NaPPP_i$ blocked clathrin-triskelion binding; in contrast, interactions with cages persisted in the presence of 25 mM $NaPPP_i$. To begin to identify the region(s) of the clathrin molecule important in recognition by AP-2, clathrin cages were proteolyzed to remove heavy chain terminal domains and portions of the distal leg as well as all of the light chains. AP-2 bound to these "clipped cages"; however, unlike the interaction with native cages, binding of AP-2 to clipped cages was sensitive to the lower concentrations of both Tris-HCl and $NaPPP_i$ which disrupt interactions of AP-2 with clathrin trimers. Reconstitution of the clipped cages with clathrin light chains did not restore resistance of AP-2 binding to Tris-HCl. We conclude that one binding site for AP-2 resides on the hub and/or proximal part of the clathrin triskelion whereas a second site is likely to involve the terminal domain and/or distal leg; the second site is manifested only in the assembled lattice structure. We suggest that these two distinct binding interactions may be mediated by the two unique large subunits within the AP-2 complex, acting sequentially during assembly.

Clathrin-coated membranes are subcellular "organelles" that perform specialized functions relating to the sorting and dynamics of intracellular membranes and their protein components. At the plasma membrane, clathrin-coated pits and vesicles mediate the receptor-mediated endocytosis of numerous cell surface receptors and their ligands (reviewed in Goldstein *et al.*, 1985). In the Golgi region, clathrin-coated membranes are presumably involved in similar sorting events, of which the processing of lysosomal enzymes is perhaps the best characterized (reviewed in Dahms *et al.*, 1989). To provide a molecular basis for understanding the biological events with which coated membranes are involved, we have been engaged in an analysis of the protein-protein interactions involved in assembling and stabilizing the clathrin lattice structure.

The coat structure has been shown to be composed of two principal protein components (reviewed in Keen, 1990a, 1990b). The major component is clathrin, a protein with a characteristic triskelion shape which contains three 191,000-Da heavy chains (Kirchhausen *et al.*, 1989) and three M_r 23,000–27,000 light chains (Jackson *et al.*, 1987; Kirchhausen *et al.*, 1987). Each leg of the clathrin triskelion is divided into a proximal and a distal segment, ending in a globular terminal domain (Kirchhausen and Harrison, 1984). Biochemical and ultrastructural studies suggest that the hub, the proximal leg, and a portion of the distal leg are localized within the surface lattice of the coat structure whereas the remainder of the distal leg and terminal domain protrude radially inward toward the enclosed membrane surface (Heuser and Kirchhausen, 1985; Kirchhausen and Harrison, 1984;, Vigers *et al.*, 1986a).

The second component of the coat is referred to as assembly (Zaremba and Keen, 1983), adaptor (Pearse, 1988), or associated proteins (AP).[1] Extensive *in vitro* experiments have shown that APs promote the assembly of clathrin trimers into lattices, and they have also been shown to be structural components of completed coat structures both *in situ* and in purified coated vesicle preparations (reviewed in Keen, 1990a). The major assembly protein isolated from bovine brain-coated vesicles, AP-2 (Keen, 1987; Pearse and Robinson, 1984), is composed of M_r 17,000, 50,000, and two distinct 100,000–115,000 polypeptides denoted α and β (Ahle *et al.* 1988) and possesses a tripartite morphology with a large central domain and two smaller appendages (Heuser and Keen, 1988). AP-1 is a more minor component of bovine brain-coated vesicles (Keen, 1987; Pearse and Robinson, 1984) and contains M_r 19,000, 47,000, and two distinct 100,000–115,000 polypeptides denoted γ and β′ (Ahle *et al.*, 1988).

Keen and Beck (1989) demonstrated that the N-terminal core of the AP-2 molecule possesses clathrin binding activity, a result that has been confirmed by Matsui and Kirchhausen (1990). However, conflicting results characterize our understanding of the region of the clathrin triskelion that is recognized by AP-2. On the one hand, cryoelectron microscopic image reconstruction studies of cages, empty coats, and isolated coated vesicles suggest that the AP molecules form a

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[1] The abbreviations used are: AP, associated protein(s); MES, 4-morpholinepropanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; $NaPPP_i$, sodium polytriphosphate.

protein shell entirely within the surrounding clathrin lattice and inwardly protruding terminal domains (Vigers *et al.*, 1986b). Such a localization would be consistent with interaction of AP-2 with the terminal domains of clathrin trimers. In seeming contradiction to these observations are biochemical studies that indicated that proteolyzed clathrin triskelions, lacking terminal domains and most of the distal legs, were indistinguishable from native clathrin triskelions in their ability to bind to the surface of stripped coated vesicles (Hanspal *et al.*, 1984). Since circumstantial evidence supported the involvement of APs as the binding agents on the surface of these stripped vesicles (Mosley and Branton, 1988; Unanue *et al.*, 1981) and these proteolyzed clathrin triskelions lack terminal domains and most of the distal leg, these results would indicate that it is the hub and/or proximal segments of the clathrin triskelion that are involved in AP-2 recognition.

To address this question from a different perspective, we initiated studies of clathrin-AP interactions using a novel cage binding assay in which APs are added after cages have been assembled. After demonstrating the validity of this technique as an assay, we used proteolytically treated cages and free trimers to prove that one binding site for AP-2 exists on the hub and/or proximal leg of the clathrin triskelion. In addition we find that this site is biochemically distinct from binding sites in the intact assembled cage, which likely involve the terminal domains and/or distal legs of clathrin. Recent studies report fractionation of the large subunits of AP-2 and provide evidence that the separated[2] α and β (Ahle and Ungewickell, 1989) subunits of the complex exhibit clathrin binding activity although neither individually is active in promoting clathrin assembly. These and related observations (see "Discussion") suggest that the binding sites on the clathrin molecule which we report here may be distinct recognition sites for the α and β subunits of AP-2.

MATERIALS AND METHODS

Bovine brain clathrin and APs purified to the stage of passage through a Superose 6 (Pharmacia LKB Biotechnology Inc.) gel filtration column were prepared as described previously (Keen, 1987). Pure clathrin cages were assembled by overnight dialysis against 5 mM sodium MES, 1 mM $CaCl_2$, pH 6.10, at 4 °C; coats containing clathrin and AP (in equal amounts) were prepared by dialysis against buffer A (0.1 M sodium MES, pH 6.50) supplemented with 1 mM EGTA, 0.5 mM magnesium chloride, and 0.02% sodium azide. Clathrin light chains were prepared by boiling clathrin (Brodsky *et al.*, 1983; Lisanti *et al.*, 1982) for 5 min and recovering the supernatant after centrifugation at 13,000 × *g* for 10 min in an Eppendorf microcentrifuge. "Clipped" cages were prepared by incubating pure clathrin cages in buffer A with tosylphenylalanyl chloromethyl ketone-treated trypsin (15:1, w/w) for 60 min at 20 °C; a 1.5-fold molar excess (to trypsin) of soybean trypsin inhibitor was added, the solution was centrifuged for 10 min at 20 °C at 75,000 rpm in a Beckman TL-100 rotor, and the clipped cages were resuspended in buffer A. Clipped cages in buffer A (750 μg) were reconstituted with clathrin light chains (500 μg) by incubation for 30 min at 4 °C followed by centrifugation for 10 min at 20 °C at 75,000 rpm in a Beckman TL-100 rotor. Complete binding was attained, ascertained by comparison of the light chain content with an equivalent amount of unproteolyzed clathrin trimers.

To measure binding of AP to intact or clipped clathrin cages, the cage preparations were sedimented to remove dissociated protein and resuspended in buffer A, generally at a concentration of 0.2–0.4 mg/ml. A small volume of 1 M Tris-HCl, pH 6.5, was added followed by the addition of AP in 0.5 M Tris-HCl, pH 6.5. Both additions were performed with constant stirring, and the final concentration of Tris-HCl was 50 mM or more so that aggregation was avoided. The samples were incubated on ice for 30 min and were applied to gradients of 5–20% glycerol in buffer A unless otherwise indicated. Ultracentrifugation at 4 °C was performed using an SW 28.1 rotor (Beckman Instruments) at a speed of 27,000 rpm for the time indicated in the appropriate figure legend. Gradients were fractionated and analyzed as described previously, collecting 1.0-ml fractions (Keen, 1987). Apparent $s_{20,w}$ values of initial and shifted protein peaks recovered from the gradients were calculated from their radial position, and the ω^2t value of the run (Beckman Instruments application data DS-528A). When cage binding was measured by differential sedimentation (see Fig. 4) low speed pellet samples were obtained by centrifugation for 2 min in an Eppendorf centrifuge; the low speed supernatant was then centrifuged at 75,000 rpm for 7 min in the TL-100.

Samples for polyacrylamide gel electrophoresis, concentrated by precipitation with carrier cytochrome *c* where necessary, were run on 5–8.5% gels as previously described (Keen, 1987). Quantitation of Coomassie Blue-stained bands was performed by densitometry (Keen and Beck, 1989).

RESULTS

We have devised a technique for studying regions on the clathrin molecule which are important for interactions with AP-2 using pre-assembled clathrin cages[3] as a macromolecular "substrate" in a binding assay for exogenously added assembly protein. AP-2 has a strong tendency to self-associate under the conditions in which the clathrin cages are prepared (Beck and Keen, 1991a). For the assay to be useful, conditions must be defined which both ensure the stability of the cage substrate and the solubility of AP-2. Since Tris-HCl is active in maintaining AP-2 in a dissociated state, we evaluated its effect on cage stability.

Pure clathrin cage structures were prepared as described under "Materials and Methods" and analyzed by ultracentrifugation. Consistent with earlier observations (Zaremba and Keen, 1983) two distinct sizes of cages with apparent $s_{20,w}$ values of approximately 230 and 410 S were obtained. These structures were entirely stable to buffer A containing 50 mM Tris-HCl, pH 6.5, for the time required (≈90–130 min) for binding and glycerol gradient ultracentrifugation (Fig. 1*A*, *dashed line*). When AP preparations were added to preassembled clathrin cages in buffer A containing 50 mM Tris-HCl, pH 6.5, ultracentrifugation revealed a shift of both the small and larger clathrin cages to more rapidly sedimenting peaks, with apparent $s_{20,w}$ values of approximately 320 and 650 S (Fig. 1*A*, *solid line*). Polyacrylamide gel electrophoresis of these samples demonstrated that AP was associated with the preassembled cage structures in both of the shifted peaks and was not present in the initial clathrin cages (polypeptide composition of the major peaks are shown in Fig. 2*A*, *lanes 1* and *3*). It should be noted that the AP-2, identified by its 50-kDa polypeptide, was preferentially bound to the cages in comparison with AP-1, distinguishable by its 47-kDa polypeptide (compare Fig. 2*A*, *lanes 2* and *3*). Although the cause of this preference is not known, the result parallels our earlier observations that under these buffer conditions AP-2 is more effective than AP-1 in both binding to clathrin-Sepharose and in coassembling into coat structures (Keen, 1987). These properties and the ability to identify the presence of the AP-2 by virtue of its 50-kDa polypeptide allowed us to employ partially purified preparations of brain AP rather than purified AP-2 for these studies.

Before using this procedure as an assay, the nature of the reaction was evaluated in several ways. The amount of AP-2 bound to preassembled cages (measured by electrophoresis of the shifted peaks and densitometry) was consistently about 50% of that found in coassembled coats (Table I). These results (in Fig. 1 and 2) were obtained using ratios similar to those used in coassembly of clathrin and AP (see "Materials

[2] K. Prasad and J. H. Keen, submitted for publication.

[3] Assembled structures containing clathrin alone are termed *cages* whereas those containing both clathrin and APs are referred to as *coats*. Unlike coated vesicles, both are empty in that they do not enclose a membrane bilayer.

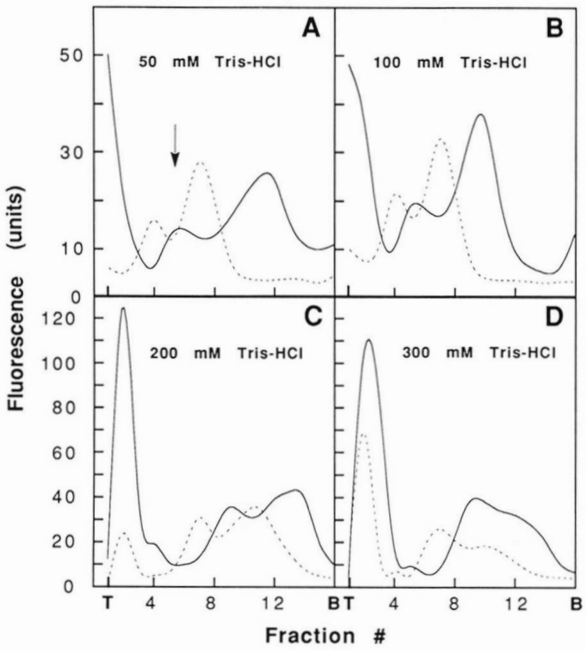

FIG. 1. **AP binds to preassembled clathrin cages and shifts their sedimentation in glycerol gradients.** Preassembled clathrin cages were incubated alone (*dashed lines*) or with added AP in buffer A containing the indicated concentrations of Tris-HCl. Samples in *panels A* and *B* were prepared with 0.15 mg/ml each of clathrin and AP, were applied to 5–20% glycerol gradients in buffer A, and centrifuged for 52 min. Samples in *panels C* and *D* were prepared with 0.30 mg/ml each of clathrin and AP and were applied to a gradient containing 0.5 ml of 200 mM Tris-HCl, 5% glycerol in buffer A which had been layered upon a linear 10–30% glycerol gradient in buffer A. These samples were centrifuged as above but for 120 min.

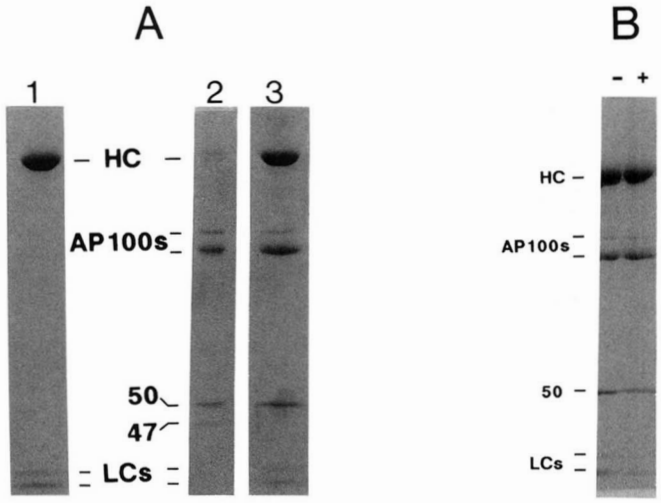

FIG. 2. **AP-2 binds to preassembled clathrin cages but not to complete clathrin and AP-containing coats.** *A*, polyacrylamide gel electrophoresis of AP bound to preassembled cages and fractionated by gradient ultracentrifugation. *Lane 1*, preassembled clathrin cages alone (Fig. 1*A*, *dashed line*, fraction 7). *Lanes 2* and *3*, preassembled cages supplemented with APs (Fig. 1*A*, *solid line*). *Lane 2*, nonsedimenting protein (Fig. 1*A*, fraction 1) showing the presence of AP-1 and excess AP-2. *Lane 3*, cages containing bound AP-2 (Fig. 1*A*, fraction 11). *B*, aliquots of coassembled coats alone (0.19 mg/ml) containing clathrin and AP (*lane 1*) or (*lane 2*) coats supplemented with additional AP (0.19 mg/ml) were analyzed by polyacrylamide gel electrophoresis. *LCs*, clathrin light chains.

TABLE I

Effect of Tris-HCl on the Binding of AP-2 to Intact and Clipped Cages

Composition	AP:clathrin ratio[a]
Co-assembled coats (clathrin + AP)	1.11
Intact clathrin cages + AP (50 mM Tris-HCl)	0.55 (0.52–0.58)
Intact clathrin cages + AP (200 mM Tris-HCl)	0.40 (0.37–0.42)
Clipped clathrin cages + AP (50 mM Tris-HCl)	0.41 (0.34–0.55)
Clipped clathrin cages + AP (200 mM Tris-HCl)	<0.04

[a] Mass ratio of AP50 to clathrin trimer in cage or coat structures recovered from gradients and determined by densitometry of AP50 and clathrin light chain LC_b bands in stained gels. Clipped cages were supplemented with light chains as described under "Materials and Methods." Average and range of three complete experiments are shown.

and Methods"). At higher levels of AP (>1.5:1 by weight) no greater incorporation was observed although aggregated material containing both clathrin and AP pelleted under the conditions of the experiment, probably reflecting the formation of AP-2-cross-linked coat structures or nonspecific binding (data not shown).

The possibility that the apparent binding of AP to preexisting clathrin cages could actually reflect dissociation of the cages and their *de novo* reassembly directed by the added AP can be ruled out for the more rapidly sedimenting major protein peak by the following reasoning. The major peak observed on AP addition to clathrin cages has a higher apparent sedimentation coefficient than the pure clathrin cages alone, consistent with an increase in mass. In contrast, the coats that would be formed by disassembly of the cages and subsequent AP-directed reassembly of the depolymerized clathrin have a smaller sedimentation coefficient (≈250 S) than pure clathrin cages (Zaremba and Keen, 1983). As a result these coassembled coats, under the centrifugation conditions employed in Fig. 1, would be present in fractions 5–6 at the position indicated by the *arrow*. Thus, the major peak observed on AP addition to clathrin cages does not sediment in the position expected for *de novo* assembled coat structures.

To test whether AP-2 decoration of cages is a consequence of binding to the same site or sites that are occupied by AP-2 in the coat structure formed by coassembly of clathrin with AP-2, we tested for the ability of these preformed complete coats to bind additional AP. Incubation of these structures with AP did not result in a shift in sedimentation on ultracentrifugation but rather in the appearance of a major peak of nonsedimenting material at the top of the gradient that contained unincorporated AP (data not shown). Furthermore, analysis by gel electrophoresis provided no evidence for an increase in the amount of AP-2 polypeptides relative to clathrin (Fig. 2*B*). We conclude that exogenous AP-2 added to preassembled clathrin cages binds to discrete sites that are blocked in coassembled coats and which are very likely to be similar or identical to those that are recognized by AP-2 during the *de novo* coat assembly process.

Having established that AP-2 bound to preassembled cages in the presence of 50 mM Tris-HCl, pH 6.5, it was of interest to determine if the interaction could be detected in the presence of higher concentrations of Tris-HCl. To begin to evaluate this possibility, the stability of clathrin cages alone to buffer A containing increasing Tris-HCl concentrations, pH 6.5, was studied (Fig. 1, *B-D*, *dashed lines*). Cages could be incubated in substantial concentrations of Tris-HCl (*e.g.* 200 mM, Fig. 1*C*) without inducing significant dissociation; even

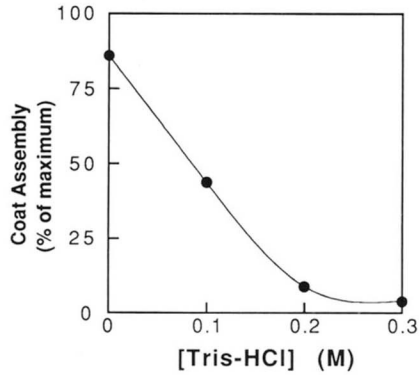

FIG. 3. **Inhibition of AP-mediated clathrin coat assembly by increasing Tris-HCl concentrations.** Clathrin and AP (0.19 mg/ml each) were dialyzed against buffer A supplemented with 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% sodium azide at pH 6.5 and containing the indicated concentrations of Tris-HCl. Coat assembly was measured by gradient ultracentrifugation as described under "Materials and Methods." At maximal assembly, 86% of the total protein was incorporated into coat structures with apparent $s_{20,w}$ values of ≈ 250 S (Zaremba and Keen, 1983).

at 300 mM Tris-HCl some intact cages were still detectable after a lengthy centrifugation although much of the protein had dissociated (Fig. 1*D*, *dashed line*).[4] Similar observations have been made in experiments with coated vesicle preparations (Wiedenmann *et al.*, 1985; Zaremba and Keen, 1983). The absence of a substantial effect on cage stability in solutions containing ≥ 200 mM Tris-HCl is in contrast to the complete inhibition of *de novo* coat assembly under these solution conditions (Fig. 3). We conclude that once formed the cages are comparatively stable under these conditions and can be used as sedimentable clathrin substrates in a binding assay.

Binding of AP to preassembled intact clathrin cages was only slightly affected by increasing Tris-HCl; binding was strong in 100–200 mM Tris-HCl and was detectable even in the presence of 300 mM Tris-HCl (Fig. 1, *B-D*). Quantitation of the amount of AP bound to cages in buffer A containing 200 mM Tris-HCl revealed < 25% decrease in comparison with that bound in 50 mM Tris-HCl (Table I) and generated coats with an apparent $s_{20,w}$ of 455 S. This behavior is in sharp contrast to the interactions of AP-2 with dissociated clathrin trimers, as AP-2-mediated assembly of clathrin trimers into coat structures is essentially completely inhibited by 0.2 M Tris-HCl (Fig. 3). Consistent with this observation, the interaction between AP-2 bound to immobilized clathrin trimers on Sepharose has also been shown to be disrupted by much lower concentrations of Tris-HCl, in the range of 100 mM (Keen, 1987). The observation that Tris-HCl has widely differing effects on the binding of AP-2 to clathrin trimers and to assembled cages provided initial indications that these interactions might be qualitatively different reactions.

Discrimination between these two binding events has also been accomplished through the use of polyphosphates. Elsewhere we report that polyphosphates bind to a specific site on the α subunit of the AP-2 (Beck and Keen, 1991b). Of particular relevance to this report is the observation that sodium tripolyphosphate ($NaPPP_i$) at a 1–5 mM concentrations completely blocked AP-2 interactions with dissociated clathrin trimers, measured by inhibition of AP-2 binding to clathrin triskelions immobilized on Sepharose and of AP-2-mediated clathrin coat assembly (Beck and Keen, 1991b). In these respects then, the $NaPPP_i$ effect is similar to that of Tris-HCl although it is chemically unrelated, and the effective concentrations are much lower.

$NaPPP_i$, like Tris-HCl, was much less effective at blocking the binding of AP-2 to assembled clathrin cages (Fig. 4*A*) than in blocking the interaction of AP-2 with clathrin triskelions. There was only a ≈25% decrease in the amount of AP-2 bound to intact cages in the concentration range of 0–25 mM $NaPPP_i$ (Fig. 4*A*, *lanes P2*, and Fig. 5, *open circles*) although $NaPPP_i$ did solubilize small amounts of aggregated AP (compare Fig. 4*A*, *lanes P1* and *S*), consistent with the effects of this reagent on blocking AP-2 aggregation (Beck and Keen, 1991a, 1991b). Collectively, these observations indicate that the binding of AP-2 to dissociated clathrin trimers is experimentally distinguishable from the binding of AP-2 to clathrin lattices as assessed by sensitivity to both Tris-HCl and $NaPPP_i$.

To resolve further the domains on the clathrin molecule important for AP-2 recognition, we prepared proteolytically treated clathrin cages for use as binding substrates. These clipped cages are devoid of clathrin light chains and contain truncated heavy chains of approximately 100–115 kDa which lack the terminal domain regions as well as part of the distal leg (Kirchhausen and Harrison, 1984). On ultracentrifugation (Fig. 6, *dotted line*), the clipped cages exhibited a sedimentation pattern containing two peaks, similar to that of intact cages although, as expected, they had smaller apparent sedimentation constants (≈150 S and 260 S). The clipped cages

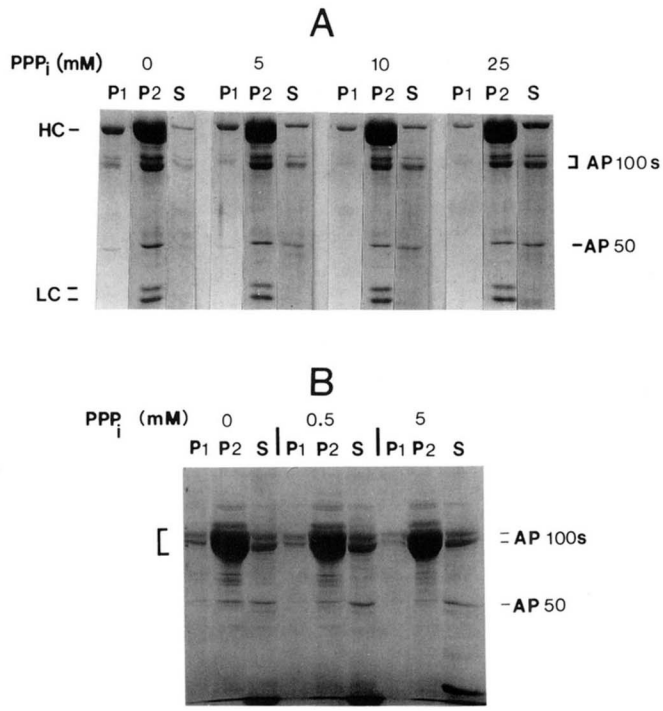

FIG. 4. **Sodium tripolyphosphate does not block AP-2 binding to intact clathrin cages but does inhibit binding to clipped cages.** *A*, clathrin cages (150 μg/ml) were incubated with AP (76 μg/ml) in buffer A containing 50 mM Tris-HCl in the presence of the indicated concentration of sodium tripolyphosphate. Low speed pellets (*P1*), high speed pellets (*P2*), and high speed supernatants (*S*) were prepared as described under "Materials and Methods." Note that with increasing [PPP_i] the AP-2 polypeptides (*AP100s* and *AP50*) are found in the cage pellet although their appearance in the low speed pellet due to aggregation is reduced. *HC* and *LC* denote the heavy and light chains of clathrin, respectively. *B*, as in *A* except that clipped clathrin cages (213 μg/ml) were incubated with AP (77 μg/ml).

[4] In Fig. 1 note that the centrifugation conditions for *panels C* and *D* are different from those in *panels A* and *B*; however, the cage peaks all have similar apparent $s_{20,w}$ values (*e.g.* 235 S for the smaller cages).

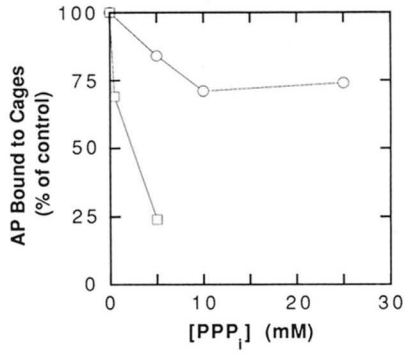

FIG. 5. **Quantitation of AP-2 binding to intact and clipped clathrin cages in the presence of sodium tripolyphosphate.** AP binding to intact clathrin cages (○) was measured densitometrically as the amount of AP50 polypeptide associated with clathrin cages (Fig. 4*A*, *lane P2*), normalized by the amount of clathrin light chain in each sample. AP binding to clipped clathrin cages (□) was measured similarly. In this case, as clipped cages lack clathrin light chains, the amount of AP50 bound was normalized by the total amount recovered in the three fractions (Fig. 4*B*, *lanes P1*, *P2*, and *S*).

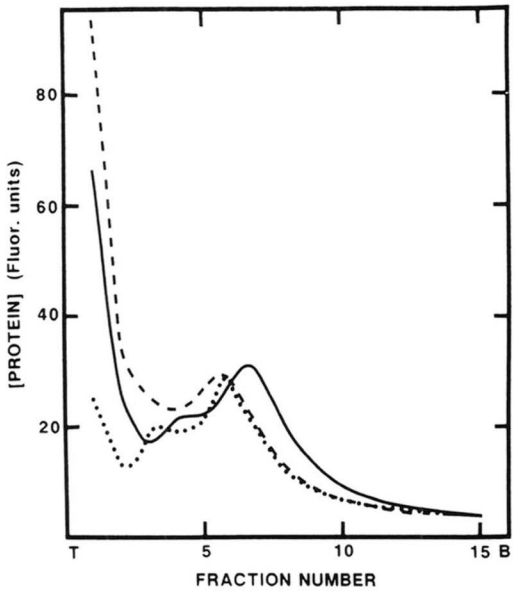

FIG. 6. **AP binds to clipped clathrin cages in the presence of 50 mM, but not 200 mM, Tris-HCl.** Clipped clathrin cages (equivalent in amount to 0.30 mg/ml intact cages) were incubated with AP (0.30 mg/ml) in 50 mM Tris-HCl (*solid line*) or in 200 mM Tris-HCl (*dashed line*) or with 200 mM Tris-HCl alone (*dotted line*) and subjected to glycerol gradient ultracentrifugation for 105 min.

bound added AP in the presence of buffer A containing 50 mM Tris-HCl, as evidenced by a small but reproducible shift (to approximately 300 S for the larger species) in sedimentation pattern (Fig. 6, *solid line*). Gel electrophoresis (data not shown, but see Fig. 7 for a related experiment that yielded a similar result) and densitometry of the polypeptides in the shifted peak revealed binding of amounts of AP-2 to proteolyzed cages similar to that of intact cage structures (Table I) despite the fact that the clipped cages contain ≤ 5% intact clathrin heavy chain. AP binding to clipped cages is apparently accompanied by a significant increase in frictional coefficient, as clipped cages do not show as great a proportional increase in sedimentation rate as do intact cages on AP binding. In other respects the binding of AP-2 to clipped cages shares several properties with binding to intact cages, including a preference for AP-2 in comparison with AP-1 (see Fig. 7), stability to ultracentrifugation, and saturable binding to

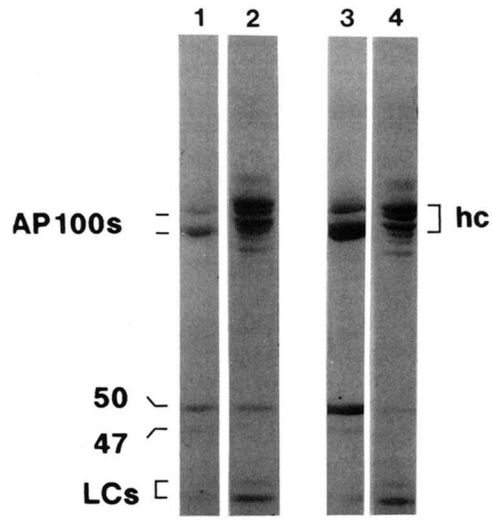

FIG. 7. **Polyacrylamide gel analysis of AP-2 binding to clipped clathrin cages reconstituted with clathrin light chains.** Fractions from glycerol gradients of clipped clathrin cages containing rebound light chains (*LCs*) that were incubated with AP (as in Fig. 6) in the presence of 50 mM (*lanes 1* and *2*) or 200 mM (*lanes 3* and *4*) Tris-HCl. *Lanes 1* and *3*, unassembled protein from the top of the gradients. *Lanes 2* and *4*, peak fractions containing assembled cage species (*e.g.* Fig. 6, fractions 6–7). As the truncated clathrin heavy chains (*hc*) comigrate with the AP100s, AP binding is revealed by the presence of the 50-kDa (*50*) and 47-kDa (*47*) polypeptides of AP-2 and AP-1, respectively.

similar stoichiometry, as excess AP-2 remains at the top of the gradient.

We ascertained that under the conditions required for the cage binding assay the clipped cages, like their intact counterparts, were entirely stable in buffer A containing 200 mM Tris-HCl. However, when AP was incubated with clipped cages in this solution no binding could be detected as indicated by the absence of a perceptible shift on sedimentation and an increase in the amount of unincorporated protein remaining at the top of the gradient (Fig. 6, *dashed line*). Gel electrophoresis of the gradient fractions (not shown, but see Fig. 7 for a related experiment) and densitometry (Table I) support the conclusion that AP binding to clipped cages is blocked entirely by 200 mM Tris-HCl.

$NaPPP_i$ was also tested for its effect on AP-2 interactions with clipped cages. The binding of AP-2 to clipped cages was inhibited substantially by 0.5 mM $NaPPP_i$ and was almost completely blocked by 5.0 mM $NaPPP_i$ (Fig. 4*B*). In contrast, similar concentrations of $NaPPP_i$ had no substantial effect on the binding of AP-2 to intact cages (Fig. 4*A*). The densitometry of the effects of $NaPPP_i$ on the binding of AP-2 to intact and clipped cages is presented in Fig. 5. Finally, in related experiments we also found that 0.5–1.0 mM $NaPPP_i$ would release AP-2 that had been prebound to clipped cages but had no effect on AP-2 prebound to intact cages (data not shown).

Two structural factors distinguish the proteolyzed cages from their intact counterparts: (a) the absence of light chains and (b) a truncated heavy chain that lacks the distal segment and the globular terminal domain region. To determine if light chains are responsible for the resistant binding of AP-2 to intact clathrin cages we took advantage of the observation that they can be added back to truncated cages (Ungewickell, 1983; Winkler and Stanley, 1983). Light chain-supplemented proteolyzed cages bound added AP in the presence of 50 mM Tris-HCl but not in 200 mM Tris-HCl, yielding gradient profiles similar to those in Fig. 6 (a related experiment with

clipped cages lacking light chains). Gel electrophoresis confirmed the binding of AP-2 polypeptides under low salt conditions (Fig. 7, *lanes 1* and *2*). In higher salt negligible AP-2 was bound with the remainder appearing at the top of the gradient (Fig. 7, *lanes 3* and *4*). These results suggest that the Tris-HCl/$NaPPP_i$-resistant binding of AP-2 which is seen with intact clathrin cages is not due to the clathrin light chains but rather reflects AP interaction with the distal leg, terminal domains, and/or the integrity of the clathrin heavy chain itself.

DISCUSSION

Information on the interactions of clathrin with AP-2 has been obtained using several approaches. Keen and Beck (1989) used a cage binding technique to show that the central core of the AP-2 molecule contains a recognition domain and can bind to clathrin, a result supported by subsequent experiments employing proteolysis of intact coat structures (Matsui and Kirchhausen, 1990). Cage binding has also been used to show that the isolated β polypeptide of AP-2 binds to intact clathrin cages (Ahle and Ungewickell, 1989). Here we use this technique as an assay to identify domains in the clathrin molecule which are important for AP-2 binding. Our results point to the existence of at least two discrete binding interactions. One interaction can be localized to the hub and/or proximal legs of the clathrin molecule; AP-2 interaction with this site appears to be similar or identical to that with dissociated clathrin trimers. A second site with biochemically distinct properties is detectable only in the assembled clathrin lattice. The latter site is likely to be associated with the terminal domain and/or distal leg regions of the clathrin heavy chain.

AP-2 was capable of binding to proteolytically clipped assembled clathrin cages. This interaction is similar to clathrin-AP interactions characterized previously in respect to saturability, stoichiometry, stability to ultracentrifugation, preference for AP-2, and sensitivity to two chemically dissimilar effectors (Tris-HCl and $NaPPP_i$). As these clipped cages lack both light chains and the distal segment and terminal domain regions of the heavy chains, these observations indicate that a recognition site for AP-2 must exist on the proximal leg and/or hub region of the clathrin trimer, supporting the earlier observations of Branton and colleagues (Hanspal *et al.*, 1984).

AP-2 also bound to intact assembled clathrin cages with biochemical properties reflecting a specific and relevant reaction although readily distinguishable from interactions with clipped cages or free clathrin triskelions. Binding of AP-2 to intact cages occurred even in the presence of comparatively high concentrations of Tris-HCl (>200 mM). In fact, concentrations of Tris-HCl required to block AP-2 binding completely were comparable to those that result in the complete dissociation of the assembled cage structure (Keen *et al.*, 1979; Wiedenmann *et al.*, 1985). An entirely analogous situation exists with respect to $NaPPP_i$ in that most of the binding of AP-2 to intact cages was resistant to concentrations of $NaPPP_i$ (≈25 mM) considerably greater than those required to block AP-2 binding to clathrin trimers or to clipped cages (≤5 mM). Note however that relatively low concentrations of Tris-HCl and $NaPPP_i$ could block approximately 25% of AP-2 binding. This suggests that a fraction of the AP-2 added to intact cages may be binding to sites that are similar to those in clipped cages, *i.e.* to proximal legs and/or terminal domains.

These results provide evidence that the binding of AP-2 to intact cages is at least in part a function of an assembled lattice structure. The interactions were altered by truncation of the clathrin heavy chain, becoming indistinguishable from clathrin trimer-AP-2 interactions. Since they are not recovered on readdition of clathrin light chains, it seems likely that it is the terminal domain/distal leg region of the clathrin heavy chain which plays an important role in the interaction of AP-2 with the assembled coat. If the terminal domain/distal leg regions of the heavy chain point inward, as suggested by previous ultrastructural studies (Heuser and Kirchhausen, 1985; Kirchhausen and Harrison, 1984; Vigers *et al.*, 1986a), the binding we detect on addition of AP to preassembled cages is consistent with the central localization of AP-2 within the surrounding clathrin lattice which has been detected in intact coated vesicles by cryoelectron microscopy (Vigers *et al.*, 1986b). Collectively, our results provide independent support for the suggestion that AP-2 interacts with both clathrin hubs (Hanspal *et al.*, 1984) and terminal domains (Vigers *et al.*, 1986b) by providing evidence for two biochemically distinct binding reactions, each of which corresponds to interactions with different portions of the clathrin molecule.

It is known that only the α and β (100–115-kDa) polypeptides of the AP-2 complex are needed for clathrin binding and assembly, as preparations lacking the smaller 50- and 17-kDa subunits are active in coat assembly[2] (Keen *et al.*, 1986; Prasad *et al.*, 1986). What might the actions of the individual subunits be? One possibility suggested by the results presented here is that the α subunit recognizes the hub/proximal leg of free clathrin triskelions whereas the β subunit binds the terminal domain and/or distal leg regions of clathrin heavy chains in an assembling lattice. Several lines of evidence support this view. Low concentrations of polyphosphates block the interaction of AP-2 with clathrin trimers (Beck and Keen, 1991b) and with truncated cages lacking terminal domains (this report); this sensitivity correlates with the observation that the N-terminal domain of the α subunit contains a polyphosphate binding site (Beck and Keen, 1991b). Furthermore, recent studies to be reported elsewhere provide direct evidence that α polypeptide preparations bind to free clathrin trimers although they do not by themselves promote lattice assembly.[2]

AP-2 binding to intact cages, unlike binding to clipped cages, is insensitive to polyphosphates (Fig. 4). This suggests that the β subunit is primarily responsible for AP-2 binding in the assembled lattice, as it does not show evidence of having a polyphosphate binding site (Beck and Keen, 1991b). Ahle and Ungewickell (1989) have provided direct evidence that the isolated β subunit binds to intact cages although it does not retain assembly activity. We have confirmed these observations, and preliminary findings in our laboratory indicate further that β preparations do not bind to clathrin trimers.[5] Collectively, these results suggest that the α subunit binds to the hub/proximal leg regions of the clathrin molecule whereas the β subunit binds to the terminal domain/distal leg region in the assembled lattice.

According to this reasoning, the initial AP-2-clathrin interaction in coat assembly may be mediated by interaction of the hub/proximal legs of a clathrin trimer with the α subunit of AP-2. Binding of a second and subsequent trimers to this complex, in the proper orientation for growth of the lattice, would be stabilized by interaction of the β subunit of this AP-2 with the terminal domains and/or distal legs of the trimers. As the second interaction appears to be the stronger, at least by the criterion of resistance to two diverse chemical reagents, it may direct the AP-2 to its final localization within the surrounding clathrin lattice, consistent with the localization seen by cryoelectron microscopy (Vigers *et al.*, 1986b). Further studies will be necessary to test these hypotheses, to relate

[5] K. Prasad and J. H. Keen, unpublished observations.

more precisely structural domains to discrete steps in the assembly process, and to evaluate how these interactions may relate to other proposed activities of the plasma membrane AP-2 such as the recognition of transmembrane receptor tails (Pearse, 1988).

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