Criss, W. E., Yamamoto, M., Takai, Y., Nishizuka, Y., & Morris, J. P. (1978) *Cancer Res. 38*, 3532-3539.
Dahmus, M. E. (1981) *J. Biol. Chem. 256*, 3319-3325.
Feige, J. J., Pirollet, F., Cochet, C., & Chambaz, E. M. (1980) *FEBS Lett. 121*, 139-142.
Hathaway, G. M., & Traugh, J. A. (1979) *J. Biol. Chem. 254*, 762-768.
Hathaway, G. M., & Traugh, J. A. (1982) *Curr. Top. Cell. Regul. 21*, 101-127.
Hathaway, G. M., & Traugh, J. A. (1984a) *J. Biol. Chem. 259*, 2850-2855.
Hathaway, G. M., & Traugh, J. A. (1984b) *J. Biol. Chem. 259*, 7011-7015.
Hathaway, G. M., Lundak, T. S., Tahara, S. M., & Traugh, J. A. (1979) *Methods Enzymol. 60*, 495-511.
Hathaway, G. M., Lubben, T. H., & Traugh, J. A. (1980) *J. Biol. Chem. 255*, 8038-8041.
Jakobi, R., Voss, H., & Pyerin, W. (1989) *Eur. J. Biochem. 183*, 227-233.
Lin, W. J., Tuazon, P. T., & Traugh, J. A. (1991) *J. Biol. Chem. 266*, 5664-5669.
Mamrack, M. D. (1989) *Mol. Cell. Biochem. 85*, 147-157.
Meggio, F., & Pinna, L. A. (1984) *Eur. J. Biochem. 145*, 593-599.
Meggio, F., Brunati, A. M., & Pinna, L. A. (1983) *FEBS Lett. 160*, 203-208.
Meggio, F., Marchiori, F., Borin, G., Chessa, G., & Pinna, L. A. (1984) *J. Biol. Chem. 259*, 14576-14579.
Meggio, F., Brunati, A. M., & Pinna, L. A. (1987) *FEBS Lett. 215*, 241-246.
Morley, S. J., & Traugh, J. A. (1989) *J. Biol. Chem. 264*, 2401-2404.
Morley, S. J., & Traugh, J. A. (1990) *J. Biol. Chem. 265*, 10611-10616.
Mulner-Lorillon, O., Cormier, P., Labbê, J.-C., Dorée, M., Poulhe, R., Osborne, H., & Bellé, R. (1990) *Eur. J. Biochem. 193*, 529-534.
Oberholtzer, J. C., Buettger, C., Summers, M. C., & Matschinsky, F. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3387-3390.
Nakajo, S., Masuda, Y., Nakaya, K., & Nakamura, Y. (1988) *J. Biochem. 104*, 946-951.
Palen, E., Huang, T. T., & Traugh, J. A. (1991) *FEBS Lett. 264*, 12-14.
Sacks, D. B., Traugh, J. A., & McDonald, J. M. (1988) *J. Cell Biol. 107*, 54a.
Saxena, A., Padmanabba, R., & Glover, C. V. C. (1987) *Mol. Cell. Biol. 7*, 3409-3417.
Sommercorn, J., & Krebs, E. G. (1987) *J. Biol. Chem. 262*, 3839-3843.
Sommercorn, J., Mulligan, J. A., Lozeman, F. J., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8834-8838.
Tao, M., Conway, R., & Cheta, S. (1980) *J. Biol. Chem. 255*, 2563-2568.
Traugh, J. A., Lin, W. J., Takada-Axelrod, F., & Tuazon, P. T. (1990) in *The Biology and Medicine of Signal Transduction* (Nishizuka, Y., Endo, M., & Tanaka, C., Eds.) pp 224-229, Raven Press, New York.
Tuazon, P. T., & Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprotein Res. 23*, 123-164.

Interaction of Assembly Protein AP-2 and Its Isolated Subunits with Clathrin†

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ABSTRACT: The clathrin assembly protein complex AP-2 is a multimeric subunit complex consisting of two 100–115-kDa subunits known as α and β and 50- and 16-kDa subunits. The subunits have been dissociated and separated by ion-exchange chromatography in 7.5 M urea. Fractions highly enriched in either the α or β subunit were obtained. The α fraction interacted with clathrin as evidenced by its ability to bind to preassembled clathrin cages. It also reacted with dissociated clathrin trimers under conditions that favor assembly of coat structures, but did not yield discrete clathrin polygonal lattices. The enriched β fraction (containing small amounts of α) reacted with clathrin to yield intact coats with the incorporation of approximately equivalent amounts of α and β subunits into the polymerized species; excess free β subunit was unreactive. The AP-2 complex was also completely dissociated in a highly denaturing solvent, 6 M Gdn·HCl, and the constituent subunits of 100–115, 50, and 16 kDa were separated by gel filtration. In a coassembly assay with clathrin, the clathrin polymerizing activity was exclusively associated with the 100-kDa subunit fraction with stoichiometric incorporation of both α and β subunits of 100 kDa into the polymerized coats, and with no requirement for 50- or 16-kDa subunits. These observations demonstrate that the assembly activity of the complex is associated with the α and β subunits and suggest that both subunits, through independent interactions with clathrin, are required for expression of complete lattice assembly activity.

Clathrin coated vesicles are involved in a variety of cellular processes, the best characterized among them being receptor-mediated endocytosis (Brodsky, 1988; Goldstein et al., 1985). In addition to clathrin, the principal component, the coat of in vitro isolated coated vesicles consists of several other proteins known as assembly, associated, or adaptor proteins,

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referred to here as APs[1] [reviewed in Keen (1990) and Pearse and Crowther (1987)]. To date, four forms of the assembly proteins are known, and they have been designated as AP-1 (or HA I), AP-2 (or HA II) (Ahle et al., 1988; Keen, 1987; Manfredi & Bazari, 1987; Pearse & Robinson, 1984), AP-3 (or AP_{180}) (Ahle & Ungewickell, 1986; Murphy et al., 1991), and auxilin (Ahle & Ungewickell, 1990), the last two being confined to brain tissue only. Immunofluorescence studies revealed that AP-1 is predominantly in the Golgi region, whereas AP-2 has been localized to plasma membrane (Ahle et al., 1988; Robinson, 1987). Both AP-1 and AP-2 have also been reported to bind to cytoplasmic tails of receptors (Glickman et al., 1989; Pearse, 1988). While AP-3 and auxilin are monomeric proteins (Ahle & Ungewickell, 1986, 1990) with strong in vitro clathrin polymerizing activity, AP-1 and AP-2 are multimeric proteins possessing similar activity (Keen, 1987; Manfredi & Bazari, 1987; Pearse & Robinson, 1984). AP-1 consists of two 100-kDa subunits known as β and γ and probably one or two copies each of 47- and 19-kDa subunits. Similarly, AP-2 consists of two 100-kDa subunits designated as α and β and one or two copies each of 50- and 16-kDa subunits.

On the basis of the observation that the α subunit of AP-2 is localized to plasma membrane and the γ subunit of AP-1 is localized to Golgi and also that β and β' subunits of these two complexes share a number of common tryptic peptides, it has been suggested that the common domains in these two complexes that recognize clathrin are represented by β and β' subunits (Ahle et al., 1988). However, purification of the β subunit of AP-2 under mild dissociating conditions and a study of its properties revealed that it does not polymerize clathrin, though it did bind to preformed cages (Ahle & Ungewickell, 1989). Furthermore, recent studies in this laboratory have provided evidence for the interaction of AP-2 with two distinct binding sites on clathrin, raising the possibility that both large subunits of the AP-2 complex might interact with clathrin (Keen et al., 1991).

An examination of the amino acid sequences of the α and β subunits of AP-2 complex (Kirchhausen et al., 1989; Ponnambalam et al., 1990; Robinson, 1989) revealed that they possess considerable charge difference. We made use of this observation to fractionate them on an ion-exchange chromatography column after dissociating the subunits in concentrated urea solutions (7.5 M). We also fractionated the 100-kDa (α and β subunits together), 50-kDa, and 16-kDa subunits of AP-2 complex by gel filtration after dissociating them from each other in concentrated (6 M) guanidine hydrochloride (Gdn·HCl) solutions. From these two studies we have attempted to define the domain of AP-2 responsible for clathrin polymerization activity.

Experimental Procedures

Materials

Calf brains were obtained from a local slaughterhouse and processed immediately. MES, Tris, and guanidine hydrochloride (Gdn·HCl) were from Sigma. Urea was from Bethesda Research Laboratories. Hydroxylapatite (fast flow) was from Calbiochem, and DE-52 was from Whatman. All other chemical reagents were of highest quality available.

Methods

Purification of Coated Vesicles and AP-2 Assembly Protein Complex. Coated vesicles were purified by a slightly modified procedure of the existing methods (Keen et al., 1979; Nandi et al., 1982; Pearse, 1975) involving no gradients. For the purpose of obtaining pure clathrin and AP-2 protein complex, the method was quite satisfactory. Briefly, the method is as follows: six calf brains (1500 g) after removing the meninges and grey matter were mixed with an equal weight of preparation buffer (0.1 M MES–NaOH, 1 mm EGTA, 0.5 mM $MgCl_2$, and 0.02% sodium azide, pH 6.5) and homogenized in a Waring blender with three 15-s bursts at high speed. The homogenate was then centrifuged in a GS-3 rotor at 9000 rpm for 40 min. The supernatant containing CVs was pelleted in a 35 Ti rotor by centrifuging at 34000 rpm for 1 h. The pellets consisting of crude CVs were dispersed in preparation buffer (30 mL/pellet) by using a Dounce homogenizer with 8–10 strokes. The resulting suspension was clarified by centrifuging at 10000 rpm for 10 min in an SS-34 rotor. The supernatant was then centrifuged in a 35 Ti rotor at 34000 rpm for 1 h. The above steps of high- and low-speed centrifugation were repeated two more times. The final CV pellets were suspended in 0.5 M Tris-HCl, pH 7.0, overnight and then centrifuged at 100000*g* for 3 h. The resulting supernatant containing clathrin and APs was precipitated with ammonium sulfate and centrifuged at 10000*g* for 30 min. The pelleted protein was resuspended in 0.5 M Tris-HCl, pH 7.0, and was then gel filtered on a Superose-6 column (2.6 × 59.5 cm) equilibrated in the same buffer. Elution of the fractions was done at a flow rate of 0.5 mL/min.

The AP-containing fractions were pooled and adsorbed directly onto a hydroxylapatite column (0.5 × 6 cm) equilibrated in the same buffer. The column was then washed with 150 mM sodium phosphate buffer containing 0.5 M Tris-HCl, pH 7.0. After washing with 4–6 column volumes, the column was eluted with 0.5 M sodium phosphate containing 0.5 M Tris-HCl, pH 7.0. AP-2 eluted with greater than 95% purity, and the yield was typically about 0.4 mg/100 g of calf brain tissue.

AP-2 Fractionation in Gdn·HCl. AP-2 was dialyzed against 6 M Gdn·HCl, 0.1 M Tris-HCl, and 2 mM DTT, pH 8.0, and loaded on a Superose-6 column (1.8 × 56 cm) equilibrated in the same buffer. Fractions were eluted at a rate of 0.25 mL/min. Fractions containing 100- and 50-kDa subunits were separately pooled and dialyzed into 2 M urea, 0.1 M Tris-HCl, and 2 mM DTT, pH 8.0, before electrophoresis or clathrin assembly assay.

AP-2 Fractionation in Urea. AP-2 fraction (4 mg in 8 mL of solution) was dialyzed into 7.5 M urea, 0.02 M Tris-HCl, and 2 mM DTT, pH 8.0, and loaded onto a DE-52 column (0.5 × 1.6 cm) equilibrated with the same buffer. The column was then washed with 10 mL of the same buffer first, and then in ~15–20 mL each of the same buffer but containing 50 mM, 80 mM, and then 0.5 M Tris-HCl, successively. Eluted fractions (0.75 mL/fraction) were dialyzed separately into 2 M urea and 0.1 M Tris-HCl, pH 8.0, buffer and used as such.

Coat Assembly. Coat assembly was assayed essentially as described previously (Keen, 1987) except that gradients containing 5–20% glycerol (v/v) in 0.1 M MES–NaOH, pH 6.5, were centrifuged at 27000 rpm for 90 min at 4 °C in an SW 28.1 rotor (Beckman Instruments Inc.). In one instance, 10–30% sucrose gradients (w/w) in the same buffer were employed, in which case the samples were centrifuged for 110 min. Typically, 800–1000 μL of sample was layered on gra-

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; DE-52, (diethylaminoethyl)cellulose; AP, assembly protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CV, coated vesicle.

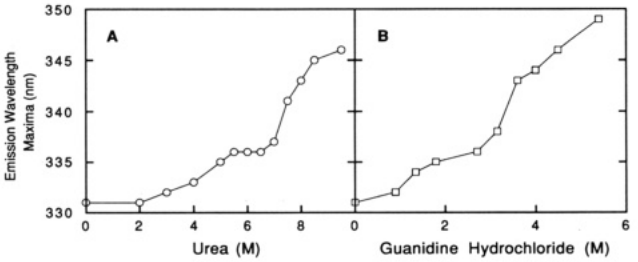

FIGURE 1: Denaturation profiles of AP-2 complex in concentrated urea and Gdn·HCl solutions as probed by fluorescence emission maxima. A concentrated solution (1–2 mg/mL) of AP-2 complex in 0.1 M Tris-HCl, pH 8.0, was diluted with a concentrated solution of urea (10 M) or Gdn·HCl (8 M) in the same buffer to give the desired final concentration of the denaturant. Panel A, urea-induced denaturation; panel B, Gdn·HCl-induced denaturation.

dients before centrifugation. Protein concentration was estimated by measuring the intrinsic tryptophan fluorescence by exciting at 280 nm and reading the emission at 331 nm.

Cage Binding Assay. Clathrin cages (3 mg/mL) were made by dialyzing pure clathrin against 0.1 M MES–NaOH, pH 6.0, overnight at 4 °C. A small aliquot of the cages was then diluted into 0.2 M MES–NaOH, pH 6.5, buffer to a final concentration of 0.3–0.4 mg/mL. Assembly protein components, $\alpha_{a/c}$ or β (see Results), at a protein concentration of 60–130 μg/mL, were dialyzed against 0.01 M Tris-HCl, pH 8.0, overnight to remove urea. Equal volumes of cages and assembly protein components were mixed and incubated for 30 min before layering on 5–20% glycerol gradients. Further details are provided in Keen et al. (1991). Gradients were spun at 27000 rpm for 1 h at 4 °C in a SW28.1 rotor. One-milliter fractions were collected and assayed for protein concentration by measuring the intrinsic tryptophan fluorescence.

Fluorescence Measurements. Fluorescence intensities were measured in a Perkin-Elmer 650-10S spectrofluorometer. Spectra in urea and Gdn·HCl solutions (excitation wavelength 280 nm) were recorded after incubating the samples in these buffers for 15 min; protein concentration was 50 μg/mL. Relative protein concentrations in other experiments were determined by means of the intrinsic tryptophan fluorescence by exciting at 280 nm and measuring the emission at 331 nm.

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was done as described previously (Keen & Beck, 1989). Seven and a half percent polyacrylamide gels were used. For separation of α and β subunits in AP-2 complex, the resolving gel contained 6 M urea in addition to the other components (Ahle et al., 1988).

Results

Quaternary and tertiary structural interactions among the proteins making up the assembly protein complex AP-2 were probed by fluorescence emission spectra in concentrated denaturant solutions of urea and Gdn·HCl. The emission spectrum of the complex when excited at 280 nm has a peak at 331 nm typical of most native proteins containing buried tryptophan and tyrosine residues. The spectrum shifts to 346 nm in 9.5 M urea and to 350 nm in 6 M Gdn·HCl, indicating a generally complete exposure of the residues presumably caused by complete denaturation of the proteins. The concentration dependence of the emission spectra on urea and Gdn·HCl concentration shows two structural transitions, one below 6 M urea or 3 M Gdn·HCl and the other above 6 M urea or 3 M Gdn·HCl solutions (Figure 1). In either case removal of denaturant by dialysis resulted in emission spectra indistinguishable from that of the native protein, indicating

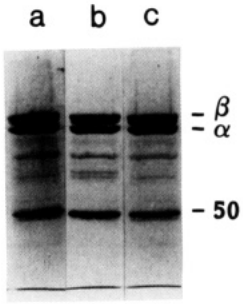

FIGURE 2: Protein composition of urea and Gdn·HCl-treated AP-2 complex. AP-2 (200 μg/mL) in 0.5 M Tris-HCl, pH 8.0, was dialyzed into 0.1 M Tris-HCl, pH 8.0 (a), 7.5 M urea and 0.1 M Tris-HCl, pH 8.0 (b), or 0.1 M Tris-HCl and 6 M Gnd·HCl, pH 8.0 (c), for 12 h and then dialyzed back into 2 M urea and 0.1 M Tris-HCl, pH 8.0, for 18 h. Samples were spun for 7 min in TL-100 at 70000 rpm to remove any aggregated material. The positions of the resolved α and β polypeptides and 50-kDa subunit are indicated at right.

that by this criterion the protein had refolded. However, a detailed study in urea solutions indicated that while the transition below 6 M urea is completely reversible with respect to the solubility of the proteins and complete recovery of clathrin polymerizing ability of the complex, the transition above 6 M urea is reversible only to an extent of 60–80% with respect to the solubility of the proteins and clathrin polymerizing activity of the complex (data not shown). It has been shown earlier that the subunits in AP-2 complex dissociate in 3 M urea solutions and in this condition were partially separable (Ahle & Ungewickell, 1989; Prasad et al., 1986). It therefore appears likely that the first transition corresponds to the dissociation of the proteins of the AP-2 complex, probably also accompanying some degree of polypeptide unfolding.

Fractionation in 7.5 M Urea Solutions. Attempts at fractionating the proteins of AP-2 complex in solutions containing less than 6 M urea using either hydroxylapatite or DE-52 were unsuccessful in yielding a homogeneous preparation of the α subunit, though the β fraction could be obtained in completely pure form. Accordingly, we chose a urea concentration of 7.5 M to dissociate the proteins of the AP-2 complex and fractionate them. Typically, AP-2 was dialyzed against 7.5 M urea, 0.02 M Tris-HCl, and 2 mM DTT, pH 8.0, for 15 h. A small aliquot was dialyzed back into 2 M urea, 0.1 M Tris-HCl, and 2 mM DTT, pH 8.0, to evaluate the reversibility of denaturation of the dissociated and denatured proteins. About 80% of the total protein remained soluble and closely resembled native AP-2 in its composition when examined on SDS gel electrophoresis (Figure 2), indicating that none of the subunits had been preferentially lost. Clathrin in 2 M urea and 0.1 M Tris-HCl, pH 8.0, was added to this fraction, and assembly into coats was tested by glycerol gradient analysis after dialysis into 0.1 M MES–NaOH, pH 6.5, buffer. When the protein fractions were analyzed, a peak of coats comparable to the control appeared in the gradients, and examination of the polypeptide composition of the reassembled coats by SDS gel electrophoresis revealed incorporation of the $\approx$100-kDa α and β subunits along with slightly lower amounts of 50- and 16-kDa polypeptides (data not shown). This served as a control experiment indicating that if fractionation of the proteins of the AP-2 complex is done under these conditions, reassembly of a functional complex should be possible with the fractionated proteins.

After dialysis of AP-2 (4 mg in 8 mL) into 7.5 M urea, 20 mM Tris-HCl and 2 mM DTT, pH 8.0, it was applied to a DE-52 column equilibrated in the same buffer (Figure 3A). Approximately 400–600 μg of protein eluted in the flow

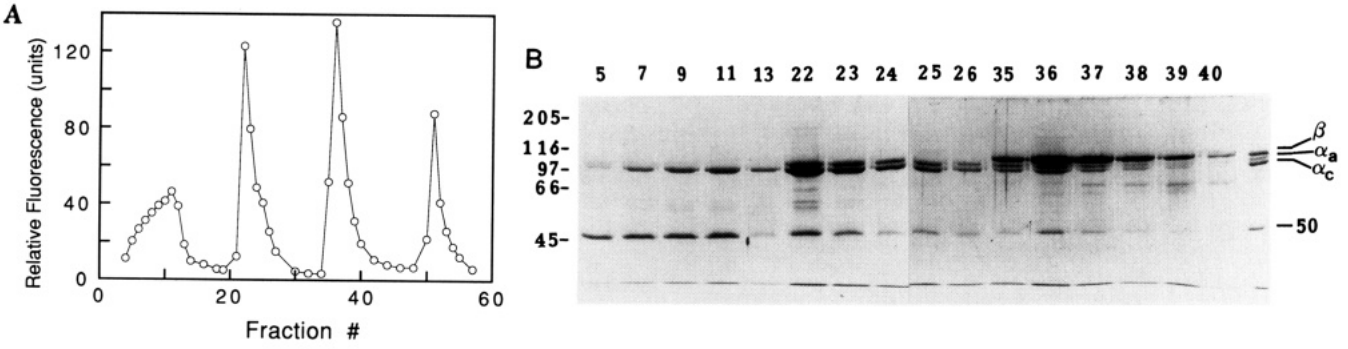

FIGURE 3: Fractionation of AP-2 into α- and β-containing fractions by DE-52 ion-exchange chromatography in 7.5 M urea solutions. AP-2 in 20 mM Tris-HCl and 7.5 M urea, pH 8.0, solution was loaded onto a DE-52 column equilibrated in the same buffer (as described under Methods), and elution was performed with increasing concentrations of Tris-HCl (in 7.5 M urea). Fractions of 750 μL were collected, and protein concentration was monitored by tryptophan fluorescence. (A) Elution profile of the column fractions. Fractions 0–18 correspond to flow through as well as column wash in 20 mM Tris-HCl; fractions 19–35 correspond to elution with 50 mM Tris-HCl; fractions 36–48 correspond to elution with 80 mM Tris-HCl; and fractions 49–57 correspond to elution with 0.5 M Tris-HCl. (B) SDS gel electrophoretic analysis of the column fractions. Fraction numbers corresponding to the lanes are indicated on the top of the gel. The extreme left lane indicates the protein molecular weight markers, and the extreme right lane indicates the AP-2 preparation applied to the column. The α, β, and 50-kDa polypeptides of AP-2 are indicated at the right end of the gel.

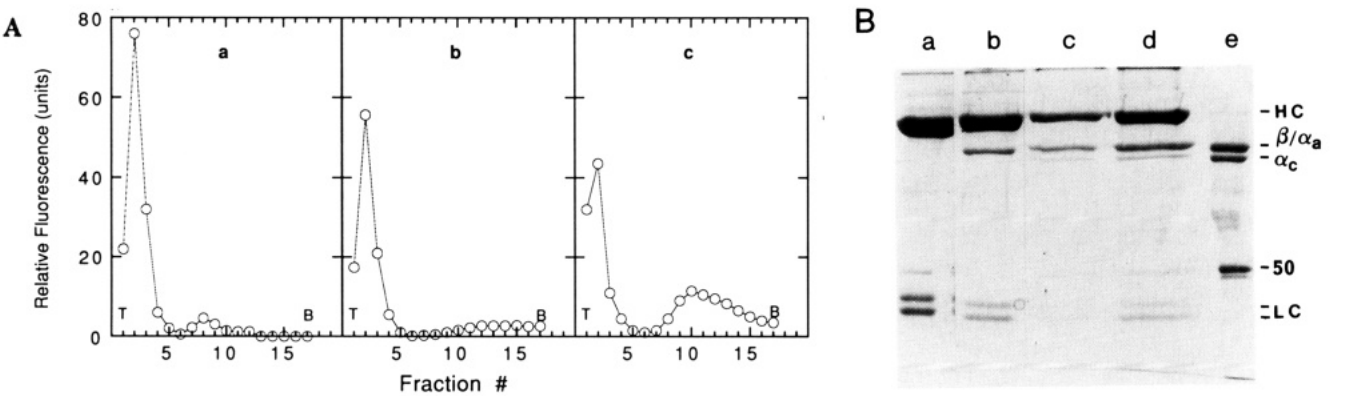

FIGURE 4: Assembly of clathrin by $\alpha_{a/c}$ and β fractions. $\alpha_{a/c}$ and β fractions were separately pooled (as described in the text) and mixed with clathrin in 2 M urea and 0.1 M Tris-HCl, pH 8.0, and dialyzed against 0.1 M MES-NaOH, pH 6.5, overnight. (A) Aliquots were analyzed by glycerol gradient centrifugation as described under Methods. Clathrin alone (380 μg) (panel a), clathrin (380 μg) plus $\alpha_{a/c}$ (110 μg) (panel b), and clathrin (380 μg) plus β (120 μg) (panel c). Note the clearly discernible coat peak with β. Also to be noted is the reduction in the clathrin peak in the gradients with $\alpha_{a/c}$ in comparison with clathrin control. (B) Analysis of unsedimented protein and assembled coats by SDS gel electrophoresis. Lane a, Unsedimented protein of gradient with $\alpha_{a/c}$ fraction (panel a, fraction 2); lane b, unsedimented protein of gradient with β (panel b, fraction 2); lanes c and d, at two loadings, coat fractions of gadient with β (panel b, fractions 10–12); lane e, AP-2 complex used as a standard.

through. This contained mostly the 50-kDa and α_c subunits (Figure 3B). After washing the column with 3–4 column volumes of buffer, the column was eluted with 7.5 M urea, 50 mM Tris-HCl and 2 mM DTT, pH 8.0. Another 600–800 μg of the protein eluted with this buffer, and these fractions were devoid of β but contained mostly α_a and α_c with some 50- and 16-kDa subunits. Again after substantial washing of the column with this buffer, the salt concentration was raised to 80 mM Tris-HCl, in the same buffer. At this stage β subunit containing fractions totaling about 600–800 μg with variable amounts of α subunits eluted. The initial fractions (e.g., 35) had a large proportion of α, while the latter fractions (e.g., 36 and following) contained at most 10% of α in β. After prolonged elution with this buffer, the column was eluted with 7.5 M urea, 500 mM Tris-HCl, and 2 mM DTT, pH 8.0, to see whether anything still remained bound to the column. About 400–600 μg of protein eluted (Figure 3A, fractions 50 and following), containing polypeptides indistinguishable from the native AP-2 complex together with several minor contaminants (data not shown). Why this fraction bound to the column without eluting in lower salt concentrations is not clear. The pattern obtained has been reproduced several times, with only minor variation from one fractionation to the other. The procedure is thus successful in yielding three fractions: an α_c and 50-kDa subunit fraction (denoted α_c/50); another α fraction with α_a, α_c, and 50- and 16-kDa subunits but devoid of β (denoted $\alpha_{a/c}$); and finally a β fraction with only minor amounts of α contamination (termed β). In subsequent experiments described here only β fractions with the lowest amounts of α (e.g., 36 and following in Figure 3B) were used.

Interactions with Clathrin. The α_c/50, $\alpha_{a/c}$, and β fractions were separately pooled and dialyzed into 2 M urea, 0.1 M Tris-HCl, pH 8.0, and 2 mM DTT to lower the urea concentration to a level that has previously been shown to be compatible with clathrin in assembly assays (Prasad et al., 1986; Schook et al., 1979; Woodward & Roth, 1978). Since our interest at the moment is in determining the domain of AP-2 responsible for polymerization of clathrin, various amounts (110–120 μg) of these fractions (with the exception of the α_c/50 which became turbid and hence could not be evaluated), after centrifuging at 100000g to remove the aggregated materials, were mixed with clathrin (380 μg) and dialyzed into 0.1 M MES–NaOH, pH 6.5, overnight. Aliquots were layered on 5–20% glycerol gradients, and assembly was examined. Figure 4A shows the gradient patterns. Clathrin alone generated only a negligible amount of assembled structures (Figure 4A, panel a). In the gradients with $\alpha_{a/c}$ (Figure 4A, panel b), substantial amounts of clathrin were

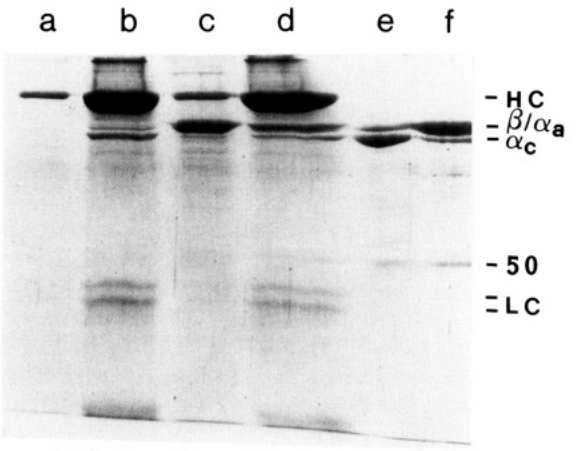

FIGURE 5: Binding of $\alpha_{a/c}$ and β to preassembled cages. Clathrin cages alone (200 μg/mL) or cages (200 μg/mL) with $\alpha_{a/c}$ (30 μg/mL) or β fractions (65 μg/mL) were incubated and analyzed by glycerol gradient sedimentation and SDS gel electrophoresis. Lanes a and b: Unsedimented and cage peak fractions, respectively, from gradient with $\alpha_{a/c}$. Lanes c and d: Unsedimented and cage peak fractions, respectively, from gradient with β. Note that a substantial amount of β remains unsedimented. Lanes e and f: $\alpha_{a/c}$ and β preparations. Migration positions are indicated at the right (note that α_a and β were not resolved at this loading); HC and LC indicate positions of clathrin heavy and light chains, respectively.

removed from the unpolymerized pool remaining at the top of the gradient and were found to have pelleted, but no discernible peak of coats was detected on the gradients. In contrast, in the gradients with β (containing minor amounts of α) a substantial peak corresponding to coat structures appeared while the clathrin peak became smaller (Figure 4A, panel c). Examination of the top of the gradients and peak fractions corresponding to coats by SDS gel electrophoresis revealed that, in the case of the $\alpha_{a/c}$ fraction, no α protein remained at the top of the gradient (Figure 4B, lane a). In the case of the β fraction, the reassembled coats were found to contain both α and β polypeptides (Figure 4B, lanes c and d). The ratio of these polypeptides was quite distinct from that of the starting β fraction (which had minimal amounts of α: Figure 3B, fraction 37) but was similar to that in coats prepared with native AP. Also, unincorporated protein remaining at the top of the gradient contained β but was devoid of α (Figure 4B, lane b). As an internal control the material eluted with 0.5 M Tris-HCl, pH 8.0, 7.5 M urea, and 2 mM DTT (Figure 3A, fraction 52) was examined for polymerization activity. This fraction is completely active like the native AP-2 complex, suggesting that neither the DE-52 column nor treatment with 7.5 M urea irreversibly destroyed the activity of the AP-2 complex (data not shown). This was consistent with the other control experiment performed with unfractionated AP-2 in 7.5 M urea solution, referred to earlier.

In view of the indication that the $\alpha_{a/c}$ fraction did interact in some way with clathrin although it did not promote coat assembly activity, we evaluated its interaction with clathrin using two additional approaches. First, by use of a cage binding assay reported in more detail elsewhere (Keen et al., 1991), the $\alpha_{a/c}$ preparation was found to bind to preassembled clathrin cages, detected as a reproducible shift in the sedimentation profile of the cages (see supplementary materials, Figure 1) and by gel electrophoresis of the shifted peak (Figure 5, lane b). As a control, the β-rich fraction was also evaluated for its cage binding activity. This fraction also produced a detectable shift of the cages on the gradients. Interestingly, SDS gel electrophoretic comparison of the unbound protein (Figure 5, lane c) and shifted peak (Figure 5, lane d) showed that relatively stoichiometric amounts of β and α bound to the cages while excess β subunit remained unbound. This pattern is similar to that seen in the coat assembly experiment (Figure 4).

To further probe the interactions between clathrin trimers and the $\alpha_{a/c}$ fraction that occur under assembly conditions, the two preparations in 0.01 M Tris-HCl, pH 8.0, were mixed and rapidly diluted into assembly buffer 0.1 M MES–NaOH, pH 6.5. When analyzed by negative staining electron microscopy, roughly globular particles of approximately 15–25 nm and aggregates thereof were detected. By virtue of their apparent surface uniformity these particles were readily distinguishable from true clathrin lattices formed by mixing clathrin and native AP-2 and were absent from preparations of either pure clathrin, native AP-2, or the $\alpha_{a/c}$ fraction (data not shown).

Thus, by several criteria the $\alpha_{a/c}$ preparation is capable of binding to both free clathrin trimers and assembled cages, although under the present conditions it alone does not display the complete assembly activity of the parent AP-2 complex. The isolated β subunit has similar properties, from studies described here and previously published work (Ahle & Ungewickell, 1989). However, we observe important differences between the $\alpha_{a/c}$ and β fractions. The $\alpha_{a/c}$ fraction readily aggregated under clathrin polymerization conditions (0.1 M MES–NaOH, pH 6.5), similar to native AP-2 which we have shown undergoes a specific and reversible self-association reaction under these conditions (Beck & Keen, 1991); in contrast, the β fraction remained totally soluble. Furthermore, the $\alpha_{a/c}$ fraction binds and aggregates synthetic liposomes as does the native AP-2 molecule (Beck and Keen, manuscript in preparation); the β fraction again does not promote this reversible reaction. The similarity in the behavior of the isolated $\alpha_{a/c}$ and the native AP-2 protein in these respects provides support to the view that the $\alpha_{a/c}$ fraction purified here is in a native conformation.

Fractionation of AP-2 Complex into 100-, 50-, and 16-kDa Subunits. (a) Denaturation and Renaturation of the AP-2 Subunits. In the above experiments the proteins making up the AP-2 complex were broadly fractionated into α and β fractions with variable amounts of 50- and 16-kDa proteins associated with them, particularly in the case of the α fraction. Do the 50- and 16-kDa subunits play a required role in the assembly of α and β subunits into the native AP-2 complex, or in the clathrin polymerization activity of AP-2? To address these questions, we sought to fractionate the AP-2 polypeptides by size, using 6 M Gdn·HCl to fully dissociate the complex. As shown earlier (Figure 1), the fluorescence emission spectrum of AP-2 in 6 M Gdn·HCl corresponds to that of unfolded protein and therefore likely reflects full dissociation of the polypeptides. However, before fractionating them, we determined whether the denatured and dissociated proteins would recombine in a renatured form and be able to induce polymerization of clathrin. In several different experiments, 100–150 μg/mL of AP-2 was dialyzed overnight into 6 M Gdn·HCl, 0.1 M Tris-HCl, and 2 mM DTT, pH 8.0, and the denatured sample was then dialyzed against 2 M urea, 0.1 M Tris-HCl, and 2 mM DTT, pH 8.0, to remove Gdn·HCl. About 20–25% of the sample was lost when the resulting sample was briefly centrifuged at 100000*g* to remove aggregated protein. The soluble renatured AP-2 complex had a similar composition to that of the native AP-2 complex (FIgure 2, compare lanes a and c). Clathrin was added to the renatured AP-2 complex, the total sample was dialyzed into 0.1 M MES–NaOH, pH 6.5, buffer, and the polymerization was assayed by gradient analysis and SDS gel electrophoresis. The extent of assembly

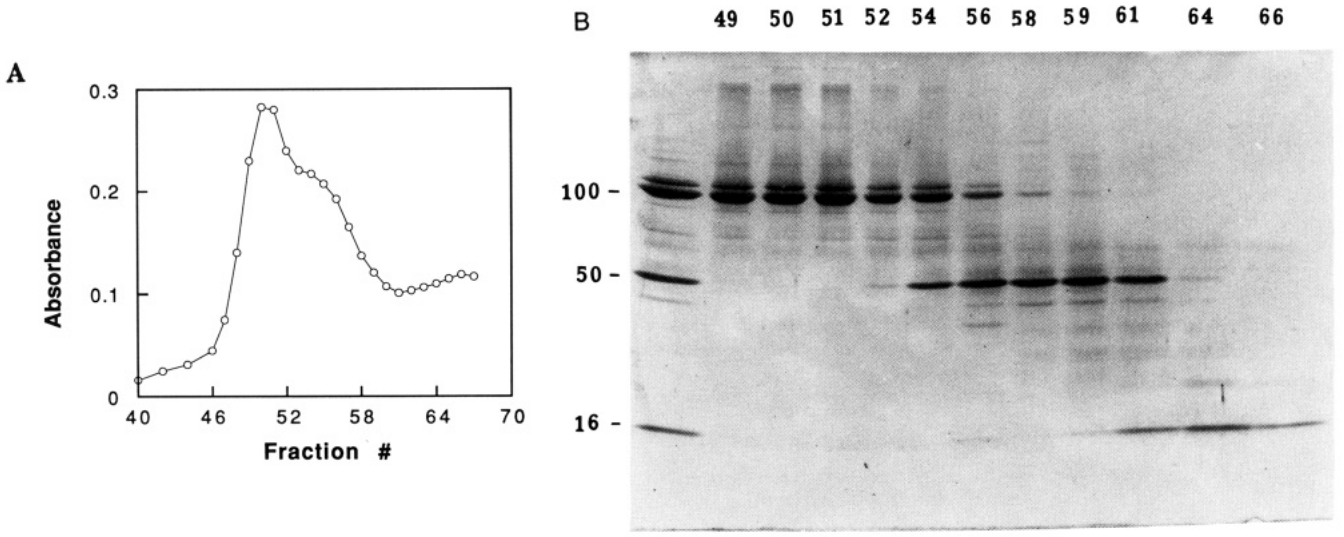

FIGURE 6: Separation of AP-2 subunits by gel filtration in 6 M Gdn·HCl solution. AP-2 complex in 6 M Gdn·HCl, 0.1 M Tris-HCl, pH 8.0, and 2 mM DTT was gel filtered on Superose-6 equilibrated in the same buffer as described under Methods. Fractions of 1.5 mL were collected. (A) Elution profile. Absorbance at 280 nm is plotted as a function of the fraction number of the column eluate. (B) SDS gel electrophoretic pattern of the column profile; fraction numbers denoted at top. The extreme left lane is the AP-2 sample loaded on the column. The molecular weight markers are indicated on the left. This gel does not contain 6 M urea and hence does not resolve the α and β subunits.

of the 6 M Gdn·HCl-treated protein was very similar to that of the native AP-2 complex, and gel electrophoresis revealed incorporation of substantial levels of the 100-kDa α and β subunits into the coat structures, with variable and often lesser amounts of the 50- and 16-kDa subunits (data not shown but included during review).

Having thus ascertained that the assembly activity of the complex could be regained after treatment with high concentrations of Gdn·HCl, we then dialyzed about 3–5 mg/mL AP-2 into 6 M Gdn·HCl, 0.1 M Tris-HCl, and 2 mM DTT, pH 8.0, and subjected the sample to gel filtration on a Superose-6 column equilibrated in the same buffer. A major peak (fractions 49–52), a broad trailing shoulder (fractions 53–61), and a minor shoulder in the leading side of the bed volume peak (fractions 61–66; particularly evident when measured by fluorescence) were recovered (Figure 6A). SDS gel electrophoretic analysis demonstrated that the first peak contained 100-kDa subunits, the trailing shoulder contained the 50-kDa subunit, and the small shoulder contained the 16-kDa subunit (Figure 6B). Fractions containing purified 100-kDa (fractions 49–51) and 50-kDa (fractions 58–60) subunits were pooled separately and dialyzed into 2 M urea, 0.1 M Tris-HCl, and 2 mM DTT, pH 8.0. A small amount of protein aggregated in each case, which was removed by briefly centrifuging at 100000*g*.

(*b*) *Polymerization Activity*. Clathrin was added to separate aliquots of the 100- and 50-kDa subunit fractions (all in 2 M urea and 0.1 M Tris-HCl, pH 8.0), and the resulting samples were dialyzed against 0.1 M MES–NaOH, pH 6.5. When these samples were analyzed by sucrose gradients, a distinct peak corresponding to assembled coats was obtained with fractions corresponding to the 100-kDa proteins and there was a corresponding reduction in unpolymerized protein remaining at the top of the gradient (see supplementary material, Figure 2). No difference was found with the 50-kDa subunit fraction relative to the clathrin control (data not shown). SDS gel electrophoretic analysis of the peak fractions confirmed the assembly of coat structures by revealing the presence of 100-kDa subunits, with stoichiometric incorporation of both α and β subunits (Figure 7). In the case of the 50-kDa subunit fraction, substantial amounts of it remained at the top of the gradient (not shown). The effect of the 16-kDa subunit could not be reliably evaluated in view of its availability in only low concentrations.

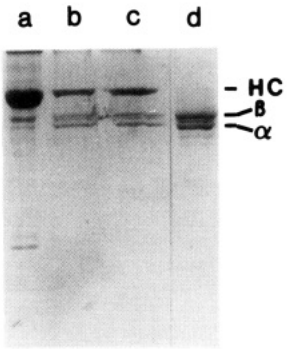

FIGURE 7: 100-kDa subunits of the AP-2 complex polymerize clathrin into coats. Fractions containing 100-kDa subunits (165 μg) obtained by Superose-6 gel filtration were pooled and dialyzed against 2 M urea to remove Gdn·HCl. The renatured protein was mixed with clathrin (375 μg) and dialyzed against 0.1 M MES–NaOH, pH 6.5. As a control, clathrin alone (375 μg) was dialyzed against the same buffer. The two samples were analyzed by sucrose gradient centrifugation and SDS gel electrophoresis as described under Methods. Lane a, unsedimenting protein; lanes b and c, assembled coats from two adjacent portions of the peak of the gradient, lane d, 100-kDa fraction used for assembly. On the right side of the gel, the clathrin heavy chain (HC) and α and β subunit polypeptides are marked. This gel contained 6 M urea to resolve α and β subunits.

Discussion

One of the goals of the present study is to more precisely understand the functional organization of the clathrin assembly protein complexes AP-2 and AP-1. These proteins have analogous structures with each containing two larger ≈100-kDa subunits and two smaller subunits of 50 or 47 kDa and 16 or 19 kDa. The two ≈100-kDa subunits in AP-2 are designated as β and α while the ones in AP-1 are designated as β' and γ, in each case the former representing the slowly moving protein and the latter the faster moving protein on urea–SDS gels (Ahle et al., 1988). α subunit has been shown to be localized to plasma membrane whereas γ was exclusively identified with Golgi membranes (Ahle et al., 1988; Robinson, 1987). Further, it was shown through tryptic mapping that α and γ subunits are completely unrelated whereas β and β' subunits share a common number of peptides and also share

a common property of being localized to both plasma and Golgi membranes (Ahle et al., 1988). On the basis of this evidence Ahle et al. speculated that the common subunits in AP-2 and AP-1 that attach these complexes to clathrin are β and β'. However, purification of the β subunit of the complex AP-2 after its dissociation in 3 M urea revealed that it itself had no polymerization activity, though it did bind to preformed cages (Ahle & Ungewickell, 1989).

If β subunit does not polymerize clathrin, then what is the polymerization inducing domain in the AP-2 complex? Does it consists of α subunit(s) alone or both α and β subunits? To address these questions, we took an approach complementary to that of earlier studies and fractionated α subunit(s) in a highly denaturing medium (7.5 M urea) where a substantial degree of dissociation of the subunits of the AP-2 complex can be expected. We also obtained a fraction that is highly enriched in β subunit but had a small amount of α subunit(s) associated with it. Prior to the fractionation it was ascertained that the unfractioned but renatured proteins retained clathrin polymerization activity. In the reassembly experiments involving purified fractions, it was found that the purified α subunit interacts with clathrin in a number of different assays but does not give rise to discrete coat structures. Confirming the results of Ahle and Ungewickell (1989), it was also found in other experiments that β subunit did not induce clathrin polymerization. However, fractions containing both β and α subunits did polymerize clathrin into discrete coats, with approximately equivalent incorporation of β and α subunits into coats. Excess β subunit remained unreactive at the top of the gradients.

The lack of detectable polymerization seen with the α fraction as compared with that caused by the β-rich fraction may be explained in several ways. (1) The α fraction is irreversibly denatured. However, by several criteria this fraction appears to retain discrete clathrin binding interactions. Furthermore, the fluorescence spectrum of the renatured α fraction has an emission maximum of 331 nm, similar to that of the native AP-2 complex, indicating a substantial degree of renaturation. (2) Inactive and active forms of α exist, corresponding to the α_a/α_c fraction and that in the β fraction, respectively. However, as a large fraction of the AP-2 was active in polymerizing clathrin after treatment with 7.5 M urea, and the amount of α in the β fraction constitutes only a small percentage of the total, this possibility is extremely unlikely. The only view we favor at this time is that while α subunit(s) can independently interact with clathrin, a stoichiometric amount of β subunit is required to promote the assembly reaction.

The above tentative conclusion is also consistent with structural information obtained so far by electron microscopy (Heuser & Keen, 1988), cDNA cloning (Kirchhausen et al., 1989; Ponnambalam et al., 1990; Robinson, 1989), proteolytic studies (Kirchhausen et al., 1989; Zaremba & Keen, 1985), and binding to immobilized clathrin (Keen & Beck, 1989). These studies indicated that AP-2 has a symmetrical structure with the N-terminal end of both α and β subunits along with 50- and 16-kDa proteins forming the larger core portion of the structure. The C-terminal ends point away from the central mass of the molecule. The core containing the larger domains of the α and β subunits along with intact 50- and 16-kDa subunits is found to retain the property of binding to immobilized clathrin trimers and preformed clathrin cages but had no assembly activity (Keen & Beck, 1989; Zaremba & Keen, 1985). Proteolysis of both the α and β subunits also yields similar results in that both are clipped to yield 60–66- and 30–40-kDa fragments, with cleavage in regions that are mostly acidic in both sequences (Kirchhausen et al., 1989).

It therefore is logical to hypothesize that polymerization in some way involves a domain of AP-2 that retains the symmetric dimeric nature of the original complex. As our results reported here provide the first evidence that isolated α subunits interact with clathrin trimers, and since it has been shown that β subunit does not interact with unassembled clathrin, we propose that the interaction of α subunit with clathrin precedes that with β, though both α and β subunits are required for correct polymerization of clathrin. The binding studies of β subunit or AP-2 to preformed cages may be reflecting the latter part of the polymerization process. This view is supported by the observation that assembled clathrin cages and free clathrin triskelions manifest two distinct binding interactions with AP-2, as reported elsewhere (Keen et al., 1991).

One of us earlier reported the purification of a 114-kDa subunit of the AP-2 assembly protein complex after its dissociation in 3 M urea (Prasad et al., 1986). As this urea concentration can give at least partial dissociation of all the subunits of the AP-2 complex (Ahle & Ungewickell, 1989), we suspect that both α and β subunits were present, resulting in expression of assembly activity and the poor agreement with a single-component system by sedimentation equilibrium analysis that was observed.

Our fractionation in 6 M Gdn·HCl solution also shows that the fractions containing α and β subunits with no 50- or 16-kDa subunit can induce clathrin polymerization. The composition of the renatured and active species in polymerizing clathrin indicated a stoichiometric nature of interaction of α and β proteins, thus reinforcing the earlier observation that both are required for polymerization of clathrin. It is not clear at this time what functions the 50- or 16-kDa subunits may serve.

Finally, it is interesting to note that most of the subunits of the AP-2 complex, particularly the larger α and β 100-kDa subunits, can be reversibly folded with great efficiency into a form representative of the native complex. While it should not be surprising that such is the case for smaller monomeric proteins, it clearly is impressive to see this for subunits of the size of 100 kDa possessing considerable amount of structure. The information that these subunits can be reversibly denatured should have application when cloned cDNAs for these proteins are expressed in *Escherichia coli*. Further, their manipulation may also assist in understanding how the different subunits of AP-2 associate with each other as well as with clathrin, receptors, membrane, etc. In addition, such an approach of reversible denaturation may also be useful to dissociate the proteins of the AP-1 complex and identify the domains responsible for clathrin interaction.

Supplementary Material Available

Figures demonstrating the binding of $\alpha_{a/c}$ and β to clathrin cages and the resultant increase in the latter's sedimentation on glycerol gradient ultracentrifugation (Figure 1) and clathrin coat assembly by Gdn·HCl 100-kDa polypeptides on glycerol gradient ultracentrifugation (Figure 2) (2 pages). Ordering information is given on any current masthead page.

References

Ahle, S., & Ungewickell, E. (1986) *EMBO J. 5* (12), 3143–9.
Ahle, S., & Ungewickell, E. (1989) *J. Biol. Chem. 264* (33), 20089–93.
Ahle, S., & Ungewickell, E. (1990) *J. Cell Biol. 111*, 19–29.
Ahle, S., Mann, A., Eichelsbacher, U., & Ungewickell, E. (1988) *EMBO J. 7* (4), 919–29.

Beck, K. A., & Keen, J. H. (1991) *J. Biol. Chem.* (in press).
Brodsky, F. M. (1988) *Science 242* (4884), 1396-402.
Glickman, J. N., Conibear, E., and Pearse, B. M. (1989) *EMBO J. 8* (4), 1041-7.
Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W., & Schneider, W. J. (1985) *Annu. Rev. Cell Biol. 1*, 1-39.
Heuser, J. E., & Keen, J. (1988) *J. Cell Biol. 107* (3), 877-86.
Keen, J. H. (1987) *J. Cell Biol. 105* (5), 1989-98.
Keen, J. H. (1990) *Annu. Rev. Biochem. 59*, 415-438.
Keen, J. H., & Beck, K. A. (1989) *Biochem. Biophys. Res. Commun. 158* (1), 17-23.
Keen, J. H., Willingham, M. C., & Pastan, I. (1979) *Cell 16*, 303-12.
Keen, J. H., Beck, K. A., Kirchhausen, T., and Jarrett, T. (1991) *J. Biol. Chem.* (in press).
Kirchhausen, T., Nathanson, K. L., Matsui, W., Vaisberg, A., Chow, E. P., Burne, C., Keen, J. H., & Davis, A. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86* (8), 2612-6.
Manfredi, J. J., & Bazari, W. L. (1987) *J. Biol. Chem. 262* (25), 12182-8.
Murphy, J.-E., Pleasure, I. T., Puszkin, S., Prasad, K., & Keen, J. H. (1991) *J. Biol. Chem.* (in press).
Nandi, P., Irace, G., Van Jaarsveld, P. P., Lippoldt, R. E., & Edelhoch, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5881.
Pearse, B. M. F. (1975) *J. Mol. Biol. 97*, 93-98.
Pearse, B. M. (1988) *EMBO J. 7* (11), 3331-6.
Pearse, B. M., & Robinson, M. S. (1984) *EMBO J. 3* (9), 1951-7.
Pearse, B. M., & Crowther, R. A. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 49-68.
Ponnambalam, S., Robinson, M. S., Jackson, A. P., Peiperl, L., & Parham, P. (1990) *J. Biol. Chem. 265*, 4814-20.
Prasad, K., Yora, T., Yano, O., Lippoldt, R. E., Edelhoch, H., & Saroff, H. (1986) *Biochemistry 25* (22), 6942-7.
Robinson, M. S. (1987) *J. Cell Biol. 104* (4), 887-95.
Robinson, M. S. (1989) *J. Cell Biol. 108* (3), 833-42.
Schook, W., Puszkin, S., Bloom, W., Ores, E., & Kochwa, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 116-20.
Woodward, M. P., & Roth, T. P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4394-8.
Zaremba, S., & Keen, J. H. (1985) *J. Cell Biochem. 28* (1), 47-58.

Protein Kinase C in Tumoricidal Activation of Mouse Macrophage Cell Lines†

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ABSTRACT: A potential role of protein kinase C (PKC) in lipopolysaccharide- (LPS-) induced tumoricidal activation of macrophages was investigated by using two mouse macrophage cell lines ($P388D_1$ and J774). J774 cells are stimulated by LPS to kill target P815 mastocytoma cells, whereas $P388D_1$ cells fail to develop such an ability. Pretreatment of J774 cells with H-7 or phorbol myristate acetate resulted in a significant inhibition of LPS-induced cytotoxicity, whereas pretreatment with H-8, ML-7, HA1004, or W-7 did not. Since these results suggested a critical role of PKC in the activation process, the properties of PKC in the two cell lines were compared. Western blotting with rabbit antiserum specific for the PKCβ regulatory domain allowed detection of a protein of 79 kilodaltons (kDa) in the detergent lysates of both cell lines that were not stimulated by LPS. However, LPS treatment resulted in the appearance of a second protein of 40 kDa only in J774 cells and not in $P388D_1$ cells. Furthermore, two forms of protein kinase (one basic and the other acidic) were identified in the cytosol of J774 cells by HPLC on DEAE-5PW, whereas only the basic form was found in $P388D_1$ cells. On the basis of the response of the basic and acidic form protein kinases to phosphatidylserine (PS), diolein, and Ca^{2+}, the basic form was found to contain both regulatory and catalytic domains of PKC, whereas the acidic form was suggested to represent the PKC catalytic domain. This was confirmed by Western blotting with the rabbit anti-PKC regulatory domain serum, which whowed the presence of two proteins of 79 and 40 kDa in the basic form kinase of J774 cells. No protein was recognized by this antiserum in the acidic form kinase of J774 cells. To confirm the presence of the catalytic domain in both basic and acidic forms of kinase, J774 cytosols were incubated with [^{3}H]staurosporine (SS) and analyzed by Sephadex G-150 gel filtration, which separated [^{3}H]SS-binding proteins into two major peaks. When the first and second [^{3}H]SS-binding proteins were separately chromatographed on DEAE-5PW, they were eluted with the basic and acidic protein kinase active fractions, respectively. The acidic form of kinase therefore contains the catalytic but not the regulatory domain of PKC. Collectively, these results suggest that proteolytic cleavage of PKC to generate the catalytic domain fragment may serve an important role in LPS-induced tumoricidal activation of macrophages.

Mouse macrophages and macrophage-like cell lines acquire the ability to kill tumor cells and bacteria following the interaction with IFN-γ and/or LPS (Hibbs et al., 1981; Pace & Russell, 1981; Adams, 1989). The LPS-triggered biochemical sequence of events leading to tumoricidal activation of macrophages has been the subject of intense studies. Jakway and DeFranco (1986) suggested that a pertussis toxin sensitive Gi protein, which inhibits adenylate cyclase, may play a critical role in mediating the effects of LPS on $P388D_1$ cells

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