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Light-Chain-Independent Binding of Adaptors, AP180, and Auxilin to Clathrin

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ABSTRACT: Binding of coated vesicle assembly proteins to clathrin causes it to assemble into regular coat structures. The assembly protein fraction of bovine brain coated vesicles comprises AP180, auxilin, and HA1 and HA2 adaptors. Clathrin heavy chains, separated from their light chains, polymerize with unimpaired efficiency when assembly proteins are added. The reassembled coats were purified by sucrose gradient centrifugation and examined for composition by SDS–PAGE and immunoblotting. We found that all four major coat proteins are incorporated in the presence and absence of light chains. Moreover, each of the purified coat proteins is able to associate directly with clathrin heavy chains in preassembled cages as efficiently as with intact clathrin. We conclude that light chains are not essential for the interaction of AP180, auxilin, and HA1 and HA2 with clathrin.

Clathrin-coated membranes are the vehicles of receptor-mediated endocytosis and are responsible for routing lysosomal proteins from the trans-Golgi network to prelysosomal compartments (Goldstein et al., 1985; Brodsky, 1988; Morris et al., 1989). Cells capable of regulated exocytotic activities utilize clathrin also for the formation of secretory vesicles (Orci et al., 1984) and the retrieval of their membrane after exocytosis (Heuser, 1989). Known peripheral membrane components of clathrin-coated vesicles from bovine brain are the pinwheel-shaped protein clathrin, composed of three heavy and three light chains (Ungewickell & Branton, 1981; Kirchhausen & Harrison, 1981), the adaptor complexes HA1 and HA2, AP180, and auxilin (Morris et al., 1989; Ahle & Ungewickell, 1990). Clathrin represents the structural unit of the polygonal

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surface lattice of coated vesicles, while the adaptors are believed to mediate its interaction with specific components of the membrane (Unanue et al., 1981). Evidence for direct interaction between adaptors and cytoplasmic segments of certain receptors comes from in vitro binding studies (Pearse, 1988; Glickman et al., 1989). The adaptors are heterotetrameric protein complexes which, like clathrin, also occur in probably all mammalian cell types (Manfredi & Bazari, 1987; Ahle et al., 1988; Virshup & Bennett, 1988). HA1 is a specific component of Golgi-derived clathrin-coated membranes, while HA2 appears to be specific to endocytic coated membranes (Robinson, 1987; Ahle et al., 1988). A common feature of adaptors, AP180, and auxilin is their ability to interact with clathrin and thereby to promote its polymerization into polyhedral cages resembling the surface lattice of coated vesicles (Keen et al., 1979; Ahle & Ungewickell, 1986; Manfredi & Bazari, 1987).

As a first step toward identifying the binding site(s) of clathrin-associated proteins on clathrin, we set out to determine whether light chains are part of those sites. Our results show that light chains are not required for efficient binding of adaptors, AP180, and auxilin to clathrin.

Materials and Methods

Materials. Fresh bovine brains were obtained from a local abattoir and processed within 2 h of slaughter. Superose 6 preparation-grade gel filtration medium, MonoQ anion-exchange resin, and PD10 desalting columns were from Deutsche Pharmacia, Freiburg (FRG); Centriprep 30 and Centricon 30 ultraconcentrators were from Amicon GmbH (Witten, FRG); [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA)[1] was from Serva, Heidelberg (FRG); reagents for SDS–PAGE and urea were from LKB Instrument GmbH, Gräfelfing (FRG); MES, DTT, and PMSF were from Sigma Chemie GmbH, Deisenhofen (FRG). Peroxidase-conjugated IgGs to mouse antibodies were from Dakopatts GmbH, Hamburg (FRG); nitrocellulose transfer membrane (BA 83, 0.2 μm) was from Schleicher & Schuell, Dassel (FRG); all other reagents were Merck or Roth analytical grade.

Purification of Clathrin. Coated vesicles from bovine brain tissue were prepared according to Campbell et al. (1984). Clathrin and associated proteins were dissociated from the membrane with 0.5 M Tris-HCl (Keen et al., 1979) and purified by gel filtration as described in detail elsewhere (Ahle & Ungewickell, 1990). Clathrin and the assembly proteins were concentrated by low-speed centrifugation in a Centriprep 30 device to final concentrations of 3–5 mg/mL.

Purification of Clathrin Heavy Chains. Light chains were dissociated from heavy chains with 1.3 M sodium thiocyanate, following closely the procedure of Winkler and Stanley (1983). In brief, intact clathrin at a concentration of 3–5 mg/mL in 50 mM Tris-HCl, 2 mM EDTA, 0.02% NaN_3, and 1 mM DTT, pH 8.0, was applied in 2.5-mL aliquots to a PD 10 desalting column, equilibrated with 1.3 M sodium thiocyanate, 50 mM Tris-HCl, 2 mM EDTA, 0.02% NaN_3, and 1 mM DTT, pH 8.0. The column was eluted according to the manufacturer's instructions. Three-milliliter aliquots of the clathrin were then applied to a preparation-grade Superose 6 gel filtration column (1.6 × 50 cm) to separate heavy from light chains. The eluant was 1.3 M sodium thiocyanate, 50 mM Tris-HCl, 2 mM EDTA, 0.02% NaN_3, and 1 mM DTT, pH 8.0. Fractions containing heavy chains were pooled and dialyzed with one change against ~40 volumes each of 0.5 M Tris-HCl, 2 mM EDTA, and 0.02% NaN_3, pH 7.0, and concentrated by low-speed centrifugation in a Centriprep device to a final concentration of 2–3 mg/mL.

Purification of AP180, Auxilin, HA1, and HA2. The pool of assembly proteins was further fractionated by hydroxyapatite chromatography as described in detail previously (Ahle & Ungewickell, 1989). Fractions enriched in AP180, auxilin, HA1, and HA2 were individually pooled and further processed [for a typical elution profile, see Figure 1 in Ahle and Ungewickell (1989)]. AP180 was further purified by gel filtration on Superose 6 to remove contaminating auxilin, as described by Ahle and Ungewickell (1990). Other minor contaminating proteins precipitated during dialysis of AP180 against 0.1 M MES, 1 mM EGTA, and 0.5 mM $MgCl_2$, pH 6.4 (isolation buffer 6.4). AP180 was then dialyzed against 5 mM Tris-HCl, 2 mM EDTA, and 0.02% NaN_3, pH 8.0. Hydroxyapatite fractions containing the HA1 adaptor were dialyzed against 20 mM Tris-HCl, 2 mM EDTA, and 0.02% NaN_3, pH 8.0, and applied to a MonoQ anion-exchange column. Protein eluted with a salt gradient between 240 and 280 mM NaCl was pooled and concentrated in a Centricon 30 device. Minor contaminants were subsequently removed by sucrose density gradient centrifugation using 4.4-mL gradients of 5–15% sucrose in 0.5 M Tris-HCl, 2 mM EDTA, and 0.02% NaN_3, pH 7.0. The gradients were spun for 15 h at 45 000 rpm in an SW 60 rotor (Beckman). HA1 was pooled and concentrated in a Centricon 30 device. Auxilin was purified by conventional chromatographic techniques as described in detail elsewhere (Ahle & Ungewickell, 1990). Traces of contaminating AP180 were removed from auxilin preparations by passage through an affinity column which contained Sepharose-coupled mAb AP180-1 (Ahle & Ungewickell, 1986). HA2 as obtained from the hydroxyapatite column step was also passed through an anti-AP180 affinity column to eliminate residual AP180. For clathrin binding experiments, both proteins were dialyzed against 10 mM HEPES, 100 mM sodium tartrate, 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% NaN_3, pH 7.0 (tartrate buffer).

Concentration Determinations. Protein concentrations were determined spectrophotometrically. The extinction coefficients for intact clathrin and clathrin heavy chains were calculated from the tryptophan and tyrosine contents obtained from the sequences of heavy and light chains (Kirchhausen et al., 1987a,b; Jackson et al., 1987) according to the relationship $E^{1cm}_{280nm}(10\ mg/mL) = 10[(5700n)Trp + (1300n)Tyr]/M$, where M is the molecular weight and n is the number of residues (Cantor & Schimmel, 1980). The values thus obtained were $E^{1cm}_{280nm}(10\ mg/mL) = 10.4$ for intact clathrin and $E^{1cm}_{280nm}(10\ mg/mL) = 10.3$ for heavy chains. Extinction coefficients for adaptors, AP180, and auxilin were determined according to Waddell (1956). The respective values were $E^{1cm}_{280nm}(10\ mg/mL) = 7.9$ for auxilin (Ahle & Ungewickell, 1990), 5 for AP180 (S. Ahle, personal communication), 5.6 for HA1, and 6 for HA2 (Lindner and Ungewickell, unpublished results).

Assembly Experiments. Intact clathrin or heavy chains in 0.5 M Tris buffer at concentrations of 0.4 and 0.35 mg/mL, respectively, were titrated with assembly protein (0–0.8 mg/mL) and then dialyzed overnight against isolation buffer, pH 6.7. After removal of aggregated material by centrifugation for 3 min at 14 000 rpm in an Eppendorf centrifuge,

[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol.

assembly was analyzed by pelleting 50-μL aliquots of the cages using a fixed-angle TLA.45 rotor (13 min, 40000 rpm) in the Beckman TL100 ultracentrifuge. The pellets were redissolved in the original volume with 0.5 M Tris-HCl, pH 7.0, and examined, together with the supernatants, by SDS–PAGE. The fraction of clathrin in the pellet was determined by densitometry of the Coomassie-stained heavy-chain zone. For qualitative analysis of assembly, 1.0 mg/mL intact clathrin or 0.88 mg/mL heavy chain in 0.5 M Tris buffer was assembled in the presence of 0.7 mg/mL assembly protein by dialysis against isolation buffer, pH 6.7. Aliquots of 250 μL were layered onto a 4.1-mL 5–30% sucrose density gradient (in isolation buffer) and centrifuged at 38000 rpm for 1 h using an SW 60 rotor (Beckman Instruments). Thirteen fractions were collected manually and analyzed by SDS–PAGE.

Cage Binding Experiments. Both light-chain-free and light-chain-containing cages were assembled from heavy chains and intact clathrin by dialyzing the proteins at concentrations ranging from 2.0 to 5.0 mg/mL against isolation buffer, pH 6.5, in the presence of 3 mM $CaCl_2$. An additional dialysis step served to transfer the cages into either isolation buffer, pH 6.7, or 10 mM HEPES, 100 mM sodium tartrate, 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% NaN_3, pH 7.0 (tartrate buffer). Binding experiments were performed by incubating 20 μg of intact and 17.6 μg of heavy-chain cages with 4 μg of either AP180 or HA1 in 50 μL of isolation buffer for 1 h on ice. Binding of HA2 and auxilin to clathrin was measured in tartrate buffer to suppress self-aggregation of these proteins, which becomes a serious problem in isolation buffer (Lindner and Ungewickell, unpublished results). Incubations were as for HA1 and AP180: Identical amounts of appropriate cages were incubated with 20 μg of HA2 or approximately 8 μg of auxilin in a volume of 50 μL for 1 h at 4 °C. All the proteins were dialyzed into tartrate buffer before incubation. In all cases, binding was analyzed by ultracentrifugation in a fixed-angle rotor and SDS–PAGE as above.

Antibodies. Immunoblotting with monoclonal antibodies was performed essentially as described in detail previously (Ahle et al., 1988). In brief, AP180 was stained with mAb AP180-I (Ahle & Ungewickell, 1986), HA1 and HA2 with mAbs 100/3 and 100/2, respectively (Ahle et al., 1988), and auxilin with mAb 100/4 (Ahle & Ungewickell, 1990) at concentrations of 5–10 μg/mL. Antibody–antigen complexes were reacted with peroxidase-conjugated secondary rabbit antibodies to mouse IgG (Dako) according to the manufacturer's recommendation (usually at dilutions of 1:500) and developed with 4-chloro-1-naphthol.

Miscellaneous Techniques. SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (1970), except for using 1.5% instead of the standard 2.6% *N,N'*-methylenebis(acrylamide) (relative to the total acrylamide concentration) in the separation gel. Gels with a low *N,N'*-methylenebis(acrylamide) content were found to resolve AP180 from the clathrin heavy chain, as originally observed when the PAGE method of Neville was employed (Ungewickell & Oestergaard, 1989). Electrophoresis was performed in 7.5 × 8.0 × 0.075 cm minigel slabs in a Hoefer Mighty Small II unit. The gels were stained with Coomassie Brilliant Blue and scanned with an Elscript instrument from Hirschmann (Unterhaching, FRG).

Results and Discussion

To explore the role of light chains in the interaction of adaptor proteins (HA1 and HA2), AP180, and auxilin with clathrin, we began by comparing the effects of these proteins on clathrin assembly in the presence and absence of light chains. To this end, the clathrin protomers, each consisting of three heavy and three light chains, were separated from the adaptors, AP180, and auxilin by gel filtration (Figure 1, track 1). The latter elute together behind the clathrin in a broad peak, which has been collectively referred to as the assembly protein fraction (Figure 1, track 3). Light-chain-free clathrin heavy chains were prepared from intact clathrin by the thiocyanate method of Stanley and Winkler (1983) (Figure 1, track 2). For assembly protein driven clathrin assembly, intact clathrin (with light chains) at a concentration of 0.4 mg/mL and purified heavy chains at equivalent molar concentration (0.35 mg/mL) were titrated with assembly proteins and then dialyzed overnight against isolation buffer at pH 6.7. The proportion of assembled coats in the reaction mixture was determined by pelleting the cages and quantifying the amount of clathrin in the pellets and supernatants by SDS–PAGE. Under our experimental conditions (isolation buffer at pH 6.7), polymerization of intact clathrin was dependent on the concentration of assembly proteins in the reaction mixture (Figure 2). Moreover, heavy chains, which also do fail to assemble efficiently by themselves in isolation buffer at pH 6.7, showed the same concentration dependence on assembly proteins as intact clathrin (Figure 2). These results indicate that light chains are not required for uptake of those assembly proteins by clathrin that engender its polymerization into cages. To determine if the composition of assembly proteins in these cages is altered by the absence of light chains, we reconstituted cages from intact clathrin or heavy chains in the presence of assembly proteins. The weight ratio of clathrin to assembly proteins was adjusted to be the same as in the original Tris-soluble coat protein fraction and therefore limiting to the extent

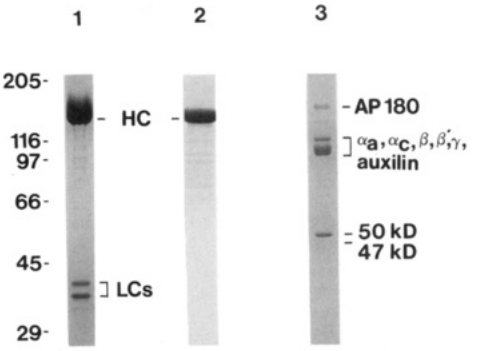

FIGURE 1: SDS gel electrophoretic analysis of the proteins used for assembly experiments: intact clathrin (track 1); purified clathrin heavy chain (track 2); assembly protein fraction (track 3).

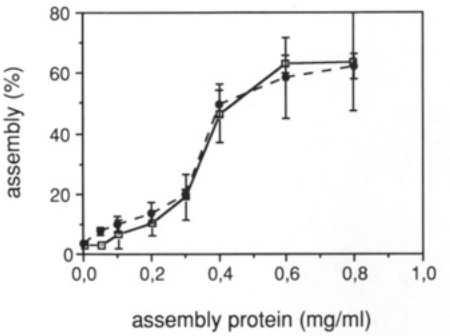

FIGURE 2: Assembly of intact clathrin (●) and of heavy chain (□) as a function of the concentration of assembly protein. Equimolar amounts of intact clathrin and of heavy chains in 0.5 M Tris-HCl buffer (0.40 and 0.35 mg/mL, respectively) were titrated with assembly protein (0–0.8 mg/mL). Samples in triplicate were dialyzed into isolation buffer, and assembly was quantitated as described under Materials and Methods. Values in the diagram represent the means with error bars indicating the standard deviation.

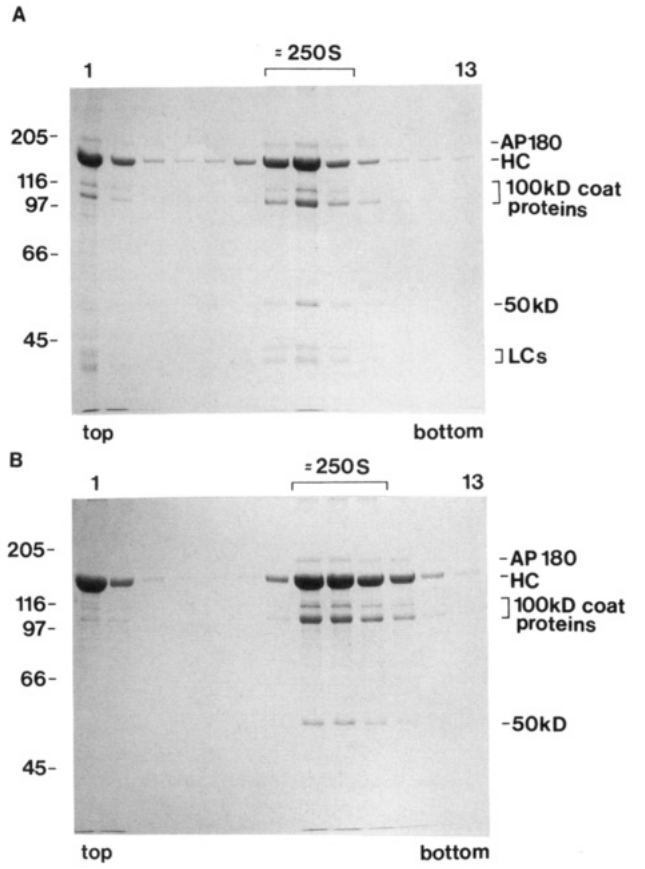

FIGURE 3: Purification of reassembled coats by sucrose density gradient centrifugation. Cages were assembled from either 1.0 mg/mL intact clathrin (A) or 0.88 mg/mL heavy chains (B) in the presence of 0.7 mg/mL assembly protein and then fractionated on 5–30% sucrose gradients. All fractions were examined by SDS–PAGE using polyacrylamide gels with a reduced *N,N'*-methylenebis(acrylamide) concentration to facilitate separation of AP180 from the clathrin heavy chain (see Materials and Methods). The loading of the gel shown in (A) is two-thirds of that in (B). Note coassembly of AP180 and the 100-kDa coat polypeptides in both (A) and (B). The brackets indicate pooled peak fractions.

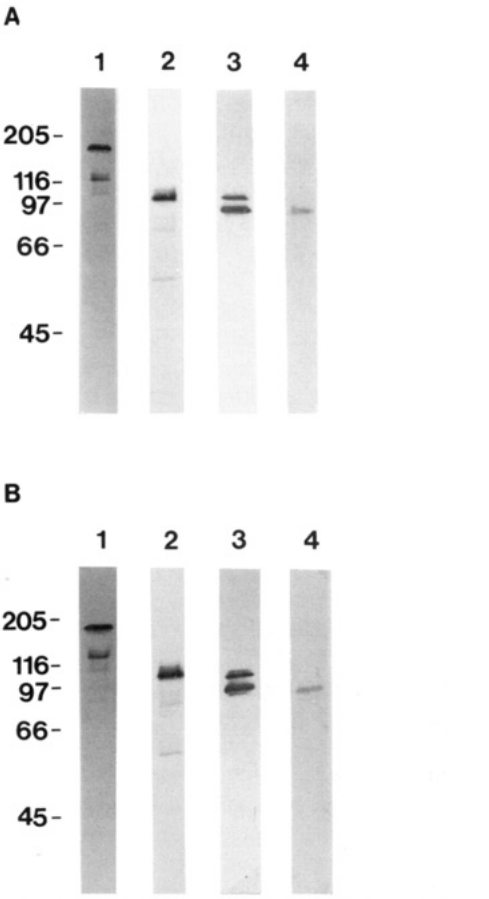

FIGURE 4: Immunoblot analysis of reassembled heavy chain or clathrin coats. Fractions were pooled as indicated by the brackets in Figure 3 and then subjected to SDS–PAGE and followed by immunoblotting with monoclonal antibodies to AP180 (track 1), auxilin (track 2), HA2 (track 3), and HA1 (track 4). Clathrin coats (A); heavy-chain coats (B).

Table I: Binding of Purified Coat Proteins to Cages Preassembled from Intact Clathrin or Heavy Chains

	% coat protein bound to cages[a]	
	intact clathrin cages	heavy-chain cages
AP180	74	61
auxilin	97	97
HA1	71	71
HA2	21	22

[a] Values were corrected for aggregation.

of assembly. The cages were purified by sucrose gradient centrifugation, and the fractions were analyzed by SDS–PAGE (Figure 3). Those containing only cages were pooled for further examination by immunoblotting using monoclonal antibodies to adaptor polypeptides, AP180, and auxilin. Regardless of whether light chains were present, the clathrin cages incorporated HA2 and HA1 adaptors, as shown by the presence of the α- and γ-type subunits, as well as AP180 and auxilin (Figure 4). The amounts of incorporated AP180 and of the 100-kDa coat proteins as determined by densitometry were found to be nearly identical for light-chain-free and light-chain-containing cages (data not shown). We have considered the possibility that one or the other minor coat protein may require light chains for its direct interaction with clathrin but can still associate with heavy-chain cages through other coat proteins that act as bridges. To exclude this, we investigated the binding of purified HA2, HA1, AP180, and auxilin to clathrin heavy chains. For this experiment, we used preassembled cages (with and without light chains) as binding substrates. These cages were obtained by dialyzing intact clathrin or heavy chains against isolation buffer containing millimolar calcium. For binding experiments with AP180 and HA1, the cages were dialyzed against isolation buffer, pH 6.7. For binding auxilin and HA2 to cages, we used tartrate buffer because these proteins are prone to self-aggregation in isolation buffer. After 1 h of incubation with the purified coat proteins, the cages were separated from the reaction mixture by ultracentrifugation, and the amount of coat protein in the supernatants and pellets was quantified by SDS–PAGE (Figure 5). In all cases, we observed binding of the individual assembly proteins to clathrin cages, irrespective of the presence or absence of light chains. Moreover, there were no significant quantitative differences in the extent, and therefore by implication the affinity, of binding (Table I). In sum, our results strongly suggest that HA1, HA2, AP180, and auxilin bind directly to the clathrin heavy chain and that light chains are not required for this interaction. Consistent with our biochemical data are morphological studies of Heuser and Keen (1988) and Vigers et al. (1986), who place the adaptors close to the terminal domain of clathrin. This is known not to bind light chains (Ungewickell, 1983). It remains now to be shown by biochemical techniques which heavy-chain domains are involved in binding the various coat proteins. The generation of appropriate heavy-chain fragments using controlled proteolysis may help to define their binding site(s) on clathrin

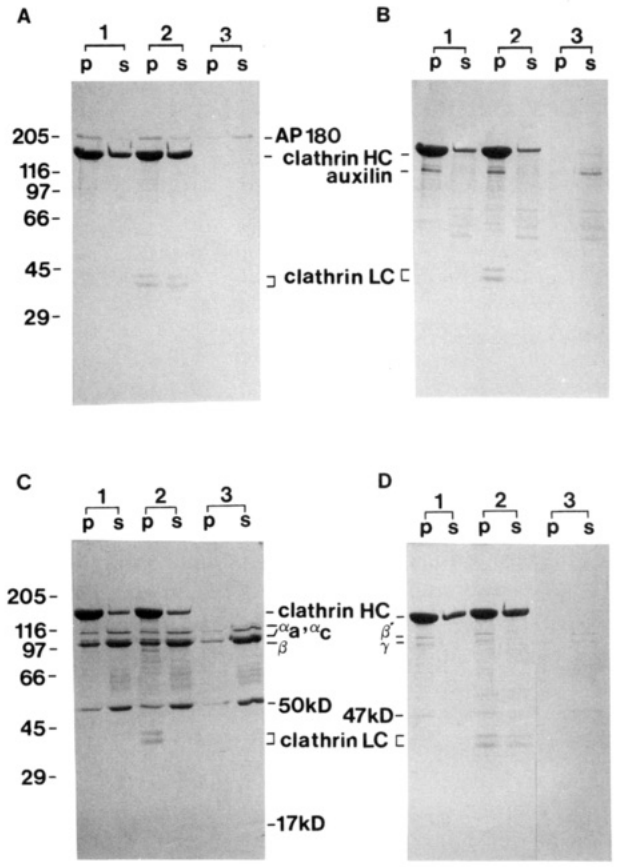

FIGURE 5: Binding of purified coat proteins to preformed clathrin and heavy-chain cages. After incubation with the indicated purified coat protein, the cages (0.4 mg/mL clathrin cages or 0.35 mg/mL heavy-chain cages) were pelleted by ultracentrifugation. Aliquots from supernatant (s) and pellet fractions (p) were examined by SDS–PAGE. Binding of AP180 (0.08 mg/mL) is shown in (A), auxilin (0.16 mg/mL) in (B), HA2 (0.4 mg/mL) in (C), and HA1 (0.08 mg/mL) in (D). The binding substrates were (1) heavy-chain cages and (2) intact clathrin. (3) Control incubations of coat protein in the absence of clathrin. Aggregation of coat protein under our assay conditions is lower than 10%.

more precisely.

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