

Clathrin Assembly Protein AP-2 Induces Aggregation of Membrane Vesicles: A Possible Role for AP-2 in Endosome Formation

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Abstract. We have examined the *in vitro* behavior of clathrin-coated vesicles that have been stripped of their surface coats such that the majority of the clathrin is removed but substantial amounts of clathrin assembly proteins (AP) remain membrane-associated. Aggregation of these stripped coated vesicles (s-CV) is observed when they are placed under conditions that approximate the pH and ionic strength of the cell interior (pH 7.2, ~100 mM salt). This s-CV aggregation reaction is rapid ($t_{1/2} \leq 0.5$ min), independent of temperature within a range of 4–37°C, and unaffected by ATP, guanosine-5'-*O*-(3-thiophosphate), and in particular EGTA, distinguishing it from Ca²⁺-dependent membrane aggregation reactions. The process is driven by the action of membrane-associated AP molecules since partial proteolysis results in a full loss of activity and since aggregation is abolished by pretreatment of the s-CVs with a monoclonal antibody that reacts with the α subunit of AP-2. However, vesicle aggregation is not inhibited by PPP_i, indicating that the previously characterized polyphosphate-sensitive AP-2 self-association is not responsible for the reaction. The vesicle

aggregation reaction can be reconstituted: liposomes of phospholipid composition approximating that found on the cytoplasmic surfaces of the plasma membrane and of coated vesicles (70% L- α -phosphatidylethanolamine (type I-A), 15% L- α -phosphatidyl-L-serine, and 15% L- α -phosphatidylinositol) aggregated after addition of AP-2, but not of AP-1, AP-3 (AP180), or pure clathrin triskelions. Aggregation of liposomes is abolished by limited proteolysis of AP-2 with trypsin. In addition, a highly purified AP-2 α preparation devoid of β causes liposome aggregation, whereas pure β subunit does not, consistent with results obtained in the s-CV assay which also indicate the involvement of the α subunit. Using a fluorescence energy transfer assay we show that AP-2 does not cause fusion of liposomes under physiological solution conditions. However, since the fusion of membranes necessarily requires the close opposition of the two participating bilayers, the AP-2-dependent vesicle aggregation events that we have identified may represent an initial step in the formation and fusion of endosomes that occur subsequent to endocytosis and clathrin uncoating *in vivo*.

THE pathway of receptor-mediated endocytosis proceeds first through the binding of extracellular ligands to specific cell surface receptors. These ligand receptor complexes are clustered in plasma membrane clathrin-coated pits which are thought to subsequently "pinch off" from the plasma membrane, giving rise to coated vesicles. Soon after their formation these coated vesicles lose their clathrin coats and the resulting uncoated vesicles immediately participate in a series of fusion events resulting in the accumulation of ligands and receptors in an early endosome compartment. Here the ligands and receptors are sorted for delivery to either lysosomes or the plasma membrane (for review see Goldstein et al., 1985; Pastan and Willingham, 1985; Gruenberg and Howell, 1989; Rodman et al., 1990).

The plasma membrane localized clathrin assembly protein AP-2¹ is thought to be involved in two events that occur early in the endocytic pathway. First, it has been demonstrated in a number of studies that AP-2 is required for the *in vitro* assembly of clathrin coat structures under physiological solution conditions (Keen et al., 1979; Zaremba and Keen, 1983), suggesting that AP-2 functions in cells to promote clathrin lattice formation. In addition, AP-2 may play some role in the clustering of receptors in plasma membrane-coated pits (Pearse, 1988). There is also some evidence suggesting that AP-2 may participate in events that oc-

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1. *Abbreviations used in this paper:* AP, clathrin assembly protein; CV, coated vesicle; DPH, 1,6-diphenyl-1,3-hexatriene; HM-AP, heavy mero-AP; LM-AP, light mero-AP; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl- α -phosphatidylethanolamine; Rh-PE, *N*-(Lissamine rhodamine B sulfonyl)dipalmitoyl-L- α -phosphatidylethanolamine; s-CV, stripped coated vesicle.

cur at later stages in the endocytic pathway. That AP-2 is present during these late events is suggested by the observation that after *in vitro* uncoating of isolated coated vesicles by the uncoating protein (hsc 70), AP molecules maintain an association with the uncoated vesicles (Heuser and Keen, 1988). In addition, *in vivo* immunolocalization studies (Guagliardi et al., 1990) have provided evidence for the presence of AP-2 on the surfaces of endosomes, indicating that AP-2 may participate in some function associated with this compartment. In previous studies we have found that microinjection of antibodies to AP-2 into cultured cells gave rise to an inhibition of endocytosis that varied in magnitude from cell to cell (Chin et al., 1989). In those cells where a partial block in endocytic activity was observed, vesicles containing newly internalized ligand showed a more peripheral cytoplasmic distribution compared with the perinuclear localization of endosomes in control cells. These results are consistent with a blockage by the antibodies of some stage of endosome maturation, possibly early endosome fusion, and hence suggest a role for AP-2 in this process.

In this study we show that when coated vesicles are treated under conditions that strip the majority of surface-bound clathrin but leave behind substantial amounts of AP-2, the vesicles aggregate in a protein-dependent process. With the hypothesis that this stripped vesicle aggregation may reflect an initial step in the formation and fusion of endosomes we have developed quantitative assays for vesicle aggregation in order to characterize the reaction further and to assess the role of AP-2.

Materials and Methods

L- α -Phosphatidylinositol (type I, P2517), L- α -phosphatidylethanolamine (type I-A, P7523), and L- α -phosphatidyl-L-serine (P6641) were purchased from Sigma Chemical Co. as 10 mg/ml solutions in chloroform. *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) dipalmitoyl- α -phosphatidylethanolamine (NBD-PE), *N*-(Lissamine rhodamine B sulfonyl)dipalmitoyl-L- α -phosphatidylethanolamine (Rh-PE), and 1,6-diphenyl-1,3-hexatriene were from Molecular Probes Inc. (Eugene, OR). *D*-myo-inositol-1,3,4-trisphosphate was purchased from Calbiochem Corp. (La Jolla, CA). Guanosine-5'-*O*-(3-thiophosphate) was from Boehringer Mannheim Corp. (Indianapolis, IN). Trypsin (TPCK treated, 241 U/mg) was from Worthington Biochemical Corp. (Freehold, NJ). L- α -Phosphatidylinositol-4,5-bisphosphate was purchased as a solid sodium salt and was resuspended in water (2 mg/ml) and sonicated in a water bath sonicator for 30 s at room temperature. This material was then stored at -70°C . Immediately before use (no longer than 2 d after the initial preparation) the lipid solution was thawed under warm water and sonicated 60 min at room temperature. Monoclonal antibodies to clathrin light chain LC_b (Brodsky et al., 1987) and to the α subunit of AP-2 (Chin et al., 1989) were prepared and purified as described previously.

AP and clathrin were extracted from purified bovine brain coated vesicles and isolated by Superose 6B gel filtration as described previously (Keen et al., 1979; Keen, 1987). For experiments with pure AP-2 and partially purified AP-1, fractions corresponding to the trailing half of the Superose 6B AP peak (fractions 38–40 in Fig. 2 of Keen, 1987), which contain mostly AP-1 and AP-2, were pooled and fractionated by clathrin-Sepharose chromatography (Keen, 1987). AP-2 subunits were isolated by DEAE chromatography of urea-denatured AP-2 as described (Prasad and Keen, 1991).

Stripped coated vesicles were prepared as follows. A crude preparation of coated vesicles (CVs) was isolated from bovine brain according to the method of Keen et al. (1979). This preparation was further fractionated by Sephacryl S-1000 chromatography as follows. 1 g of crude CV protein in 10 ml of 0.1 M sodium MES, pH 6.5, was loaded onto an S-1000 column (66.2 \times 2.5 cm). The column was eluted with 0.1 M sodium MES, pH 6.5, at 4°C with a downward flow rate of 6 ml/h and fractions containing pure CVs, which eluted as a single peak at 185 ml, were pooled, centrifuged at 100,000 *g* for 1 h, and resuspended in 2 ml of 0.1 M sodium MES, pH 6.5. To remove their clathrin coats, the vesicles were placed in collodion dialysis

bags (UH100/25; Schleicher & Schuell, Inc., Keene, NH) and dialyzed against 1 liter of 10 mM Tris, pH 8.5, for 15 h. After dialysis the vesicle bilayers were labeled with the lipid-soluble fluorescent probe 1,6-diphenyl-1,3-hexatriene (DPH) by adding 2 μl of a 10 mM stock solution of DPH in tetrahydrofuran to 1–2 ml of vesicle suspension. The extracted coat proteins were then separated from the stripped vesicles by centrifugation (80,000 *g* for 4.5 h in an SW 28 rotor [Beckman Instruments, Palo Alto, CA]) on continuous sucrose gradients (10–30% sucrose [wt/vol] in 10 mM Tris, pH 8.5). Gradient fractions containing the sedimented vesicles, revealed by DPH fluorescence (excitation = 360 nm, emission = 430 nm), were pooled and used directly in the assays described below. A typical stripped coated vesicle (s-CV) preparation contained <1% of the original clathrin heavy chain but maintained substantial amounts of APs (20–50% of that in intact CVs).

Stripped coated vesicle aggregation experiments were conducted by dialyzing s-CVs (300 μl) against 1 liter of 0.1 M sodium MES, pH 7.2, for 15 h at 4°C. In addition, s-CV aggregation could be induced to occur over a short time course by adding 0.1 vol of 1 M sodium MES, pH 6.5 or 7.2, to a solution containing 250 $\mu\text{g}/\text{ml}$ s-CV protein in 10 mM Tris-HCl, pH 8.5. Aggregate formation was quantitated by measuring the absorbance of the s-CV solution at 350 nm due to light scatter in the turbid sample. Alternatively, suspensions containing s-CV aggregates were subjected to centrifugation at 10,000 *g* for 10 min, which pellets the aggregates, and the DPH fluorescence in the resulting supernatant and pellet fractions was measured. Proteolysis of s-CV surface proteins was conducted by incubation with trypsin (500:1, s-CV protein/trypsin, wt/wt) at 22°C. The reaction was terminated by the addition of soybean trypsin inhibitor to a twofold molar excess with respect to trypsin.

To prepare liposomes, pure phospholipids in chloroform were first mixed together in a borosilicate glass test tube in the following molal proportions: 70% PE, 15% PS, and 15% PI. DPH was added to this mixture such that its final concentration (after sonication; see below) was 10 μM . After evaporation of the solvent under argon, buffer (0.1 M sodium MES, pH 7.2) was added to the dried phospholipids and the mixture was sonicated on ice for 40 min using a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT) probe sonicator (setting # 4). The liposome suspension was then centrifuged 10,000 *g* for 5 min to remove any aggregated material. The final phospholipid concentration was typically 3–5 mM. To assay the ability of AP-2 and other purified proteins to cause the aggregation of these liposomes, a small volume (typically 1/10 the final sample volume) of concentrated protein (1–2 mg/ml in 10 mM Tris-HCl, pH 8.5) was added to a 300- μl solution containing 0.1 M sodium MES, pH 7.2, 300 μM phospholipid, and 10 mM sodium tripolyphosphate (the latter was present as an inhibitor of AP-2 self-association; Beck and Keen, 1991b). Aggregation was quantified by sample absorbance at 350 nm and by measuring the loss of DPH fluorescence in the sample after centrifugation at 10,000 *g* for 5 min.

Liposome fusion was measured using the phospholipid mixing assay of Struck et al. (1981). Liposomes (composition as above) containing NBD-PE and Rh-PE at 0.75 and 0.5 mol%, respectively, were prepared as described above. At these proportions the surface density of NBD-PE and Rh-PE is such that excitation of the NBD moiety with 450-nm light results in efficient fluorescence energy transfer to adjacent Rh-PE molecules, whereas fusion of these liposomes with liposomes containing no fluorescent phospholipids effectively lowers the surface density of NBD and Rh to a level that results in an uncoupling of energy transfer. Before they were used in the assay the labeled liposomes were passed over a 60-ml Sepharose CL-4B column at a flow rate of 0.2 ml/min to isolate unilamellar vesicles, which eluted with a K_{av} of 0.28. Fusion assays were carried out by incubating fluorescent liposomes in 0.1 M sodium MES with a 16-fold molar excess of liposomes containing no fluorophore for 30 min at 37°C in the presence of added protein. After this incubation the samples were excited with 450-nm light and the fluorescence emission spectrum between 485 and 640 nm was recorded.

Production of AP fragments for sandwich radioimmunoassay. Purified APs were digested with elastase at 1:100 enzyme/AP (wt/wt) for 30 min at 23°C and the digestion was terminated with PMSF. The digest was chromatographed on a Sephadex G-75 Superfine column and analyzed by gel electrophoresis. Peak I (fractions 19–24) contained ~60–70-kD NH₂-terminal domains of the α and β polypeptides in a complex with the intact 50- and 17-kD subunits defined as heavy mero-AP (HM-AP) (Zaremba and Keen, 1985; Keen and Beck, 1989; Kirchhausen et al., 1989), peak II (fractions 34–36) contained 30–40-kD polypeptides termed light-mero-AP (LM-AP), and peak III (fractions 52–54) contained smaller digest products.

Sandwich radioimmunoassay. The anti-AP mouse monoclonal antibodies AP.1, AP.6, and AP.7 were produced as described (Chin et al., 1989). Antibodies were purified (Brodsky, 1985) and iodinated using Iodobeads (Pierce Chemical Co., Rockford, IL), 1 mCi/25 μg antibody. Purified anti-

body (50 $\mu\text{g/ml}$) was applied to the wells of a polyvinylchloride 96-well plate, 1 h at 23°, and then washed and incubated 1 h RT with 2% BSA in phosphate-buffered saline, pH 7.4. AP fragments or intact AP were then added, 1 h at 23°. After washing in PBS-BSA (0.5%), the radioactive antibodies (350,000 cpm/well) were bound for 1 h at 23°. Wells were then washed and counted for bound radioactivity.

Protein quantitation in membrane fractions was carried out using the Pierce bicinchoninic acid assay method (Pierce Chemical Co.). Quantitative densitometry of Coomassie blue-stained gels was performed on a Hoefer GS-300 densitometer with Hoefer GS-360 Data System software (Hoefer Scientific Instruments, San Francisco, CA).

Results

Aggregation of Stripped Clathrin-coated Vesicles

In examining the physical properties of pure coated vesicles under various solution conditions, it was observed that when coat proteins are stripped from their surfaces by treatment with 10 mM Tris-HCl, pH 8.5, extensive aggregation of the vesicles occurs after exposure to conditions approximating

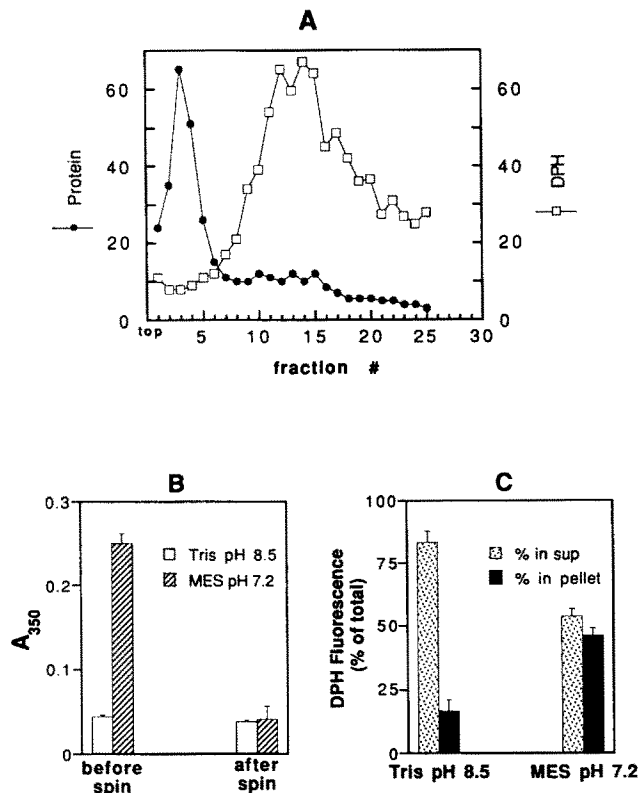


Figure 1. An assay for stripped-coated vesicle aggregation. (A) Sephacryl S-1000 purified CVs were stripped by dialysis against 10 mM Tris, pH 8.5, and the vesicle bilayers were labeled with DPH. The s-CVs were then centrifuged (100,000 g) on a 10–30% continuous sucrose gradient. s-CVs were pooled from fractions 11–16. (B) Equal aliquots of s-CVs ([AP] = 50–100 $\mu\text{g/ml}$) were dialyzed against either 10 mM Tris-HCl, pH 8.5 (control, stippled bars), or 0.1 M sodium MES, pH 7.2 (hatched bars). Sample light scatter, measured as absorbance at 350 nm (A_{350}), was recorded (before spin). The samples were then centrifuged 10,000 g for 2 min, and the supernatant A_{350} was recorded (after spin). (C) DPH fluorescence in both the supernatant and pellet fractions of these same samples was also measured. Plotted are the averages and standard deviations of triplicate samples.

physiological pH and ionic strength (pH 7.2, 100 mM salt; see Fig. 1). Since within the intact cell the event that immediately follows the uncoating of coated vesicles is their rapid fusion either with each other or with pre-existing membrane compartments, we speculated that this aggregation reaction represents an initial step in the fusion process. Accordingly, a quantitative assay for examining this s-CV aggregation reaction was developed in order to conduct a biochemical characterization of the event, identify the vesicle components responsible for it, and assess the potential for its involvement in endosome fusion.

s-CVs were prepared by dialyzing purified bovine brain coated vesicles against 10 mM Tris-HCl, pH 8.5, and were isolated by sucrose gradient centrifugation (Fig. 1 A). After this treatment the majority of the released protein was found at the top of the gradient (fractions 1–6), whereas the s-CVs (revealed by DPH fluorescence; see Materials and Methods) sedimented as a single peak between fractions 10 and 15. Analysis of the protein composition of a typical s-CV preparation (see Fig. 2 A, lane 1) showed the presence of substantial amounts of AP (20–50% of the initial coated vesicle AP) and only minimal amounts of the 180-kD clathrin heavy chain (<5% of the total coated vesicle clathrin). Upon dialysis of the s-CVs against 0.1 M sodium MES, pH 7.2, there was a marked increase in sample light scatter (measured as

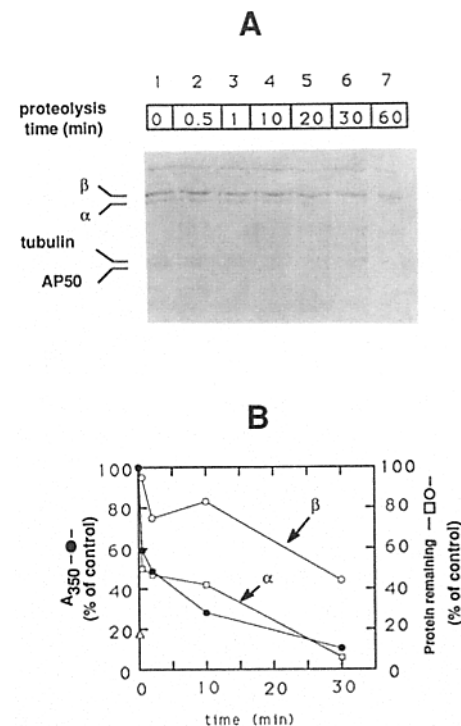


Figure 2. Limited proteolysis of s-CVs blocks their aggregation. S-CVs (in 10 mM Tris-HCl, pH 8.5) were treated with trypsin (1:500, wt/wt) for increasing times, dialyzed against 0.1 M sodium MES, pH 7.2, and assayed for vesicle aggregation by A_{350} (B, ●). The A_{350} of intact s-CVs maintained in 10 mM Tris-HCl is indicated on the ordinate (Δ). The proteolyzed vesicles were subjected to SDS-PAGE in the presence of urea (Ahle et al., 1988) to enhance separation of α and β subunits (A). The positions of the AP 100-kD (α and β) and 50-kD subunits (AP50) as well as tubulin are indicated to the left of the figure. The α and β subunits were quantitated by densitometry (B, □ and ○).

absorbance at 350 nm; Fig. 1 *B*) with respect to the control (in 10 mM Tris-HCl, pH 8.5), suggesting that the vesicles had aggregated. This is supported by the observation that after brief low-speed centrifugation the sample A_{350} was reduced to control levels (Fig. 1 *B*) and there was a substantial accumulation of pelleted vesicles as assessed by the distribution of DPH fluorescence in the supernatant and pellet fractions (Fig. 1 *C*).

Unlike other documented vesicle aggregation and fusion events (Wilschut and Hoekstra, 1986; Feigenson, 1989), s-CV aggregation occurred readily in the absence of Ca^{2+} and was not affected by the addition of EGTA (not shown). Rather, it was found that the reaction was protein mediated since aggregation of s-CVs was blocked after proteolysis of surface proteins with trypsin (Fig. 2 *B*). To identify the specific protein components responsible for s-CV aggregation, we treated s-CVs with trypsin for increasing periods of time and measured the loss of surface proteins due to proteolysis by SDS-PAGE (Fig. 2 *A*). There was a progressive loss of both the 100- and 50-kD subunits of AP with increasing time of proteolysis, a result consistent with the conclusion that surface-bound AP molecules are responsible for vesicle aggregation. Conversely, small amounts of contaminating 180- and 55-kD species (the latter presumably vesicle-bound tubulin) were unaffected.

Interestingly, of the two 100-kD AP subunits, the loss of the α subunit closely parallels loss of aggregation activity (Fig. 2 *B*), suggesting the involvement of this subunit. The preferential loss of the AP-2 α subunit compared with the β subunit on trypsinization of s-CVs is in contrast to studies examining the trypsin sensitivity of pure AP-2 performed in a different buffer, in which both subunits are equally susceptible to proteolysis (Keen and Beck, 1989). Control experiments (not shown) have demonstrated that the difference in proteolysis is due to the difference in buffer conditions used (10 mM Tris-HCl, pH 8.5, this study; 0.5 M Tris-HCl, pH 7.0, Keen and Beck, 1989) rather than the membrane association of the AP. Nevertheless, since proteolysis under both conditions causes the release of a 30-kD COOH-terminal fragment of the AP-2 α subunit (Keen and Beck, 1989; Krichhausen et al., 1989), the results shown in Fig. 2 suggest that this domain could be involved in the membrane aggregation reactions. Though the 50-kD subunit is also cleaved under the conditions used here, further proteolysis and antibody inhibition experiments indicate that it is unlikely to be involved in the aggregation reaction (see below).

To test the hypothesis that surface-bound AP-2 molecules cause the aggregation of s-CVs more rigorously, we measured the ability of a number of monoclonal antibodies raised against coated vesicle proteins (Brodsky, 1987; Chin et al., 1989) to inhibit the reaction (Fig. 3). A monoclonal antibody, AP.6, which recognizes the 30-kD COOH-terminal domain of the α subunit of AP-2 inhibited s-CV aggregation in a concentration-dependent manner, causing 80% inhibition at a molar ratio of antibody/AP-2 of ~ 1.0 . Monoclonal antibody LCB.2, which reacts with the clathrin light chain LCB, produced no effect on s-CV aggregation even at concentrations 10-fold greater than the effective range of AP.6, indicating that the result seen with AP.6 is not due to a nonspecific effect of antibody addition. Furthermore, two other mAbs to the 30-kD domain of the α subunit (LM-AP), AP.1 and AP.7, also were without effect (Fig. 3), although all

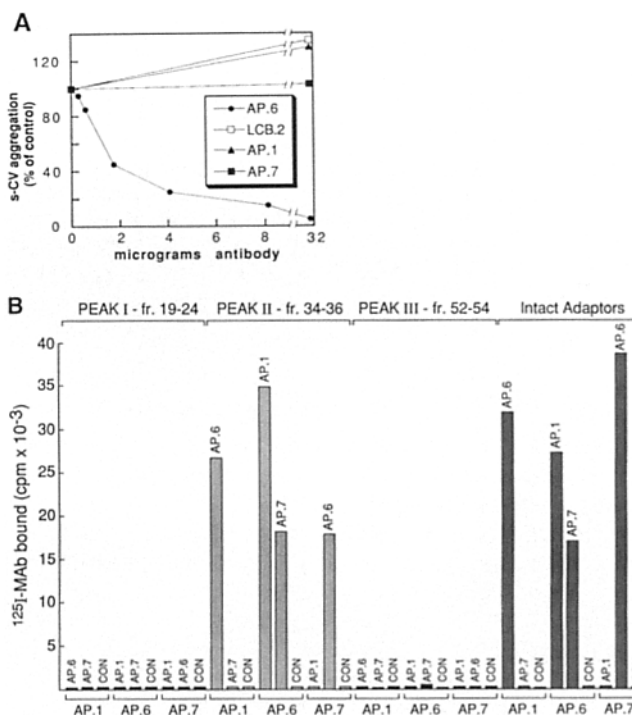


Figure 3. (A) Inhibition of s-CV aggregation by an antibody to the α subunit of AP-2. s-CVs in 10 mM Tris-HCl (300 μ l, 10 μ g AP, 31 pmol) were preincubated with the indicated amounts of mAbs for 20 min at 22°C. After this treatment the s-CVs were induced to aggregate by the addition of a small volume (30 μ l) of 0.1 M sodium MES, pH 6.5 (see Materials and Methods). Shown is the percentage of control light scatter observed 20 min after the addition of sodium MES as a function of the amount of antibody present. AP.1 (▲), AP.6 (●), and AP.7 (■) are antibodies to the α subunit of AP-2. LCB2 (□) recognizes the clathrin light chain LCB. (B) Sandwich radioimmunoassay demonstrating distinct epitopes on the LM-AP fragments. mAbs AP.1, AP.6, and AP.7 were bound to the wells of plastic plates (indicated at the bottom) and chromatographed fragments of APs and intact APs were added to the wells (indicated at the top). Then iodinated antibodies were added to each well and the binding of the iodinated antibodies (^{125}I -MAB) is indicated by the labeled bars. Peak I contains the HM-AP and peak II contains the LM-AP. The binding of a radioactive antibody will occur only if it binds to a distinct site from the antibody bound to the plate. However, the site must be on the same AP fragment. These results show that all three antibodies bind to the LM-AP (30-kD piece) and that AP.6 recognizes a distinct site from AP.1 and AP.7. The binding sites of AP.1 and AP.7 are too close for the antibodies to bind simultaneously, but they behave distinctly in immunofluorescence. CON refers to the binding of iodinated control antibody (29B5, anti-dinitrophenol).

of the AP-2-directed mAbs used were found to recognize and bind to native AP-2. When tested for binding to the 30-kD fragment of AP-2 and intact AP-2 it was found that AP.1 and AP.7 bound to different epitopes than that recognized by AP.6. This was demonstrated by a sandwich radioimmunoassay (Fig. 3 *B*) in which AP.6 and AP.1 or AP.6 and AP.7 bound simultaneously to the 30-kD fragment of AP-2; however, the binding of AP.1 and AP.7 were mutually exclusive. Finally, Fab' fragments of mAb AP.6 retained their inhibitory activity toward s-CV aggregation, although greater concentrations were required consistent with a decrease in avidity.

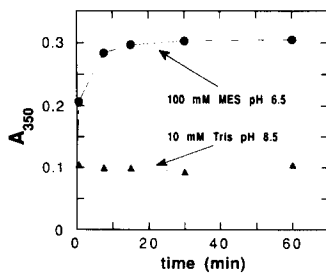


Figure 4. Time course of s-CV aggregation. s-CVs were aggregated by the addition of 30 μ l 0.1 M sodium MES, pH 6.5, to 270 μ l s-CV in 10 mM Tris-HCl, pH 8.5 (\bullet). As a control 30 μ l of 10 mM Tris-HCl, pH 8.5, was added to an identical sample (\blacktriangle). Aggregation was recorded as a function of time by measuring the A_{350} of the sample.

Again, Fab' fragments of AP.1 and AP.7 were without effect. Collectively, these results indicate that the COOH-terminal domain of the AP-2 α subunit recognized by the mAb AP.6 is involved in s-CV aggregation.

To characterize the s-CV aggregation reaction further, we developed an assay that measures the formation of vesicle aggregates after the addition of a small volume of concentrated buffer to the s-CV preparation in 10 mM Tris-HCl, pH 8.5 (see Materials and Methods). This treatment results in an immediate increase to physiological ionic strength and a simultaneous lowering of the pH to levels (pH 7.2) that support vesicle aggregation. Under these conditions rapid formation of s-CV aggregates is observed ($t_{1/2} \leq 0.5$ min; Fig. 4), indicating that the reaction occurs at a rate compatible with the time course of endosome fusion in intact cells (1–5 min).

Diaz et al. (1989) have developed a reconstituted endosome fusion assay and used it to test effectors of the process and to identify a clustered vesicle intermediate. We have measured the activities of a number of effectors of the process in the s-CV aggregation assay to determine if the two events share common properties (Table I). Like the endosome fusion intermediate, s-CV aggregation is minimal in the presence of "fusion buffer" lacking KCl, and is induced upon addition of 50 mM KCl. In addition, treatment of endosomes with high concentrations of KCl destroys their ability to undergo fusion by stripping from their surfaces proteins that are required for the fusion reaction (Colombo, M. I., and P. D. Stahl. 1990. *J. Cell Biol.* 111:80a. [Abstr.]). Similarly, s-CV aggregation is reduced in the presence of high KCl concentrations (Table I). Hence both the endosome fu-

Table I. Characterization of Stripped Vesicle Aggregation

Agent (condition)	Effect on stripped vesicle aggregation (% of control)
37°C*	100
20°C*	92
4°C*	106
Fusion buffer [†] without KCl	0
Fusion buffer [†] with 50 mM KCl	49
500 μ M GTP γ S*	90
1.5 mM ATP*	113
1.5 mM MgATP*	116
0.67 M KCl*	41

* In 0.1 M NaMES, pH 6.5.

[†] In 20 mM Hepes, 1.5 mM MgCl₂, 0.5 mM EGTA, and 1 mM DTT, pH 7.0.

sion and s-CV aggregation reactions show a requirement for moderate salt concentrations.

However, the two events differ in several important respects (Table I). For instance, incubation at 4°C, which has been shown to block the formation of the fusion intermediate (Diaz et al., 1989), has no effect on s-CV aggregation. Likewise, the fusion intermediate requires ATP, whereas s-CV aggregation is unaffected by either the free or Mg²⁺-bound form of this molecule. In addition, endosome fusion has been found to be sensitive to GTP γ S (Mayorga et al., 1989a,b), which also has no effect on s-CV aggregation. The potential significance of these differences will be discussed below.

AP-2 Induces Aggregation of Pure Phospholipid Liposomes

We were able to reconstitute the vesicle aggregation reaction using purified AP-2 and phospholipid liposomes (Fig. 5). Liposomes were prepared composed of 70% L- α -phosphatidylethanolamine (type I-A), 15% L- α -phosphatidyl-L-serine, and 15% L- α -phosphatidylinositol, approximating the phospholipid composition of the cytoplasmic surface of CVs and

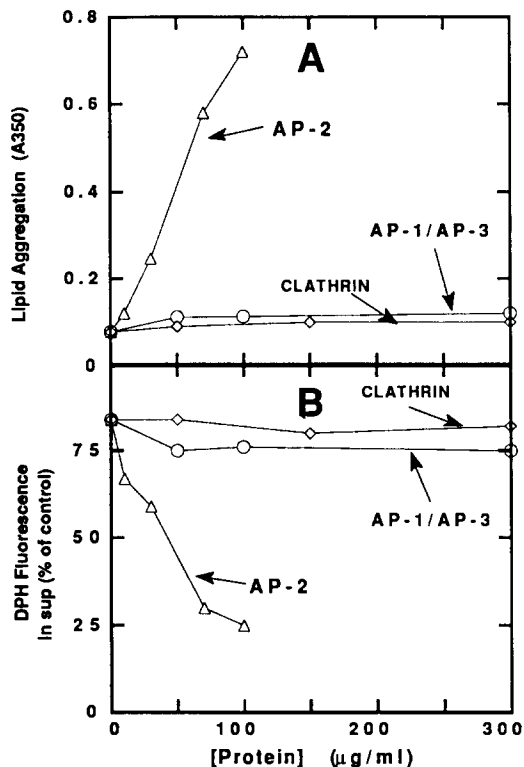


Figure 5. Aggregation of pure phospholipid liposomes by AP-2. Sonicated pure phospholipid liposomes (composed of 70% L- α -phosphatidylethanolamine (type I-A), 15% L- α -phosphatidyl-L-serine, 15% L- α -phosphatidylinositol, and 10 mM DPH; approximating the phospholipid composition of the cytoplasmic surface of CVs and the plasma membrane; Altstiel and Branton, 1983) were incubated (20 min) with the indicated concentrations of the following proteins: AP-2 (\blacktriangle), a mixture of AP-1/AP-3 (\circ), and pure clathrin triskelions (\diamond). Light scatter was measured as A_{350} (A). The samples were also centrifuged (10,000 g, 2 min) and DPH fluorescence of the supernatant fractions was measured to assess the sedimentability of the vesicles (B).

the plasma membrane (Alstiel and Branton, 1983). Incubation of liposomes with AP-2 resulted in a rapid ($t_{1/2} \approx 0.5$ min; see Fig. 6) increase in sample light scatter (A_{350} , Fig. 5 A) and a corresponding increase in sedimented liposomes (Fig. 5 B), indicating that liposome aggregates were formed. SDS-PAGE of pellet and supernatant fractions obtained after brief centrifugation of liposomes incubated in the presence of 100 $\mu\text{g}/\text{ml}$ AP-2 revealed the presence of substantial amounts of AP-2 in the pellet fraction (50% of the total), indicating that the aggregates formed are indeed composed of both AP-2 and phospholipid vesicles (data not shown).

In contrast to the result seen with AP-2, an AP preparation containing a mixture of the neuron-specific protein designated AP-3 or AP180 (Ahle and Ungewickell, 1986; Murphy et al., 1991) and the Golgi localized AP-1 (Robinson, 1987; Ahle et al., 1988) did not cause liposome aggregation (Fig. 5), a result which demonstrates that aggregation of liposomes, like that of s-CVs, is a function unique to AP-2. Pure clathrin triskelions were also unable to cause liposome aggregation; however, coassembled AP-2-clathrin coat structures did induce significant aggregation. The significance of the latter observation is unclear at this time.

The conditions used here for examining liposome aggregation are identical to those under which AP-2 alone undergoes a specific self-association reaction which gives rise to large aggregates of AP-2 (Beck and Keen, 1991a). To determine if this AP-2 self-association reaction is responsible for liposome aggregation we measured the effect of high-affinity polyphosphate inhibitors of AP-2 self-association (Beck and Keen, 1991b) to affect liposome aggregation (Fig. 6). ATP and inositol-1,4,5-trisphosphate produced no effect on liposome aggregation even at concentrations 25–50-fold greater than those required to block AP-2 self-association. It can therefore be concluded that the interactions involved in AP-2-induced liposome aggregation are distinct from those involved in the polyphosphate-sensitive AP-2 self-association reaction. Interestingly, however, PIP_2 micelles did significantly inhibit liposome aggregation at concentrations equivalent to those required for inhibition of self-association. This result is likely due to the physical size of the 1- α -phosphatidylinositol-4,5-bisphosphate micelle (~ 6 nm in diameter; Suguira, 1981), which is comparable in size to the AP-2 molecule. Occupancy of the polyphosphoinositol site by the low molecular weight polyphosphates apparently does not intrude on the region of the α subunit that is involved in liposome aggregation; however, occupancy of the same site by the relatively large 1- α -phosphatidylinositol-4,5-bisphosphate micelle may give rise to a steric effect that results in an inhibition of liposome aggregation.

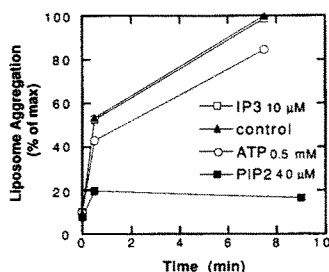


Figure 6. Effect of inhibitors of AP-2 self-association on AP-2-induced liposome aggregation. AP-2-induced aggregation of liposomes (100 $\mu\text{g}/\text{ml}$ AP) was measured in the presence of 10 μM Ins-1, 4,5- P_3 (□), 500 μM ATP (○), 40 μM 1- α -phosphatidylinositol-4,5-bisphosphate (■), or no added inhibitor (▲).

Like s-CV aggregation, AP-2-induced liposome aggregation was sensitive to limited proteolysis (Fig. 7). Incubation of soluble AP-2 with trypsin (300:1, wt/wt) completely destroyed its ability to induce liposome aggregation (Fig. 7 A, left). Since treatment under these conditions affects only the 100-kD subunits of AP-2 and not the AP50 (Zaremba and Keen, 1985; Keen and Beck, 1989) it can be concluded that either the α or the β subunits of AP-2 are responsible for the reaction. Importantly, it was also found that treatment of AP-2-liposome aggregates with protease causes the dissociation of the aggregates (Fig. 7 A, right), a result which demonstrates that AP-2 molecules act to reversibly and noncovalently cross-link the liposomes. This observation provides strong evidence that AP-2 is aggregating, rather than fusing, liposomes since fusion would not be expected to be reversed by subsequent proteolysis; more direct evidence for a lack of fusion under physiological conditions is described below.

Limited proteolysis of AP-2 under the conditions used in this experiment gives rise to two distinct domains of AP-2 (Zaremba and Keen, 1985; Heuser and Keen, 1988; Keen and Beck, 1989) termed HM-AP and LM-AP (see Materials and Methods). To determine which of these domains is

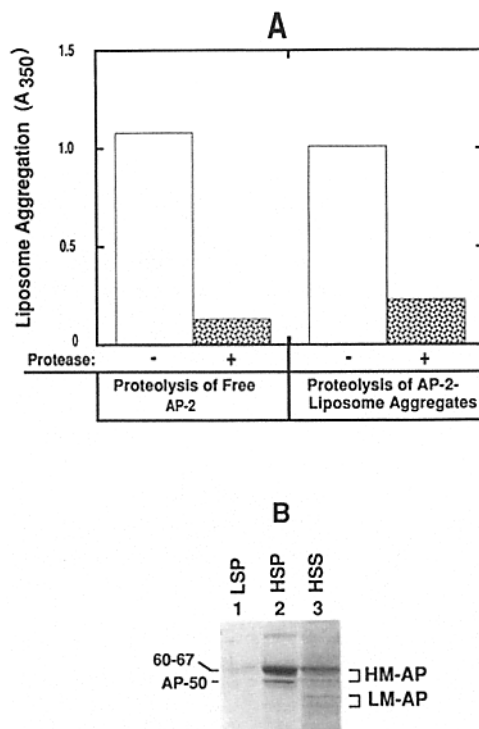


Figure 7. Effect of proteolysis of AP-2 on liposome aggregation. (A) Left, AP in 10 mM Tris-HCl, pH 8.5, was treated with (stippled bar) or without (open bar) trypsin (1:300, wt/wt) for 7 min at 27°C and tested for liposome aggregation activity. Right, AP-liposome aggregates were treated with trypsin (1:150) for 60 min and the A_{350} was measured both before (open bar) and after (stippled bar) proteolysis. (B) The product of the incubation of clipped AP with liposomes was subjected to a 10,000-g spin to pellet aggregates followed by a 150,000-g spin to pellet nonaggregated membranes. Equal amounts of the low speed pellet (LSP), high speed pellet (HSP), and high speed supernatant were electrophoresed on a 8.5% polyacrylamide gel. HM-AP and LM-AP refer to the heavy- and light-mero AP domains.

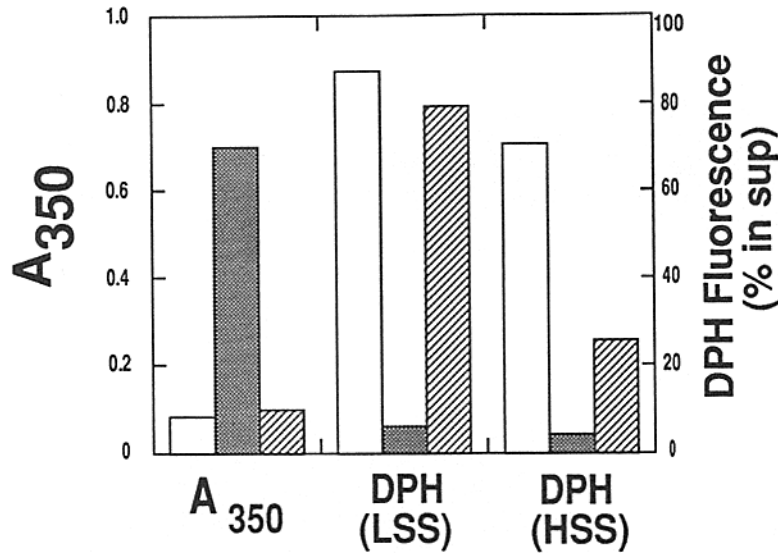
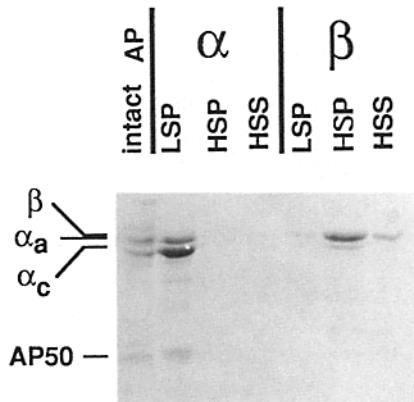
A**B**

Figure 8. α but not β subunits aggregate liposomes. 100-kD subunits of AP-2 were dissociated from the AP-2 complex by treatment with 6 M urea, partially purified by ion exchange chromatography, and renatured as described (Prasad and Keen, 1991). (A) To determine if these isolated 100-kD subunits of AP-2 can promote liposome aggregation, the protein preparations (64 μ g/ml) were incubated in the presence of liposomes and the resulting A_{350} was measured. Open bar, control (lipid alone); stippled bar, α subunit; hatched bar, β subunit. The samples were subjected to low speed centrifugation (10,000 g for 2 min) followed by a high speed centrifugation step (150,000 g) and the supernatant DPH fluorescence in the low speed supernatant (LSS) and high speed supernatant (HSS) was measured. (B) SDS-PAGE of these fractions confirmed that the α subunit was present in the low speed pellet (LSP) and that the β subunit was found in the high speed pellet (HSP). HSS, high speed supernatant.

responsible for bilayer interactions, liposomes incubated with clipped AP-2 were subjected to successive high and low speed centrifugations (Fig. 7 B). No protein was found in the low speed pellet, consistent with the absence of aggregate formation, whereas the high speed spin pelleted the majority of the HM-AP. Conversely, the LM-AP domain was found exclusively in the supernatant fraction. Since the lipids are quantitatively precipitated under these conditions (not shown), these results indicate that the isolated HM-AP domain maintains an association with the liposome bilayer. However, since aggregation does not occur under these conditions, the HM-AP domain alone cannot cause liposome aggregation.

To determine if liposome aggregation is specifically a function of the α subunit of AP-2, the following experiments were performed. First, the effects of mAbs AP.1, AP.6, and AP.7 on liposome aggregation were tested (not shown). The mAb AP.6 was able to significantly block liposome aggregation, consistent with its effects on s-CV aggregation noted above. AP.1 produced only a modest effect, whereas AP.7 and LCB.2 were completely inactive. As an additional approach to demonstrating a role for the α subunit in this pro-

cess we took advantage of the observation that the 100-kD subunits of AP-2 can be dissociated from the AP-2 complex by treatment with 6 M urea and partially purified by ion exchange chromatography (Prasad and Keen, 1991). Subsequent removal of urea from these purified proteins appears to give rise to renatured subunits, as assessed by intrinsic tryptophan fluorescence (Prasad and Keen, 1991), by the observation that the renatured subunits are capable of binding clathrin (Ahle and Ungewickell, 1989; Prasad and Keen, 1991) and in some combinations can also facilitate coat assembly (Prasad and Keen, 1991).

To determine if these separated 100-kD subunits of AP-2 can promote liposome aggregation, they were incubated in the presence of liposomes and the resulting light scatter increase was measured (A_{350} ; Fig. 8). The α subunit (which retained a small amount of 50-kD polypeptide; stippled bar) caused a substantial increase in sample A_{350} with respect to the control (open bar). The β subunit (hatched bar) produced no significant effect. The samples were then subjected to low speed centrifugation (10,000 g for 2 min) and the resulting supernatants were fractionated further by a high speed centrifugation step (150,000 g). The supernatant DPH

fluorescence of each successive step was then measured (Fig. 8 A). Consistent with the light scatter results, only the α subunit (*stippled bar*) caused the vesicles to pellet in the low speed spin. The β subunit (*hatched bar*) did, however, cause the vesicles to pellet in the high speed spin, suggesting that it was bound to the vesicles. SDS-PAGE of these fractions (Fig. 8 B) confirmed that the α subunit was present in the low speed pellet and that the β subunit was indeed found in the high speed pellet. These results, which demonstrate that of the two large subunits only the AP-2 α subunit is capable of causing liposome aggregation, are in agreement with the results obtained with s-CV aggregation which also implicate the AP-2 α subunit as the location of the relevant functional domains. Interestingly, they also indicate that the β subunit can bind to liposomes but does not cause aggregation.

Using the liposome fusion assay of Struck et al. (1981), we asked whether AP (or clathrin) causes fusion of the pure phospholipid vesicles under these conditions (Fig. 9). The fusion assay measures the loss of fluorescence energy transfer between NBD- and Rh-labeled PE that occurs upon fusion of labeled vesicles with target vesicles containing no fluorophore (see Materials and Methods). In the absence of any fusion-promoting factor (Fig. 9 A), energy transfer between excited NBD-PE and acceptor Rh-PE is observed as a strong Rh emission at 590 nm. This condition is stable over a wide pH range (5–7.2). Fusion induced by 1 mM CaCl_2 (Fig. 9 B) results in a loss of energy transfer manifested as a reduction in Rh emission intensity at 590 nm and a corresponding increase in NBD emission at 530 nm. Dispersion of the phospholipids in 0.5% Triton X-100 gives a similar result. Addition of AP or clathrin at 0.1 mg/ml under conditions where AP-induced liposome aggregation is observed (0.1 sodium MES, pH 7.2) did not cause vesicle fusion (Fig. 9 C). However, at lower pH (Fig. 9, D–F), fusion is observed with both AP and clathrin. Dilution of both proteins results in loss of activity, indicating that neither fraction is active because it is contaminated with the other (Fig. 9 F). These results confirm previous reports which showed that clathrin can induce fusion at acidic pH (Blumenthal et al., 1982; Maezawa and Yoshimura, 1990). Thus, this assay reveals that although AP and clathrin will induce fusion under

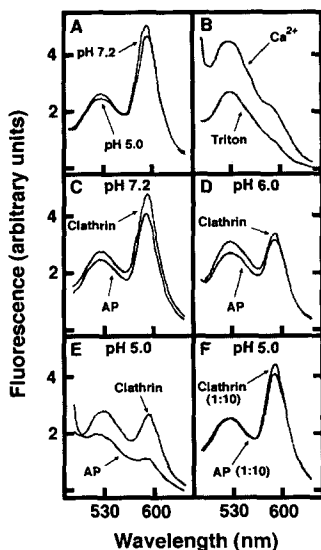


Figure 9. Fusion of liposomes by AP-2 and clathrin. The ability of pure AP-2 to cause the fusion of liposomes was assessed using a fluorescence energy transfer assay as described in Materials and Methods. Fusion of liposomes was measured in the absence of added reagents (A) or in the presence of 1 mM CaCl_2 (B), 0.5% Triton X-100 (B), 100 $\mu\text{g/ml}$ AP (C–E), 100 $\mu\text{g/ml}$ clathrin (C, D), 10 $\mu\text{g/ml}$ AP (F), or 10 $\mu\text{g/ml}$ clathrin (F). Incubations were carried out for 30 min at 37°C. The pH of the reaction mix is indicated in each panel.

acidic conditions, under the physiological conditions in which we see AP-mediated liposome aggregation, the proteins do not induce either overt fusion or mixing of the vesicle bilayer lipids.

Discussion

The available evidence indicates that very soon after the formation of detached coated vesicles within cells, the clathrin coats of these structures are removed and the resulting uncoated vesicles immediately participate in a series of fusions which ultimately result in the delivery of their contents to early endosome compartments. From *in vitro* studies which have shown that AP molecules remain associated with the vesicle surface after clathrin uncoating by hsc 70 (Heuser and Keen, 1988) and from *in vivo* immunolocalization studies which show that AP-2 is present on the surfaces of endosomes (Guagliardi et al., 1990), it appears that vesicle-bound AP-2 survives uncoating and is present on these more distal organelles of the endocytic pathway. In this study we report that upon treatment of coated vesicles under conditions that cause the removal of the majority of the clathrin from their surface but fail to remove all of the AP-2 (presumably mimicking the *in vivo* process), the resulting s-CVs undergo a rapid and extensive aggregation reaction (Fig. 1).

That this reaction is driven by the action of surface-bound AP-2 molecules is indicated by the following observations. First, proteolytic loss of s-CV aggregation follows a time course that correlates well with the time-dependent proteolysis of the α subunit (Fig. 2). Most importantly, a mAb that specifically recognizes the α subunit of AP-2 blocks s-CV aggregation (Fig. 3). The intrinsic ability of AP-2 molecules to self-associate in solution does not appear to be involved in vesicle aggregation since partial proteolysis under conditions that do not affect self-association (Beck and Keen, 1991a) destroys s-CV aggregation activity (Fig. 2). Furthermore, treatment with ATP or 1,4,5-inositoltrisphosphate to block a previously characterized AP-2 self-association reaction (Beck and Keen, 1991b) also has no effect on the aggregation of s-CVs (Table I).

Experiments with pure phospholipid liposomes have shown that they can also be aggregated by the addition of purified AP-2 (Fig. 5). The liposome aggregation reaction shares many characteristics in common with s-CV aggregation and hence is very likely to represent a partial reconstitution of this process. These characteristics include the following: (1) both reactions give rise to aggregates that pellet after brief low speed centrifugation; (2) both require intact, non-proteolyzed AP-2; (3) both reactions occur rapidly ($t_{1/2} \leq 0.5$ min) under physiologically relevant solution conditions (100 mM salt, pH 7.2); (4) both reactions occur with AP concentrations in the range of 30–100 $\mu\text{g/ml}$; (5) neither reaction requires the presence of Ca^{2+} , nor are they affected by EGTA; (6) finally and most importantly, both s-CV and liposome aggregation are inhibited by monoclonal antibody AP6 and hence are dependent on the AP-2 α subunit. In addition, s-CVs with surface-bound AP-2 will aggregate liposomes (data not shown). AP-induced liposome aggregation therefore demonstrates a minimal requirement for the aggregation reaction, namely AP-2 and phospholipid bilayers. The liposome aggregation studies also demonstrate the ability of

AP-2 to interact significantly with lipid bilayers of phospholipid composition approximating that seen on the inner leaflet of the plasma membrane (70% L- α -phosphatidylethanolamine (type I-A) and 30% acidic phospholipid), suggesting that AP-2-phospholipid interactions occur within the intact cell, perhaps contributing to the steady-state association of AP-2 with the membrane.

The observation that the mAb AP.6, which recognizes a 30-kD COOH-terminal region of the α subunit of AP-2, blocks s-CV aggregation (Fig. 3) suggests that the appendage domain of α subunit of the AP-2 (Heuser and Keen, 1988; Keen and Beck, 1989) actively participates in the reaction. This hypothesis is supported by the observation that vesicle aggregation activity is lost in parallel with cleavage of α but not β (Fig. 2) or 50K (Fig. 7) subunits. Furthermore, under conditions that cause the release of the LM-AP domain, isolated HM-AP does maintain an interaction with the liposome surface after proteolysis (Fig. 8 B). These observations suggest that AP-2 binds to the membrane surface through the HM-AP or core domain. Interaction of the appendages of AP-2 molecules bound to separate bilayers could then lead to vesicle aggregation. However, at present we have no direct evidence for such appendage-appendage interactions. Also, it is important to note that such interactions must be distinct from those responsible for AP-2-AP-2 self-association in solution (Beck and Keen, 1991a) as polyphosphate reagents that block self-association do not affect vesicle aggregation.

Under physiologically relevant conditions we found no evidence for bilayer mixing or fusion induced by AP-2 (Fig. 9). Furthermore, the observation that vesicle aggregation was readily reversible by proteolytic clipping of AP-2 provides further evidence that AP-2 does not induce gross perturbations of the vesicle bilayer under these conditions. However, the observation that fusion of the aggregated vesicles could be readily induced by mild acidification indicates that AP-2 binding does bring the bilayers into close apposition. Close approach of membrane bilayers is obviously a necessary initial step in membrane fusion processes and previous studies have shown that proteins and other agents that promote membrane apposition can increase fusion rates (Duzgunes et al., 1984; Hoekstra and Duzgunes, 1986). Thus, AP-2-dependent vesicle aggregation may represent an important initial step in vesicle fusion reactions. In this context, AP-2 may function analogously to the annexins which are thought to participate in stimulated secretion processes by promoting vesicle aggregation and thereby fusion, under some conditions (Drust and Creutz, 1988; Meers et al., 1988). Indeed, this parallel may extend further in that annexins have also been shown to form calcium channels in membranes. Recently, we (Voglmaier et al., 1992) and others (Timerman et al., 1992) have shown that the AP-2 is similar or identical to an inositolhexakisphosphate receptor protein and Timerman and colleagues have reported that this preparation will induce the formation of selective potassium channels in synthetic planar bilayers. However, an important difference between the activities of these preparations and annexin activities is that annexin-dependent aggregation of membrane vesicles requires Ca^{2+} as does secretory vesicle fusion, whereas endosome fusion and s-CV aggregation by AP-2 do not.

It may be premature to rule out totally the ability of AP-2 to act as an independent fusogen within the cell. For in-

stance, the low pH-dependent fusion observed here could result from a conformational change in AP-2 that exposes a domain that mediates the fusion process, as has been proposed for a number of fusion-promoting proteins (White and Helenius, 1980; White et al., 1981; Stegmann et al., 1989) including clathrin (Blumenthal et al., 1982; Maezawa et al., 198; Maezawa and Yoshimura, 1990). It is possible that cellular factors could bind AP-2 and induce this conformational change in a regulated fashion.

A role for AP-2 in the fusion of endosomes within the intact cell is suggested by the observation that microinjection of antibodies to AP-2 limits the accumulation of internalized ligand in late perinuclear compartments (Chin et al., 1989). Since perinuclear localization is preceded by the transport of internalized materials sequentially through multiple compartments (early-late endosomes), this observation is consistent with blockage of a number of possible steps in the endocytic pathway including membrane fusions, and hence implicates a role for AP-2 in these processes. Our results provide support for such a role for AP-2 for several reasons. First, the velocity of the s-CV aggregation reaction ($t_{1/2} \leq 0.5$ min; Fig. 4) is comparable to the time course (2-5 min) of endosome fusion in cells (Salzman and Maxfield, 1989), so that the former event could be an early but not necessarily rate-limiting step in the fusion reaction. In addition, the ionic strength dependence of s-CV aggregation is similar to that of the formation of a vesicle cluster intermediate identified by Diaz et al. (1989). However, several effectors of in vitro endosome fusion (low temperature, ATP, cytosol, and $\text{GTP}\gamma\text{S}$) either are not required for or have no effect on s-CV aggregation. It is possible that these differences may result from a loss or disruption of factors that are responsible for the sensitivity of the reaction to these agents, or, perhaps more likely, that these agents affect later steps leading to endosome fusion but not the initial stages of vesicle aggregation.

The most direct way presently available to demonstrate a role for AP-2 in endosome fusion is to measure the effect of anti-AP-2 antibodies in a reconstituted endosome fusion assay. M. Colombo and P. Stahl at Washington University School of Medicine have recently conducted such experiments and found that monoclonal antibody AP.6 caused a significant (>60%) inhibition of endosome fusion, whereas AP.1, recognizing a determinant that had little or no effect on vesicle aggregation, produced no significant effect at similar concentrations. These results, to be reported in more detail elsewhere, agree well with the results presented in this study (Fig. 3) and hence strongly support the hypothesis proposed here that AP-2 is a participant in the endosome fusion mechanism.

One problem in proposing a role for AP-2-induced vesicle aggregation in endosome fusion is the lack of specificity of the aggregation reaction, as it appears to be dependent only on the presence of phospholipid bilayers and AP-2. Furthermore, we have found that pure phosphatidylcholine vesicles also readily undergo aggregation in the presence of AP-2 (not shown), indicating both that the reaction is not due to a charge interaction with the basic AP50 subunit and that it is not specific for a particular phospholipid head group. In contrast, recent studies have demonstrated considerable temporal and spatial specificity in endosomal fusion reactions using several experimental systems (Diaz et al., 1988; Dunn

et al., 1989; Parton et al., 1989). One possibility is that AP-2 may convey a degree of intelligence to the fusion mechanism through its interaction, either direct or indirect, with the cytoplasmic domains of transmembrane "cargo molecules." That is, a requirement for an initial aggregation step involving AP-2 would ensure that only those endocytic vesicles containing cargo molecules would be aggregation prone and fusion competent. Nonspecific aggregation of this discrete subset of endocytic vesicles with a variety of intracellular membranes may be followed by the action of additional factors that can assess the appropriateness of the target membrane selected (perhaps through a G protein-mediated mechanism; Wilschut, 1989) and directly facilitate fusion. Such error-checked cargo molecule-AP-2 interactions could also conceivably stimulate a fusogenic activity intrinsic to AP-2, analogous to the fusion activity seen under slightly acidic conditions (Fig. 9 E). In such ways AP-2 would serve to bring information about vesicle content to the fusion machinery. It is possible that for other fusion events that occur within cells (i.e., secretory vesicle fusion and late endosome-lysosome formation) there may also exist additional molecules that serve a similar function.

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