The EMBO Journal vol.12 no.13 pp.5237-5244, 1993

The β1 and β2 subunits of the AP complexes are the clathrin coat assembly components

Andreas Gallusser and Tomas Kirchhausen[1]

Harvard Medical School, Department of Cell Biology and The Center for Blood Research, 200 Longwood Avenue, Boston, MA 02115, USA
[1]Corresponding author

Communicated by D.Meyer

The β1 and β2 subunits are the closely-related large chains of the *trans*-Golgi network AP-1 and the plasma membrane AP-2 clathrin-associated protein complexes, respectively. Recombinant β1 and β2 subunits have been generated in *Escherichia coli*. It was found that, in the absence of all the other AP subunits, β1 and β2 interact with clathrin and drive the efficient assembly of clathrin coats. In addition, β2 subunits and AP complexes compete for the same clathrin binding site. The appearance of the clathrin/β coats is the same as the barrel-shaped structures formed with native AP complexes. It is proposed that the principal function of the β subunits is to initiate coat formation, while the remaining subunits of the AP complexes have other roles in coated pit and coated vesicle function.

Key words: AP-clathrin interaction/clathrin-associated proteins/endocytosis/membrane traffic/recombinant AP subunits

Introduction

Clathrin coated pits and coated vesicles are organelles found at the plasma membrane and at the *trans*-Golgi network (for review see Pearse and Robinson, 1990). They play a major role in the endocytic and regulated exocytic vesicular traffic in eukaryotic cells. The formation of coated pits is initiated by the assembly of the clathrin lattice on the cytosolic side of the membrane; this process mediates the engulfment and vesiculation of that section of the membrane (Harrison and Kirchhausen, 1983). When coated vesicles bud from the membrane, they selectively retain specific membrane protein receptors, which can then be targeted to the appropriate organelle (Goldstein *et al.*, 1985).

In vitro clathrin can form lattices similar to those found in coated pits and coated vesicles, but not under physiological conditions of ionic strength (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981). However, several different proteins or complexes isolated from clathrin coated vesicles can drive coat formation under these conditions (Zaremba and Keen, 1983; Pearse and Robinson, 1984; Keen, 1987; Lindner and Ungewickell, 1992). The best studied of these are the related clathrin associated protein AP-1 and AP-2 complexes, hetero-tetrameric structures ubiquitous to all mammalian tissues that preferentially localize at the *trans*-Golgi network and at the plasma membrane (Robinson, 1987; Ahle *et al.*, 1988). It is likely that the AP complexes direct the assembly of clathrin *in vivo*. Because the Golgi and the plasma membrane selectively retain a different subset of membrane receptors within the coated pit, and because the APs are the only known difference between coated vesicles in the two membrane systems, the APs are also believed to mediate receptor selection. There is also some experimental evidence that APs have weak interactions with receptor cytoplasmic tails (Pearse, 1988; Glickman *et al.*, 1989; Beltzer and Spiess, 1991; Sosa *et al.*, 1993). For this reason, the AP complexes are also sometimes called 'adaptors'. The size and complexity of these assemblages (four subunits comprising 270 kDa) make it plausible that they may have other functions as well, for example a 'switch' to initiate clathrin polymerization and perhaps another for uncoating, and a docking component to attach the APs to the membrane. As a first step towards understanding the functions of the AP complexes, it is important to know which components are required for clathrin assembly.

AP-1 and AP-2 complexes each contain two large subunits (γ and β1 or α and β2, respectively), one medium subunit (μ1 or μ2; formerly AP47 or AP50) and one small subunit (σ1 or σ2; formerly AP19 or AP17) (Ahle *et al.*, 1988; Virshup and Bennett, 1988; Matsui and Kirchhausen, 1990). The large subunits each contain a proline-glycine-alanine-rich region that is preferentially sensitive to several proteases (Zaremba and Keen, 1985; Kirchhausen *et al.*, 1989; Schröder and Ungewickell, 1991). This portion, which is referred to as the hinge region (~100 amino acids), separates and defines the N-terminal domain or trunk (~600 amino acids) and the C-terminal domain or ear (~250 amino acids). The trunks of each large subunit, together with the medium and small subunits, remain associated in the AP core, while the ears are released (Zaremba and Keen, 1985; Kirchhausen *et al.*, 1989). Proteolyzed AP cores cannot coassemble with clathrin (Zaremba and Keen, 1985). This observation has led to the suggestion that intact large subunits are required for coat formation.

In certain buffer conditions, AP cores are released from clathrin coated vesicles while the intact complex remains attached (Schröder and Ungewickell, 1991). This release correlates with the proteolysis of the β1 or β2 subunit. It has also been shown that purified β2 subunits bind to preformed clathrin cages, but cannot initiate coat formation (Ahle and Ungewickell, 1989). The idea that the β1 and β2 subunits are responsible for binding to clathrin is consistent with the observation that the β1 and β2 subunits are closely related (84% identity overall with the sequence divergence concentrated on the hinge region) (Kirchhausen *et al.*, 1989). The α and γ subunits show a much lower level of similarity, with 25-29% sequence identity mostly concentrated in the trunk domains (Robinson, 1990). As clathrin seems to be the same throughout the cell, one might imagine that the portion of the AP-1 and AP-2 complexes that interact with clathrin is relatively conserved. However, in experiments

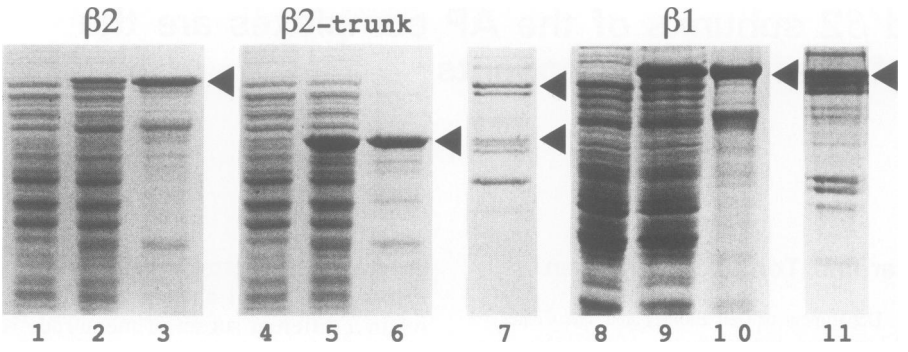

Fig. 1. β2, β2-trunk and β1 subunits expressed in *E.coli* BL21(DE3). Cultures (1 l) of *E.coli* BL21(DE3) transformed with pET5a:β2, pET5a:β2-trunk or pRSETc:β1 were grown up to an OD_{600} of ~0.7 before induction with 1 mM IPTG for an additional 2 h. Aliquots (1 ml) of the uninduced (lanes 1, 4 and 8) and induced (lanes 2, 5 and 9) cultures were spun down and resuspended in 4% SDS–sample buffer. Inclusion bodies (lanes 3, 6 and 10) were prepared as described in Materials and methods and aliquots were dissolved in 4% SDS–sample buffer. An AP-2 sample was partially digested with trypsin to indicate the electrophoretic mobility of native β2 and β2-trunk (lane 7). A mixture of AP-1 and AP-2 complexes is shown in lane 11 to indicate the mobility of native β1. All samples were fractionated by SDS–10% PAGE in the presence (lanes 1–7) or absence (lanes 8–11) of 6 M urea, and stained with Coomassie Blue. Arrow heads indicate the positions of β1, β2 and β2-trunk.

in which partially purified β2 subunits, containing ~10% of α subunit, were used to drive coat formation, the resulting coats were found to contain equal amounts of α and β2 (Prasad and Keen, 1991). Therefore, it was proposed that the α (or presumably γ) subunit is primarily responsible for clathrin binding, and that both α and β2 are necessary for lattice assembly. Given the large divergence of the α and γ subunits, this model was considered puzzling. Therefore it was decided to reinvestigate the requirements for clathrin binding, using recombinant β1 and β2 subunits to ensure that no contaminating AP-associated proteins are present. It was found that β1 and β2 not only bind directly to clathrin but also, surprisingly, are sufficient to drive coat formation.

It is proposed that intact β1 and β2 subunits are the main clathrin assembly components of the AP complexes. While our data do not directly address whether the α or γ subunit can also drive coat formation, on the basis of the sequence variation between α and γ it is proposed that α and γ do not interact with the sites on clathrin that are essential for the initiation of coat formation, but have other roles in coated pit and coated vesicle function.

Results

Expression and refolding of β2, β2-trunk and β1 subunits

The complete large β2 subunit (106 kDa) of the mammalian AP-2 complex and its 67 kDa N-terminal fragment (β2-trunk) were cloned into the bacterial expression vector pET5a under the control of the T7 promoter. *Escherichia coli* BL21(DE3) transformed with these constructs overexpressed both protein subunits on induction with IPTG (Figure 1, lanes 2 and 5). The full-length β1 subunit (105 kDa) from the mammalian rat AP-1 complex was similarly cloned and expressed (Figure 1, lane 9). The main band for the β2 and β1 subunit samples has an electrophoretic mobility similar to β2 and β1 subunits from intact bovine brain AP-2 and AP-1 complexes (Figure 1, lanes 7 and 11). The smaller and less abundant recombinant proteins of ~70–75 kDa are partial translation products of β2 and β1 subunits (data not shown). The electrophoretic mobility of the recombinant β2-trunk (Figure 1, lane 5) is indistinguishable from the β2-trunk generated by tryptic digestion of the bovine brain AP-2 complex (Figure 1, lane 7). Therefore, the recombinant

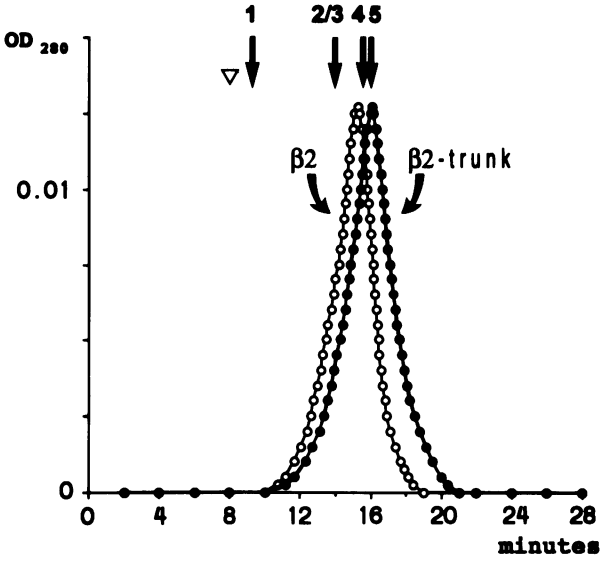

Fig. 2. Gel filtration analysis of recombinant β2 and β2-trunk subunits. Refolded β2 and β2-trunk in buffer II were dialyzed against 100 mM TEA, 100 mM NaCl, 2 mM EDTA, pH 8.0, at a protein concentration of 80–100 μg/ml. The dialyzed samples were spun in a TLA-100.4 rotor at 85 000 r.p.m. for 1 h at 4°C using a Beckman TLX ultracentrifuge and 500 μl were subjected to gel filtration on a Superose 6 (Pharmacia) column at a flow rate of 0.5 ml/min. The tracing for the β2 subunit (106 kDa) is shown with open circles, the one for the β2-trunk subunit (67 kDa) with closed circles. The column was calibrated with clathrin (1; 650 kDa), apoferritin (2; 443 kDa), APs (3; 270 kDa), alcohol dehydrogenase (4; 150 kDa) and BSA (5; 66 kDa). An open arrow head indicates the void volume of the column.

proteins are indistinguishable from their native counterparts by SDS–PAGE analysis.

Native AP-2 complexes can be dissociated into their subunits by incubation with 6 M GuHCl. Slow removal of the denaturant by dialysis allows them to reassociate into a functional complex (Prasad and Keen, 1991). Recombinant β2, β2-trunk and β1 subunits accumulate in *E.coli* as insoluble inclusion bodies (Figure 1, lanes 3, 6 and 10), and therefore a similar procedure was used to solubilize and refold them (see Materials and methods).

To establish the quality of refolding, the recombinant β2 and β2-trunk proteins were examined by gel filtration chromatography (Figure 2). The β2 subunit (106 kDa)

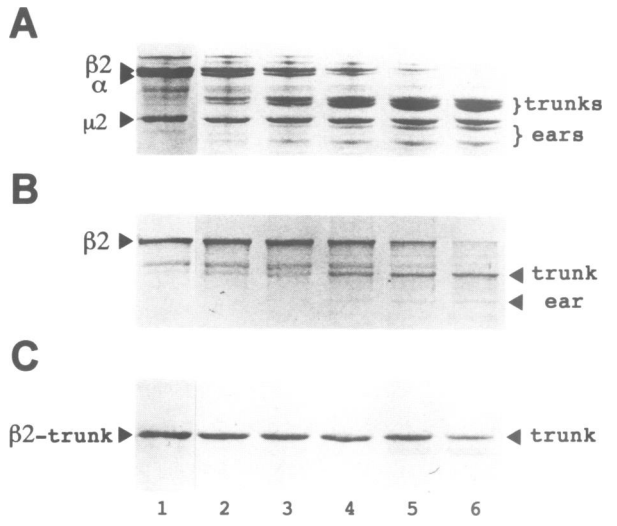

Fig. 3. Electrophoretic analysis of the tryptic digestion of recombinant β2 and β2-trunk subunits. Samples of (**A**) native AP-2 complexes (240 μg/ml), (**B**) recombinant refolded β2 subunits (40 μg/ml) and (**C**) β2-trunk subunits (80 μg/ml) were digested with different amounts of DPCC-trypsin (Sigma) for 15 min at room temperature. The reaction was stopped with 2 mM PMSF and the cleavage products were analyzed on SDS−12.5% PAGE in the presence of 6 M urea. Lane 1 of each panel shows the non-digested samples of AP-2, β2 and β2-trunk. Increasing amounts of trypsin were added (lanes 2−6) at the following enzyme:protein ratios (w/w): 1:2400 (lane 2), 1:1200 (lane 3), 1:600 (lane 4), 1:300 (lane 5) and 1:150 (lane 6). The major cleavage products are indicated on the right of each panel.

fractionates as a single symmetric peak of a Stokes radius slightly larger than the globular protein alcohol dehydrogenase (150 kDa) but smaller than native AP complexes, suggesting that the recombinant β2 is monomeric and has an elongated shape. Partially purified native β2 subunit has a similar Stokes radius (Ahle and Ungewickell, 1989). The refolding in buffer II was performed at low protein concentrations because at higher protein concentrations (200−600 μg/ml) a second larger species is seen, which may represent β2 dimers (data not shown).

The native β2 subunit within the AP-2 complex has two relatively protease-insensitive domains, the trunk (67 kDa) and ear (39 kDa) domains, which are assumed to be compact, separated by a protease-sensitive hinge region (Kirchhausen *et al.*, 1989). The sequence corresponding to the β2-trunk produced by digestion with trypsin (67 kDa) was similarly expressed in *E.coli* and refolded at dilute concentrations (100 μg/ml). This molecule was found to fractionate as a single symmetric peak of similar size to BSA, a 66 kDa globular protein (Figure 2). Thus, unlike intact β2 subunit, β2-trunk is a relatively compact molecule.

Proteolytic analysis of recombinant β2 and β2-trunk subunits

Within native AP-2 complexes the trunk and ear domains of the large subunits are resistant to many proteases, whereas the hinge region that separates these domains is fairly accessible to proteolytic cleavage (Zaremba and Keen, 1985; Kirchhausen *et al.*, 1989; Matsui and Kirchhausen, 1990). Figure 3 compares the SDS−PAGE patterns obtained after tryptic digestion of native AP-2 complexes (Figure 3A), recombinant β2 subunit (Figure 3B) and β2-trunk (Figure 3C). It is clear that the recombinant β2 subunit is preferentially cleaved at a site within the hinge region. However, using higher amounts of trypsin, recombinant β2 is also cleaved within the trunk (not shown). Recombinant β2-trunk is also more susceptible to cleavage than β2-trunk within the AP-2 core. Because separation of native β2 subunits from AP-2 complexes requires denaturation and refolding, it is currently not possible to compare directly native β2 subunits with our recombinant subunits. However, the preferential cleavage of recombinant β2 subunit into two domains whose sizes are indistinguishable from native 'trunk' and 'ear' indicates that the overall two domain structure is similar to that of the native large subunits.

Recombinant β2 subunit binds to clathrin

It has recently been demonstrated that in the presence of tartrate-containing solutions, intact AP complexes remain associated with clathrin coated vesicles, whereas proteolyzed AP cores are released from similar clathrin coated structures. In the same buffer, intact AP complexes can also bind to preformed clathrin cages (Schröder and Ungewickell, 1991). There is a strong correlation between the loss of intact β1 and β2 subunits and the loss of ability to associate with clathrin in both of these assays.

The availability of isolated recombinant β2 subunits allows the examination of the interaction of β2 subunit with clathrin directly. Recombinant β2 and β2-trunk subunits were tested for binding to preformed clathrin cages in a buffer containing 100 mM K^+-tartrate (buffer T) (Schröder and Ungewickell, 1991) using differential centrifugation, with native AP-2 complexes as a positive control. Isolated β2 subunits indeed bind to clathrin cages in amounts similar to native AP-2 complexes (Figure 4, compare β2 subunit bands indicated by arrow heads in lane 8 and lane 10). In the absence of clathrin cages, AP-2 complexes and recombinant β2 subunit remain in the supernatant fraction (Figure 4, lanes 1 and 3); thus association is specific. The β2-trunk is never found in the high speed pellet (Figure 4, lane 12), indicating that the interaction of β2-trunk with clathrin cages is negligible, at least at the concentrations used here.

Thus, recombinant β2 subunit can bind directly to preformed clathrin cages, whereas recombinant β2-trunk does not. By extrapolation, it is likely that the β1 subunit of AP-1 can also bind directly to clathrin cages.

The recombinant β1 and β2 subunits are sufficient for clathrin coat assembly

It is currently believed that both α and β2 subunits are needed to drive coat assembly (Prasad and Keen, 1991). To examine whether β2 subunit alone can drive coat formation, recombinant β2 subunit was added to Tris-solubilized clathrin trimers and the mixture was then dialyzed against coat assembly conditions. Unexpectedly this mixture formed coats. Furthermore, recombinant β1 subunit is also sufficient to form coats.

The clathrin coats that coassemble with β1 (Figure 5A) or β2 (Figure 5B) subunits were examined by electron microscopy and found to be structurally homogeneous and identical in size and geometry to the 60−70 nm barrel-shaped coats formed in the presence of native (Figure 5C) or renatured (Figure 5D) APs or AP-2 complexes (not shown). The absence of protrusions in the periphery of the coats suggests that, like native APs, the recombinant β1 or β2 subunits, as well as renatured APs, bind on the inside of the clathrin lattice on the membrane face of the coat (Heuser and Kirchhausen, 1985; Vigers *et al.*, 1986). One

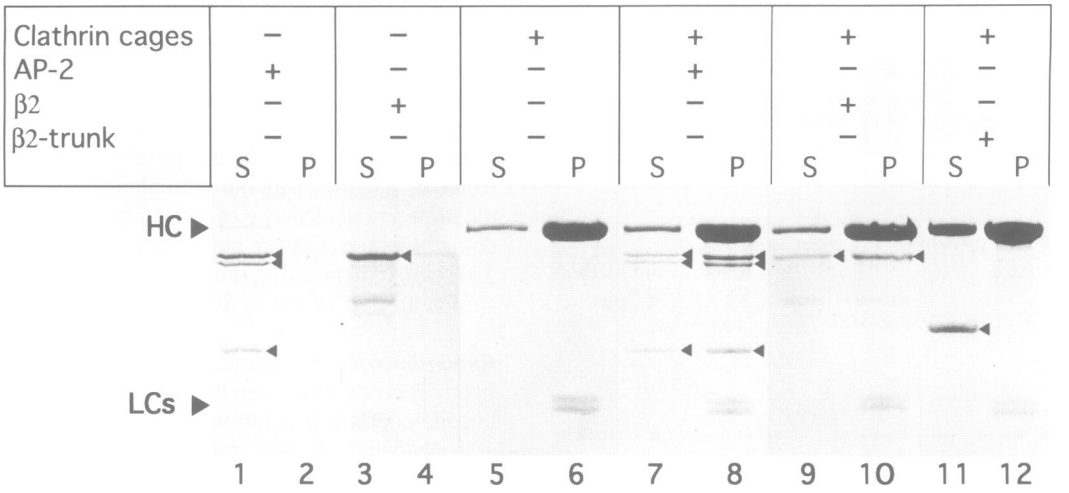

Fig. 4. β2 but not β2-trunk subunits bind to clathrin cages. Clathrin cages were assembled from purified clathrin, as described in Materials and methods, and gently resuspended in buffer T at room temperature for 30 min. Clathrin cages (final protein concentration of 400 μg/ml) were incubated for 30 min at 4°C with AP-2 complexes (150 μg/ml), recombinant β2 (150 μg/ml) and β2-trunk (150 μg/ml) subunits that were previously dialyzed against buffer T. Samples of cages alone, as well as AP-2 complexes and β2 subunits in the absence of cages, were incubated under the same conditions. The cages were separated from unbound material by ultracentrifugation. High speed supernatants (S, odd lanes) and pellets (P, even lanes) of all samples were analyzed by SDS−10% PAGE in the presence of 6 M urea. These are representative results from different experiments. Small differences in the amount of free clathrin remaining in the high speed supernatants are caused by slight variations in spontaneous depolymerization of cages in the presence of buffer T and do not reflect differences resulting from the addition of AP subunits. AP-2 subunits (α, β2 and μ2) and the recombinant β2 and β2-trunk subunits are indicated with arrow heads; HC and LCs indicate clathrin heavy and light chains respectively.

difference between the coats formed with renatured APs or recombinant β1 and β2 subunits and those formed with native AP complexes is the electron-dense material seen at the center. The origin of this material is unclear, but it may be caused by aggregation of refolded protein.

All four preparations appear to be equally efficient at driving coat formation, as measured by the total number of coats observed in electron micrographs in this and several other independent experiments. The β2-trunk, in contrast, entirely lacks this assembly activity (Figure 5F).

To confirm that recombinant β1 and β2 subunits are incorporated into coats, unassembled and assembled material from a coat assembly assay were analyzed using SDS−PAGE (Figure 6). Recombinant β1 or β2 subunits, native APs and renatured APs each copurify with clathrin and are found in the high speed pellet (Figure 6A, lanes 4, 8 and 10; Figure 6B, lanes 4 and 6). In contrast, when clathrin is incubated with the recombinant β2-trunk subunit, it remains unassembled and both clathrin and β2-trunk remain in the high speed supernatant (Figure 6A, lane 5).

To examine whether the β2 subunit binding site is specific and saturable, a quantitative coat assembly assay was used. Samples containing constant amounts of clathrin (0.5 mg/ml) and decreasing amounts of refolded β2 subunit were allowed to form coats, which were then analyzed by SDS−PAGE. It was found that the amount of coat formation is proportional to the quantity of recombinant β2 subunit in the concentration range tested (50−400 μg/ml; data not shown). For each of the coat samples, the ratio of recombinant β2 subunit to clathrin heavy or light chains was then compared and was found to be constant (Figure 7, lanes 1−4). Thus, recombinant β2 subunit binds to specific saturable sites on clathrin. The number of β2 subunits per clathrin trimer is independent of the total amount of β2 subunit in the reaction and is identical to the ratio of β2 subunit to clathrin in coats assembled using native AP complexes (Figure 7, lane 5).

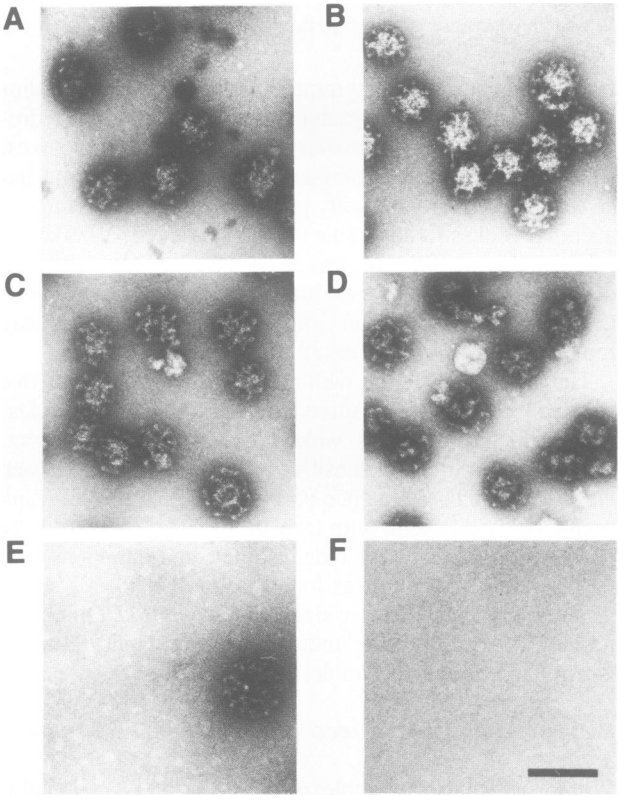

Fig. 5. Electron microscopy of clathrin coats assembled with β1 and β2 subunits. The micrographs correspond to representative fields obtained from negatively stained aliquots of the high speed pellets described in Figure 6. For each sample the efficiency of coat formation was estimated by counting the number of coats in four or five random fields. Values indicate the average number of coats and their diameter. (**A**) Clathrin/β1: 137, 70 nm. (**B**) Clathrin/β2: 111, 70 nm. (**C**) Clathrin/APs: 179, 70 nm. (**D**) Clathrin/APs renatured: 221, 70 nm. (**E**) Clathrin: 9, 70 nm; 6, 100 nm. (**F**) Clathrin/β2-trunk: 7, 70 nm. Scale bar, 100 nm.

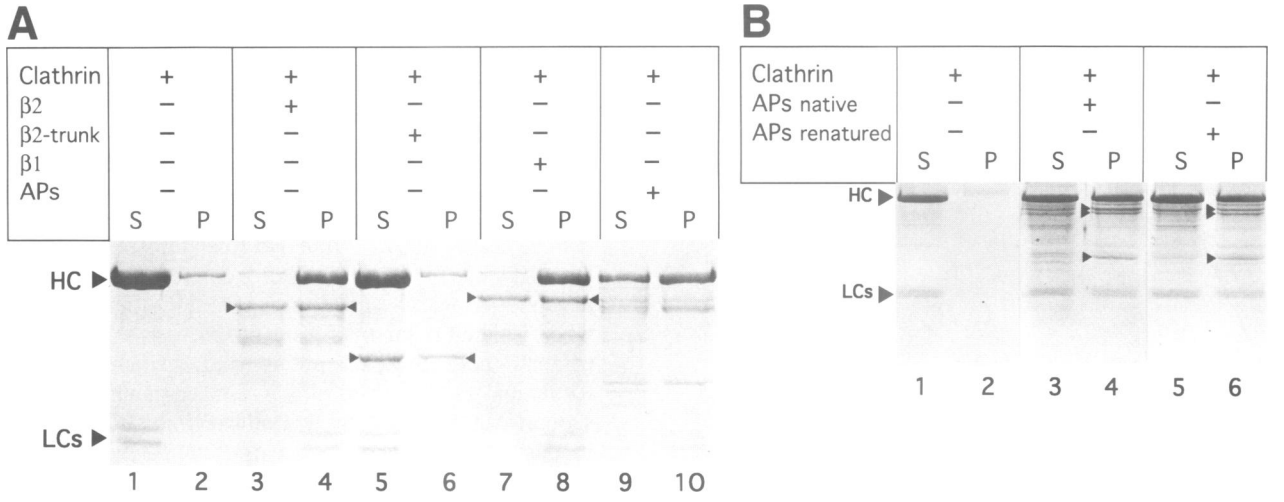

Fig. 6. Both β1 and β2 subunits assemble clathrin into coats. (**A**) Clathrin coats were assembled by mixing Tris-solubilized clathrin (0.5 mg/ml final protein concentration) with either β2, β2-trunk or β1 subunits (250 μg/ml) previously dialyzed into buffer I (2 M urea, 500 mM Tris−HCl, pH 8.0, 2 mM EDTA, 2 mM DTT) and with native AP complexes (400 μg/ml). The mixtures and a control sample containing clathrin alone were dialyzed overnight at 4°C against coat assembly conditions (buffer III). Aggregated material was removed by low speed centrifugation and the coats were then separated from unassembled material by ultracentrifugation. High speed supernatants (S, odd lanes) and pellets (P, even lanes) were analyzed by SDS−10% PAGE. (**B**) Clathrin coats were assembled and processed as described above using clathrin (0.5 mg/ml), native AP complexes and renatured AP complexes. Renatured AP complexes were first denatured by overnight dialysis at 4°C against 6 M GuHCl, 100 mM Tris−HCl pH 8.0, 2 mM EDTA, 2 mM DTT, and then renatured by overnight dialysis at 4°C against buffer I. High speed supernatants (S, odd lanes) and pellets (P, even lanes) were analyzed by SDS−12.5% PAGE in the presence of 6 M urea. HC and LCs indicate clathrin heavy and light chains respectively.

Thus, the β2 subunit binding sites are present in the same numbers as the sites used by native AP complexes. Given the homology between β2 and β1, it is likely that β1 also binds to the same specific site.

It is concluded that intact β2 or β1 subunits are sufficient for coat assembly *in vitro* and are likely to bind specifically to the same sites as do intact AP complexes.

Recombinant β2 subunits and intact APs bind to the same site in clathrin coats

AP driven assembly of clathrin coats does not, in practice, go to completion. It was found that there is always some pure clathrin under any of the coat assembly conditions, which are severely restricted by the solubility properties of AP complexes (Matsui and Kirchhausen, 1990; Beck and Keen, 1991). In contrast, β subunit driven assembly is very efficient. Therefore a straightforward competition experiment is not easy to interpret because the fraction of clathrin left unassembled in the APs is utilized for assembly by β. To circumvent these problems, a binding exclusion assay was used to demonstrate that recombinant β2 subunits and native AP complexes bind to the same site(s) in clathrin coats. For this experiment, clathrin coats were first preassembled with saturable amounts of recombinant β2 subunits by overnight dialysis against buffer III. The clathrin/β2 coats were separated from free β2 subunits by ultracentrifugation and resuspended in either buffer T or buffer T containing native AP complexes. Under these buffer conditions, APs remain in the supernatant and aggregation is minimal (Figure 8A, lanes 5 and 6), while binding to preformed clathrin cages (Schröder and Ungewickell, 1991; also see Figure 4) or coassembly into clathrin coats (data not shown) is maintained. After 1 h incubation, the mixture was subjected to high speed centrifugation and binding of AP complexes to preformed clathrin/β2 coats was determined by SDS−PAGE analysis of supernatants (Figure 8A, lanes 1 and 3) and pellets (Figure 8A, lanes 2 and 4). It was found that AP complexes remain in the supernatant (Figure 8A, lane 3) and are completely excluded from the pellet containing clathrin/β2 coats (Figure 8A, lane 4). The converse experiment, using clathrin/AP coats to which recombinant β2 subunits are added, showed the same exclusion pattern (Figure 8B).

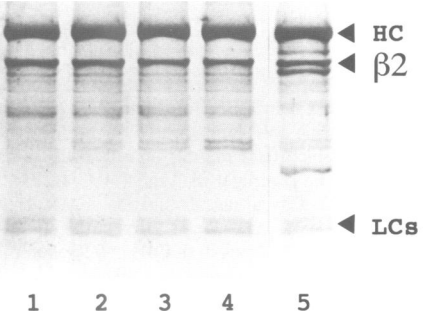

Fig. 7. The ratio of clathrin/β2 on coats is constant and independent of the amount of β2 subunit in the assembly reaction. Clathrin coats were assembled using a constant concentration of Tris-soluble clathrin (0.5 mg/ml) and the following final concentrations of recombinant β2 subunits: 400 μg/ml (lane 1), 200 μg/ml (lane 2), 100 μg/ml (lane 3) and 50 μg/ml (lane 4). For an additional assembly reaction, Tris-soluble clathrin (1.25 mg/ml) was mixed with an AP sample (125 μg/ml) (lane 5). Following an overnight dialysis at 4°C against buffer III, a small amount of aggregated material was removed by low speed centrifugation. The coats were then separated from unassembled material by ultracentrifugation at 60 000 r.p.m. for 10 min at 4°C and the pellets were subjected to SDS−10% PAGE (6 M urea) analysis. To have similar amounts of the β2 subunit in each of the lanes, they were loaded with 5 μl (lane 1), 10 μl (lane 2), 20 μl (lane 3) and 40 μl (lane 4) of sample respectively The ratio of clathrin heavy or light chains (HC and LCs respectively) to the β2 subunit was determined by densitometry of the Coomassie Blue stained bands.

Although the experiment does not resolve whether all binding sites in the preassembled coats are occupied or

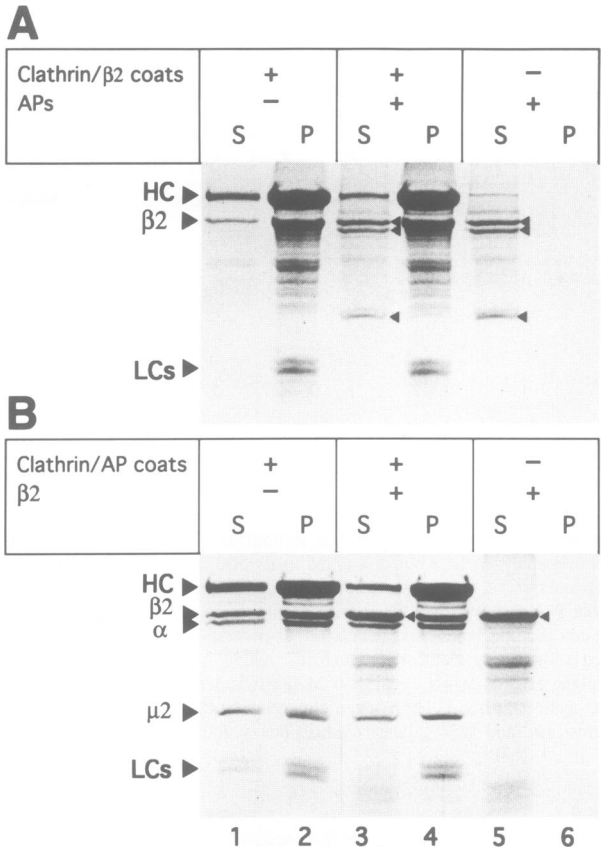

Fig. 8. β2 subunits and AP complexes compete for the same binding site in clathrin coats. Clathrin/β2 and clathrin/AP coats were preassembled by mixing soluble clathrin (0.75 mg/ml) with either recombinant β2 subunits (0.25 mg/ml) or native AP complexes (0.4 mg/ml) followed by an overnight dialysis at 4°C against buffer III. The coats were pelleted by ultracentrifugation and resuspended in either buffer T (**A** and **B**, lanes 1 and 2) or buffer T containing AP complexes (0.4 mg/ml; A, lanes 3 and 4) or β2 subunits (0.25 mg/ml; B, lanes 3 and 4). Binding of free APs to clathrin/β2 coats and of free β2 subunits to clathrin/AP coats was performed for 60 min at 4°C. The coats were then separated from non-bound material by ultracentrifugation, and supernatants (S, lanes 1 and 3) and pellets (P, lanes 2 and 4) were analyzed by SDS−10% PAGE in the presence of 6 M urea. Note that in the absence of coats, AP complexes and β2 subunits remain in the high speed supernatant (**A** and **B**, lane 5).

whether access is limited by steric hindrance, the simplest interpretation for these exclusion results is that β2 subunits, either as free recombinant protein or as part of an AP complex, occupy a similar location in the clathrin lattice.

Discussion

These experiments show that recombinant rat β1 and β2 subunits coassemble with clathrin and drive the formation of coats. The efficiency of assembly and the geometry of the coats obtained with recombinant β1 and β2 subunits are indistinguishable from those obtained using native bovine brain AP complexes. Therefore, it is proposed that the β1 and β2 subunits of the AP-1 and AP-2 complexes are primarily responsible for the interactions that facilitate the assembly of clathrin into coats.

Previous attempts to elucidate which of the large subunits in AP complexes drives clathrin coat assembly have provided incomplete or conflicting results. It was first shown by Prasad and co-workers that purified large subunits of unknown identity could drive coat formation (Prasad *et al.*, 1986). The purification procedure in this study used partial denaturation with 3 M urea to separate the large subunits from the medium and small subunits of AP-2 complexes. Ahle and Ungewickell (1989) extended this study by using an additional purification step to show that isolated β2 subunits could bind to preformed clathrin cages. In that study, isolated β2 subunits did not drive clathrin coat formation.

In a parallel study, Prasad and Keen (1991) used refolded large subunits obtained from AP-2 complexes after complete denaturation with 8 M urea or 6 M GuHCl. They found that when isolated α subunits were mixed with soluble clathrin trimers, both proteins precipitated. Although no coat formation was observed, this was taken as evidence that α subunit can bind directly to clathrin. Pure β2 subunit was harder to obtain, but a mixture of α and β2 highly enriched in β2 was found to drive coat formation. Analysis of the resulting coats showed that α subunit was also present and that the ratio of α to β2 was higher than expected, approaching 1:1. This led to the proposal that α interacts directly with clathrin, but β2 is required for coat formation. In the light of our results, it seems more likely that β2 interacts directly with clathrin, but binds α subunits stoichiometrically when a subunit is available.

The model that β1 and β2 are responsible for binding clathrin is also attractive because these subunits have the largest degree of sequence homology of all the subunits in the AP complexes (Kirchhausen *et al.*, 1989). It is known that clathrin heavy chain interacts with AP complexes (Zaremba and Keen, 1983; Pearse and Robinson, 1984) and that only a single species of heavy chain is found in mammalian cells (Kirchhausen *et al.*, 1987). Thus, from a structural point of view it can be imagined that within a clathrin coat the surface structures and protein sequences in the large subunits of AP-1 and AP-2 complexes that bind the unique clathrin heavy chain should be highly related. Protein sequence comparisons between each of the four subunits of AP-1 complexes with the homologous subunits in AP-2 complexes showed that β1 and β2 subunits are the molecules with the highest sequence identity (Kirchhausen *et al.*, 1989). The β1 and β2 subunits display 84% identity along their entire length, with no gaps along the trunk or ear domains. Most of the sequence divergence is concentrated in a small portion of ~120 amino acids, corresponding to the protease-sensitive hinge region between the trunk and ear domains (58% identity and five gaps). In contrast, the α and γ subunits are only weakly related (Robinson, 1989, 1990). The sequence similarity between α and γ is concentrated in their N-terminal trunk domains and it requires the introduction of 25−28 gaps to optimize the otherwise low level of sequence identity (30%). The ear sequences are completely unrelated, as are those of the hinges. Thus, the observation that the β1 and β2 subunits are sufficient to bind clathrin heavy subunits and initiate coat formation fits well with the notion that the structures that interact with clathrin should be similar in AP-1 and AP-2.

Earlier studies showed that AP complexes drive coat formation only when their large subunits are intact (Zaremba and Keen, 1985). Preferential cleavage of the large subunits released the large subunit ears from the AP cores and completely abolished the ability of the remaining material to promote coat formation. Thus, if intact recombinant β2 subunits participate in coat assembly in a physiologically relevant way, similar concentrations of β2-trunks should not

be able to drive coat formation. Our results using intact and truncated recombinant β2 subunits are consistent with this model.

On the face of it, our results, as well as those from earlier studies, are at variance with a recent study proposing that ear domains are not necessary for coated vesicle formation driven by APs pre-bound to plasma membranes (Peeler *et al.*, 1993). Limited proteolysis of membrane-bound APs led to the release of α subunit ears; clathrin binding and vesicle budding were unaffected. However, because of the lack of an antibody against the ear domain of the β2 subunit, proteolysis of the β2 subunit was not followed. Thus, our results do not directly conflict with those mentioned above.

Although under the coat assembly conditions utilized here AP cores can bind to clathrin and stabilize clathrin coats, they cannot drive the formation of clathrin coats (Zaremba and Keen, 1985; Keen and Beck, 1989; Matsui and Kirchhausen, 1990). Because the ear domain alone does not associate with clathrin (Keen and Beck, 1989; Matsui and Kirchhausen, 1990; Keen *et al.*, 1991), there are two possibilities: either there are two weak clathrin binding sites, one in either the hinge or ear and another in the trunk, both of which are necessary for efficient binding, or there is a single clathrin binding site within the hinge region that is destroyed on proteolysis. Our experiments are performed at micromolar concentrations of both β2 subunit and clathrin, and therefore, if there are two sites, monomeric hinge/ear and trunk dissociation constants each need only be in the millimolar range. Interestingly, there appear to be two sites for AP interaction on clathrin heavy chain, one located at the terminal domain and the other somewhere in the rest of the leg (Keen *et al.*, 1991; Murphy and Keen, 1992).

Because the recombinant products used here are obtained from totally unfolded inclusion bodies, a note of caution is necessary. It is possible that β2-trunks refolded incorrectly during renaturation and that this could account for the difference in the behavior of intact and truncated β2 subunits. We believe that this possibility can be ruled out for two reasons. First, gel filtration analysis shows that recombinant β2-trunk is relatively compact, monomeric and soluble, with a Stokes radius similar to that of albumin, a globular protein with about the same molecular weight as the β2-trunk (67 kDa). Second, protease sensitivity of β2-trunks from intact and truncated recombinant β2 subunit products is similar, although their ability to drive coat formation is different. These β2-trunks are more readily proteolyzed than their equivalents within AP-2 cores, although the most favorable cleavage is still within the hinge region. The difference in protease sensitivity could simply be caused by protection of the native β2-trunk by the other subunits in the AP-2 core. Because the digestion pattern of the trunk domain of intact β2 subunit is similar to that of recombinant trunk, if the intact subunit is correctly folded, the recombinant trunk is probably similarly folded.

The results in this paper demonstrate that the β1 and β2 subunits are sufficient for coat formation. Although it is possible that other parts of the AP-1 and AP-2 complexes also bind clathrin, that the primary components responsible for binding appear to be the β1 and β2 subunits. Soluble AP complexes do not associate with clathrin trimers in the cytosol, however, and empty clathrin coats, lacking internalized membrane, are not observed within the cell. It is possible that the AP complexes may need to bind to a membrane to become competent to drive coated vesicle formation. Thus, other components within the complex are probably responsible, directly or indirectly, for membrane binding and for regulation of binding to clathrin.

Materials and methods

Construction of bacterial expression vectors for the β2, β2-trunk and β1 subunits

A DNA fragment corresponding to β2-trunk (nucleotides 1−1773) was amplified by PCR from a full-length cDNA cloned by us containing the complete open reading frame corresponding to a previously characterized β2 (formerly AP105b or βb) rat cDNA (Kirchhausen *et al.*, 1989) using the Vent DNA polymerase (New England Biolabs) and the two primers 5′-GGGCATATGACTGACTCCAAGTAC-3′ and 5′-CGAATTCACTATTTGCGATGAATGCCATGGC-3′. The underlined sequences are unpaired bases that were added to construct an *Nde*I restriction site (CA/TATG) at the 5′ end and two stop codons, followed by an *Eco*RI restriction site (G/AATTC) at the 3′ end of the amplified fragment. In the PCR, each of the 33 cycles included 1 min at 94°C, 1 min at 50°C and 3 min at 72°C, with a 7 min extension at 72°C of the final cycle. The amplified fragment was gel purified and ligated into the Bluescript (SK+) plasmid vector which was digested with *Sma*I. From this intermediate construct a 1779 bp fragment was cut out with *Nde*I and *Eco*RI and was ligated into the bacterial expression vector pET5a (Studier *et al.*, 1990) downstream of the T7 promoter to create pET5a:β2-trunk. pET5a:β2-trunk was used as starting material to generate pET5a:β2, the expression vector containing the full-length version of the cDNA coding for the β2 subunit (nucleotides 1−2853). The cDNA clone for β2 was cut with *Cla*I (at nucleotide 1376) and *Eco*RI in the 3′ untranslated region. This *Cla*I−*Eco*RI fragment containing the 3′ end of β2 was ligated into the pET5a:β2-trunk vector previously cut with *Cla*I and *Eco*RI.

The identity of the cDNA clone of the β1 subunit, formerly called AP105a or βa (Kirchhausen *et al.*, 1989), has been established by extensive sequence comparison with peptides from β1 derived from bovine brain AP-1 (Y.Nakayama, K.Clairmont and T.Kirchhausen, unpublished observations). This clone was cut with *Nde*I within the coding region (nucleotide 403) and *Sac*I in the 3′ untranslated region (nucleotide 3524). The resulting 3121 bp fragment was ligated into the bacterial expression vector pRSETc (Invitrogen) opened with *Nde*I and *Sac*I. The 5′ end of the β1 coding region including the *Nde*I site at position 403 was amplified by PCR using the two primers 5′-GGGCATATGACTGACTCCAAGTAC-3′ and 5′-TTTGCTACCACCATGGGG-3′. The underlined sequences are unpaired bases that were added to construct an *Nde*I restriction site (CA/TATG) at the 5′ end. The PCR and the subcloning of the amplified 541 bp fragment into Bluescript (SK+) were performed as described above. To generate the expression vector pRSETc:β1, a 405 bp fragment containing the 5′ end of β1 was cut out from the Bluescript (SK+) construct with *Nde*I and ligated into the *Nde*I opened pRSETc vector already containing the remaining 3′ end of β1.

All enzyme reactions and DNA preparations were performed as described by Sambrook *et al.* (1989).

Expression of recombinant β2, β2-trunk and β1 subunits and purification of inclusion bodies

The three expression vectors pET5a:β2, pET5a:β2-trunk and pRSETc:β1 were used to transform *E.coli* strain BL21(DE3), which has the gene for the T7 RNA polymerase integrated into its chromosome under the control of the inducible lacUV promoter (Studier and Moffatt, 1986). Cultures (1 l) of the transformed bacteria were grown in M9 medium (39 mM Na_2HPO_4, 22 mM KH_2PO_4, 8 mM NaCl, 18 mM NH_4Cl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) supplemented with 0.5% fructose, 2% casamino acids (Difco) and 100 μg/ml of ampicillin up to an OD_{600} of 0.7−0.8, and the expression of either β2, β2-trunk or β1 subunit was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h. The bacteria were harvested by centrifugation (5000 *g*, 15 min, 4°C) and the pellets (~6 g/l) were resuspended in 18 ml of lysis buffer (50 mM Tris−HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). 200 μl of 50 mM PMSF and 600 μl of lysozyme (10 mg/ml) were then added and the bacteria were stirred for 20 min at room temperature. The cells were lysed by adding 25 mg of deoxycholic acid while stirring continuously at 37°C for 30 min. When the lysate became viscous, $MgCl_2$ (final concentration 8 mM) and DNase I (final concentration 20 μg/ml) were added and the lysate was slowly stirred overnight at 4°C. Inclusion bodies were spun down (5000 *g*, 10 min, 4°C) and washed three times with 20 ml of 50 mM Tris−HCl, 100 mM NaCl, 2 mM EDTA, 2 mM DTT, 1% NP-40, pH 8.0, and twice with 20 ml of the same buffer in the absence of the detergent. The washed inclusion bodies were resuspended in 50 mM Tris−HCl, 100 mM NaCl, 2 mM EDTA, 2 mM DTT, pH 7.4, and kept in aliquots at −20°C.

Refolding of the recombinant β2, β2-trunk and β1 subunits

Inclusion bodies of the β2, β2-trunk and the β1 subunits at protein concentrations of ~20 mg/ml were solubilized by a 1:10 dilution into a buffer containing 100 mM Tris−HCl, 2 mM EDTA, 2 mM DTT, pH 8.0, in the presence of 6 M GuHCl and incubated overnight with continuous rotation at 4°C. Undissolved material was removed by ultracentrifugation (Beckman TLX) at 85 000 r.p.m. for 1 h at 4°C in a TLA-100.4 rotor. Refolding was performed in two steps by sequential 12−14 h dialysis against buffer I (2 M urea, 500 mM Tris−HCl, 2 mM EDTA, 2 mM DTT, pH 8.0) followed by buffer II (100 mM TEA, 100 mM NaCl, 2 mM EDTA, 2 mM DTT, pH 8.0) at protein concentrations of 100−200 μg/ml. Aggregated protein was removed by ultracentrifugation at 85 000 r.p.m. for 1 h at 4°C. Aliquots of the supernatant were either analyzed by gel filtration using a Superose 6 HR10/30 (Pharmacia) column at a flow rate of 0.5 ml/min or subjected to limited tryptic digestion. For all coat assembly assays, the solubilized recombinant subunits were dialyzed only against buffer I prior to mixing them with clathrin.

Purification of clathrin and its associated proteins

Calf brain coated vesicles were obtained by differential centrifugation in the absence of detergent (Keen *et al.*, 1979; Campbell *et al.*, 1984). Solubilization of clathrin and APs from coated vesicles and its purification by sizing chromatography on a Sepharose CL-4B column (Pharmacia) equilibrated with 500 mM Tris−HCl, 1 mM EGTA, 0.5 mM DTT, 0.02% NaN_3, pH 7.4, has been described previously (Matsui and Kirchhausen, 1990). Clathrin-containing fractions were pooled and concentrated by centrifugation (Centriprep 30, Amicon) up to 2.5−5.0 mg/ml. AP-containing fractions (highly enriched in AP-2 complexes) were pooled and either used directly for coat assembly or subjected to further purification by ion exchange chromatography on a Sepharose Q column (Pharmacia) (Ahle *et al.*, 1988; Matsui and Kirchhausen, 1990) to obtain fractions containing exclusively AP-2 complexes.

Clathrin cage binding assay

Clathrin cages were assembled in two steps by sequential 12−14 h dialysis of concentrated clathrin (2.5−3.0 mg/ml) against 20 mM MES, 2 mM DTT, pH 6.15, at room temperature, followed by dialysis against 20 mM MES, 2 mM $CaCl_2$, 2 mM DTT, pH 6.4 (pH adjusted at room temperature) at 4°C. Aggregated clathrin was removed by centrifugation in an Eppendorf centrifuge at top speed for 10 min at 4°C and assembled cages were separated from unassembled clathrin by ultracentrifugation at 85 000 r.p.m. for 10 min at 4°C in a TLA-100.4 rotor. Clathrin cages were gently resuspended at room temperature in 100 mM K^+-tartrate, 10 mM HEPES, 1 mM EGTA, 0.5 mM $MgCl_2$, 2 mM DTT, pH 7.0 (buffer T) (Schröder and Ungewickell, 1991) to a concentration of 1.5−2.0 mg/ml, and undissolved material was removed by low speed centrifugation as before. Clathrin cages (at a final concentration of 400 μg/ml) were mixed with AP-2 complexes (150 μg/ml) and recombinant refolded β2 (150 μg/ml) and β2-trunk (100 μg/ml) subunits previously dialyzed against buffer T. Binding was allowed to proceed for 30 min at 4°C. Clathrin cages were sedimented by ultracentrifugation in the TLA-100.4 rotor at 85 000 r.p.m. for 15 min at 4°C. Supernatant and pellet fractions were analyzed by SDS−PAGE in the presence of 6 M urea (Ahle *et al.*, 1988).

Clathrin coat assembly

Clathrin coats were reconstituted by mixing concentrated Tris-soluble clathrin coming from the Sepharose CL-4B sizing column with either Tris-solubilized AP complexes or with recombinant refolded AP subunits previously dialyzed against buffer I. The mixtures were then dialyzed overnight at 4°C against coat assembly buffer (buffer III: 100 mM MES, pH 6.4, 2 mM EDTA, 2 mM DTT). Dialysis of small volumes (200−500 μl) was performed in 1.5 ml micro test tubes (BioRad) whose opening was covered by a dialysis membrane. Aggregated material was removed by low speed centrifugation (12 000 *g*, 10 min, 4°C) and coats were separated from nonassembled protein by ultracentrifugation in a TLA-100 rotor at 60 000 r.p.m. for 10 min at 4°C. The pellets were resuspended at room temperature in coat assembly buffer. Samples were removed for analysis by electron microscopy. Tris, pH 8.0, at a final concentration of 200 mM was added to the remaining samples before analysis by SDS−PAGE.

Electron microscopy

Samples of clathrin coats were adsorbed for 1 min to freshly glow-discharged carbon coated copper grids, negatively stained with 1.5% uranyl acetate and observed with a JEOL 100-CXII electron microscope at 80 kV (Kirchhausen and Harrison, 1984). Pictures were taken at a nominal magnification of 36 000.

Acknowledgements

This work was supported by grants from the National Institutes of Health and March of Dimes to T.K. and fellowships from the Swiss National Science Foundation and the Roche Research Foundation to A.G.

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Received on August 6, 1993; revised on September 3, 1993