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A membrane-associated protein complex with selective binding to the clathrin coat adaptor AP1

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SUMMARY

Adaptors are the membrane-binding components of clathrin-coated vesicles. The interaction of the *trans*-Golgi coat adaptor AP1 with membrane-associated proteins was analyzed by affinity chromatography. Proteins of 83 and 52 kDa bound specifically to the core domain of AP1 and showed no interaction with AP2 or other clathrin-coated vesicle proteins. The AP1-binding proteins were tightly membrane-associated, though behaved as peripheral membrane proteins. They were detected in membranes depleted of clathrin-coated vesicles and not in coated vesicles, suggesting that the interaction of these proteins with AP1 may precede coated vesicle budding. Co-fractionation of the AP1-binding proteins with *trans*-Golgi network membrane was also observed. Upon gel filtration, both AP1-binding proteins eluted in a high molecular mass complex which was labile at high concentrations of Tris. The 83 kDa protein bound to AP1 affinity resin in the absence of the 52 kDa protein. In contrast, the separated 52 kDa protein did not bind AP1, suggesting that the 83 kDa protein is the AP1-binding component of the complex. Characterization of this protein complex defines a novel membrane-associated component that specifically interacts with AP1 and may contribute to its function in forming clathrin-coated vesicles.

Key words: Clathrin, Adaptor, *trans*-Golgi network

INTRODUCTION

Formation of clathrin-coated pits initiates the budding of clathrin-coated vesicles from membranes. These vesicles selectively remove receptors from a donor membrane and facilitate their transport to an intracellular target membrane with which the uncoated vesicle fuses. Clathrin-coated pits form at the plasma membrane and the *trans*-Golgi network (TGN) (Brodsky, 1988; Pearse and Robinson, 1990). At each site, clathrin associates indirectly with the membrane by binding to an adaptor protein (AP) which interacts with the cytoplasmic domains of transmembrane receptors. Polymerization of clathrin then concentrates adaptors, associated receptors and their bound ligands into a coated vesicle (Pearse and Robinson, 1990). Coated pits at each intracellular location have different adaptors. The AP1 adaptor is found in the TGN and the AP2 adaptor is at the plasma membrane, where clathrin-coated vesicles play a role in biogenesis of intracellular organelles or in receptor-mediated endocytosis, respectively (Keen, 1990; Page and Robinson, 1995). Because adaptors are restricted in intracellular location, the adaptor-membrane interaction is believed to determine where clathrin-coated pits form.

In vitro studies of adaptor-membrane interaction have indicated that adaptors bind only weakly to the cytoplasmic domains of receptors and bind with higher affinity to additional determinants on intracellular membranes (Chang et al., 1993; Seaman et al., 1993; Traub et al., 1993). This mechanism would account for the fact that adaptors are tightly localized within the cell, in contrast to the receptors taken up by coated pits which are distributed widely among cellular membranes. Defining the membrane proteins that adaptors interact with is a first step in establishing how their intracellular distribution is determined and perhaps in understanding how they trap receptors. Thus far the binding of AP1 to TGN membranes has been shown to depend on the presence of ARF1 and its binding is inhibited by brefeldin A (BFA) (Robinson and Kreis, 1992; Wong and Brodsky, 1992; Stamnes and Rothman, 1993). The role of ARF1 in nucleating AP1 binding is potentially catalytic through its stimulation of phospholipase D activity (De Camilli et al., 1996), and is presumably exerted through additional proteins which have yet to be identified (Traub et al., 1993). No ARF has yet been implicated in AP2 binding to the PM, a process that is BFA-insensitive. However, AP2 binds directly to to the neuronal PM-associated protein synaptotagmin, which has been proposed as an AP2 localization determinant (Zhang et al., 1994; Li et al., 1995).

Though little is known about membrane-associated proteins that bind adaptors, the adaptor domain responsible both for receptor binding and for membrane localization has been mapped (Page and Robinson, 1995). Each adaptor is a heterotetramer, comprising two subunits of approximately 100 kDa (β1 and γ in AP1, β2 and α in AP2) and two smaller

subunits (μ1 and σ1 in AP1 of 47 and 19 kDa, μ2 and σ2 in AP2 of 50 and 17 kDa, respectively). AP1 and AP2 appear morphologically similar by electron microscopy, consisting of a core domain (head/trunk) with globular appendages (ears) (Heuser and Keen, 1988). The appendages can be removed by proteolysis (Schröder and Ungewickell, 1991), leaving behind the core domain comprising the small subunits bound to the amino-terminal fragments (approximately 70 kDa) of the large subunits. The core domains bind membranes (Peeler et al., 1993; Traub et al., 1995) and control intracellular localization through determinants on the α and γ subunits which influence association with the other subunits (Page and Robinson, 1995). The core domains also include the μ1 and μ2 subunits which can interact directly with the cytoplasmic domains of receptors (Ohno et al., 1995).

Additional membrane-associated proteins that interact with adaptor molecules need to be characterized before the mechanism of their intracellular localization and receptor sequestration can be completely understood. With this goal in mind, we sought membrane proteins that would interact with adaptors bound to an affinity matrix. This analysis led to the identification of a membrane-associated protein complex which binds specifically to AP1. This complex has some characteristics which are consistent with a role in AP1 function at the TGN. These properties include binding of the complex to AP1 and not to AP2 or other clathrin coat proteins, binding of the complex to the AP1 core domain, co-fractionation with TGN membranes and exclusion of the complex from clathrin-coated vesicles. Regardless of whether this protein complex is actually involved in localization of AP1, its identification represents a first step in analyzing membrane-associated proteins that interact with AP1 in addition to ARF and coated vesicle cargo receptors and thereby defines a new component of adaptor-membrane interactions.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies 100/1, 100/3, and GD/1 against AP1 (Ahle et al., 1988; Traub et al., 1995) and 100/2 against AP2 (Ahle et al., 1988) were from E. Ungewickell, Washington University, St Louis, MO; HC10 against HLA-B heavy chain (Stam et al., 1990) was from H. Ploegh, Massachusetts Institute of Technology, Cambridge, MA; and H68.4 against transferrin receptor (White et al., 1992) was from I. Trowbridge, The Salk Institute, La Jolla, CA. Polyclonal antibodies against the cation-independent mannose-6-phosphate receptor (Messner et al., 1989) were from S. Kornfeld, Washington University, St Louis, MO; against TGN38 were from G. Banting, University of Bristol, UK; against SRβ (Miller et al., 1995) were from P. Walter, University of California, San Francisco, CA; and against cathepsin D were from Oncogene Science. Production of monoclonal antibody TD.1 against the clathrin heavy chain was described previously (Näthke et al., 1992).

Preparation of coat protein affinity resins

Clathrin and mixed adaptors were purified from fresh bovine adrenal glands essentially as described by Manfredi and Bazari (1987). Adaptors were further chromatographed over a hydroxylapatite column (0.6 cm × 6 cm) to yield purified AP1 and AP2 (Schröder and Ungewickell, 1991). Typically, 1-2 mg AP1 was obtained from 100 adrenal glands. Coat proteins were coupled to AffiGel-10 (Bio-Rad Laboratories) at a concentration of 0.5 mg protein per ml resin, according to the instructions of the manufacturer. AP1 obtained from bovine brains was used in preliminary studies.

Biosynthetic labeling of MDBK cells

Madin-Darby bovine kidney (MDBK) cells (American Type Culture Collection) were cultured in Dulbecco's modification of Eagle's medium H-21 (Gibco BRL, Inc.) supplemented with 2 mM L-glutamine (UCSF Tissue Culture Facility) and 9% donor horse serum (Gemini Bioproducts) until approximately 80% confluent. Cells were then labeled for 16 hours with 0.5 mCi Tran^{35}S-Label/10^7 cells (ICN Biomedicals, Inc.).

Preparation of detergent-solubilized proteins

Biosynthetically labeled MDBK cells were lysed by rapid freeze/thawing. In most experiments, the ruptured cells were washed five times with 10 ml per 10 cm dish 0.6 M Tris-HCl, pH 7.0. Proteins were solubilized (5×10^6 cells/ml) in Buffer E (36.4 mM Hepes, 68.2 mM KCl, 4.1 mM Mg-acetate, 1 mM DTT, 10 μM leupeptin, 10 μM aprotinin, 0.1 mM PMSF, 2 μg/ml soybean trypsin inhibitor, 0.02% sodium azide, pH 7.3) plus 0.2% (v/v) polyoxyethylene-9-lauryl ether (polidocanol ($C_{12}E_9$), Sigma Chemical Co.). Insoluble material was removed by ultracentrifugation.

Preparation of cytosol and Tris-extracted proteins

Biosynthetically labeled cells were lysed by rapid freeze-thawing and homogenized using a Teflon/glass homogenizer. Whole-cell homogenate was centrifuged (500 ***g***, 5 minutes) and the nuclear pellet was discarded. The postnuclear supernatant was centrifuged (100,000 ***g***, 30 minutes), yielding a membrane pellet and cytosol in the supernatant. The membrane pellet was homogenized in 5 ml 0.6 M Tris-HCl, pH 7.0/10^7 cells. After 60 minutes at 4°C, the homogenate was centrifuged (100,000 ***g***, 30 minutes), yielding a stripped membrane pellet and Tris-extracted proteins in the supernatant.

Binding of proteins to affinity resins

Typically, about 1 μCi (100 μg) protein was applied to 20 μl affinity resin. Proteins were incubated with resin for 12-20 hours. After incubation, resin was washed with 5× 1 ml Buffer E with 0.1% (w/v) crystalline bovine serum albumin (Sigma Chemical Co.), then 2× 1 ml Buffer E, then 4× 1 ml 0.2 M Tris-HCl, pH 7.0. Bound proteins were eluted by incubation with 50 μl 0.7 M Tris-HCl, pH 7.0, for 1-12 hours; elution for longer than one hour was for convenience only. All manipulations were performed at 4°C, and all buffers contained 0.2% (v/v) polidocanol.

Proteolysis of AP1

To generate AP1 core domains, bovine adrenal AP1 (400 μg/ml) was incubated with 10 μg/ml TPCK-trypsin (Worthington Biochemical Corp.) for 30 minutes at 37°C. Then 200 μg/ml Soybean trypsin inhibitor (Sigma Chemical Co.) was added, and the reaction mixture was chilled to 4°C. Released appendages were separated from the AP1 core using a CentriCon-100 microfiltration device (Amicon, Inc.), which retained the core but allowed the appendages to pass through. Residual, incompletely digested AP1 was depleted from the core sample using 100/3 coupled to Protein G/Sepharose (Pharmacia Biotech, Inc.). To ensure that equimolar amounts of protein were used for subsequent affinity chromatography experiments, concentrations of intact AP1, cores, and appendages were determined by reaction with Bradford protein assay dye (Bio-Rad Laboratories) prior to coupling to AffiGel-10 resin.

Fractionation of cellular membranes

A post-nuclear supernatant was prepared from 5×10^7 or three 150 mm Petri dishes of biosynthetically labeled MDBK cells, ruptured by freeze-thaw lysis as described above. To prepare clathrin-coated vesicle enriched and depleted membranes, this supernatant was homogenized into an equal volume of 12.5% (w/v) Ficoll-400

(Pharmacia Biotech, Inc.) with 12.5% (w/v) sucrose (Campbell et al., 1984) in Buffer E. The homogenate was centrifuged at 43,700 ***g*** for 30 minutes, yielding a pellet of vesicle-depleted membranes. The supernatant was diluted five-fold with Buffer E and centrifuged (100,000 ***g***, 30 minutes) to pellet the coated vesicles. Each pellet was stripped in 0.6 M Tris-HCl, pH 7.0, and solubilized in Buffer E with 0.2% polidocanol as described above. To prepare other subcellular fractions, the post-nuclear supernatant was sequentially centrifuged at 10,000 ***g*** for 45 minutes (fraction I), 40,000 ***g*** for 60 minutes (fraction II), then 300,000 ***g*** for 90 minutes (fraction III). Pelleted membranes were resuspended in Buffer E, and aliquots of the resuspended membranes were stripped of peripheral proteins by incubation in 0.7 M Tris-HCl, pH 7.0, and then solubilized in 0.2% polidocanol and analyzed for AP1 binding proteins by application to AP1 affinity resin. Additional aliquots of the resuspended membrane fractions were analyzed for the presence of marker receptors and proteins by immunoblotting and for the following enzyme activities, as described in the reference indicated: neutral α-glucosidase (Seymour and Peters, 1977), β-hexosaminidase (Suzuki, 1987), α-mannosidase II (Tabas and Kornfeld, 1979), furin (Molloy et al., 1992) and 5′-nucleotidase (kit from Sigma Chemical Co.).

Gel filtration of MDBK proteins

Biosynthetically labeled MDBK proteins solubilized in polidocanol or affinity-purified AP1-binding proteins were resolved by gel filtration using a Superose 6 column (2 cm × 28 cm) attached to an FPLC system (Pharmacia Biotech, Inc.). Proteins were injected onto the column in 0.5 ml total volume and were eluted in Buffer E with 0.2% polidocanol at a flow rate of 1.0 ml/minute, collecting 2 ml fractions.

Triton X-114 phase partitioning

Affinity-purified AP1-binding proteins and MDBK detergent lysate were subjected separately to phase partitioning against Triton X-114 essentially as described by Bordier (1981), with the following modifications. Triton X-114 (Fluka Chemie AG) was prepared as a 12% solution (v/v) in Buffer E and was diluted to 1.5% in the protein sample. Phase partitioning was performed at 37°C. Aqueous and detergent phases from the first partitioning were partitioned twice more against fresh Triton X-114 or against Buffer E, respectively, and then analyzed.

Anion exchange of MDBK membrane proteins

MDBK membranes from 7×10^7 cells were washed in 0.6 M Tris-HCl, pH 7.0, and membrane-associated proteins were solubilized in Buffer E with 0.2% (v/v) polidocanol. Solubilized proteins in 100 μl were incubated with 10 μl Q Sepharose anion exchange resin (Pharmacia Biotech, Inc.) in batch at 4°C for 12 hours. Unbound proteins were collected. Concentrations of protein samples were estimated using Bradford protein assay dye.

Estimation of coat protein concentrations in MDBK cells by immunoblotting

Clathrin coat proteins were purified from bovine brains using standard methods (Manfredi and Bazari, 1987) and were separated by gel filtration, yielding clathrin (0.37 mg/ml) and mixed AP1/AP2 (15 μg/ml AP1 and 60 μg/ml AP2). Also, 7×10^7 MDBK cells were lysed by rapid freeze-thaw and homogenization using a Teflon/glass homogenizer. Intact cells and nuclei were removed by centrifugation at 500 ***g*** for 10 minutes, yielding 19 mg total protein in 6 ml lysate volume. Purified coat proteins were diluted to prepare SDS-PAGE samples containing clathrin (500, 250, 100, 50, and 25 ng), AP1 (100, 50, 20, 10, and 5 ng), and AP2 (400, 200, 80, 40, and 20 ng). MDBK proteins were diluted to prepare SDS-PAGE samples containing 26, 8, and 2.6 μg protein. Coat protein and MDBK protein samples were electrophoresed and transfered to nitrocellulose, and nitrocellulose was probed with monoclonal antibodies TD.1 to detect clathrin, 100/3 to detect AP1, and 100/2 to detect AP2. Coat protein levels in the MDBK lysate were estimated by visual comparison of antibody detection signals in purified coat protein and MDBK lysate samples.

SDS-PAGE, western blotting, autoradiography

Gel electrophoresis was performed according to the method of Laemmli (1970). For autoradiography, gels were incubated with Amplify (Amersham Life Science, Inc.) before drying. The dried gels then were exposed to X-OMAT AR film (Eastman Kodak Company) using an intensifying screen. For western blotting, proteins were transferred onto nitrocellulose and detected using a goat antibody to mouse immunoglobulin (Bio-Rad Laboratories) or goat antibody to rabbit immunoglobulin (Zymed Laboratories Inc.) conjugated to horseradish peroxidase, followed by ECL reagent (Amersham Life Science, Inc.) and autofluorography. Silver staining was performed according to the method of Ansorge (1985). SDS-PAGE marker proteins used were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa) (Bio-Rad Laboratories). In Fig. 1, marker proteins were prestained phosphorylase b (106 kDa), prestained bovine serum albumin (80 kDa), prestained ovalbumin (49 kDa), and prestained carbonic anhydrase (32 kDa) (Bio-Rad Laboratories).

RESULTS

Identification of AP1-binding proteins

The objective of this study was to identify membrane-associated proteins which interact with the adaptor components of clathrin-coated vesicles. Affinity resins were initially prepared from separated coat components of bovine brain clathrin-coated vesicles, AP1, AP2, clathrin and AP180. These resins were incubated with solubilized membrane fractions from biosynthetically-labeled MDBK cells. The membrane fractions were first exposed to 0.6 M Tris-HCl, pH 7.0, to remove potential competing coat proteins. It has been demonstrated previously that this treatment does not remove the binding sites for adaptors (Chang et al., 1993; Traub et al., 1993). Immobilized AP1 adaptors were the only coat component to isolate labeled proteins from solubilized MDBK membranes. Two major proteins of 83 kDa and 52 kDa were isolated, along with a number of minor bands (Fig. 1A, lane 4). To investigate the specificity of their interaction with AP1, solubilized membrane proteins were then incubated with the AP1 affinity resin in the presence of purified AP1 or purified AP2. The purified AP1 reduced binding of both the major and minor proteins to the AP1 column, while their binding was unaffected by the presence of AP2, suggesting a preferred interaction with AP1 (Fig. 1B). These preliminary results led us to investigate the specificity of the AP1-binding proteins further. First, the source of AP1 was changed to adrenal gland clathrin-coated vesicles, in which AP1 is a more abundant component. Affinity resin prepared from adrenal AP1 consistently and predominantly isolated the same 83 kDa and 52 kDa binding proteins in numerous subsequent experiments, as demonstrated in all the data shown below. Furthermore, in multiple experiments, some of which are shown below, whenever the AP1 resin eluate was reapplied to AP1 resin, only the 83 kDa and 52 kDa proteins rebound. These results convinced us that the 83 kDa and 52 kDa proteins had significant binding affinity for AP1 from two different tissue sources and prompted further analysis of their properties.

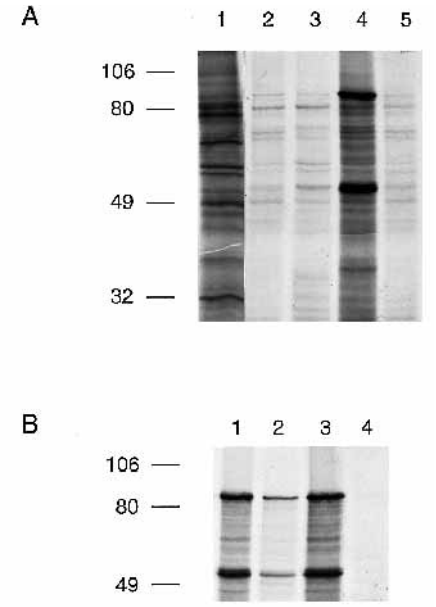

Fig. 1. Binding of MDBK membrane proteins to separated clathrin coat proteins coupled to AffiGel-10. (A) Confluent MDBK cells were biosynthetically labeled for 16 hours with Tran^{35}S-Label. Cells were lysed by freeze-thaw rupture, and residual membranes were washed extensively with 0.6 M Tris-HCl, pH 7.0. Membrane proteins were solubilized in Buffer E with 0.2% polidocanol (lane 1, diluted 50-fold; 1% of material applied to resins) and were incubated with affinity resins of AP180 (lane 2), clathrin (lane 3), AP1 (lane 4), and AP2 (lane 5). Resins were washed, and bound proteins were eluted using 0.7 M Tris-HCl, pH 7.0, and detected by SDS-PAGE and autoradiography (lanes 2-5). (B) Biosynthetically labeled MDBK membrane proteins were incubated with AP1 affinity resin (lane 1), AP1 affinity resin in the presence of equimolar free AP1 (lane 2), AP1 affinity resin in the presence of equimolar free AP2 (lane 3), and AP2 affinity resin (lane 4). Proteins eluted from the resins using 0.7 M Tris-HCl, pH 7.0, were detected by autoradiography. Migration positions of molecular mass marker proteins are indicated on the left in kDa.

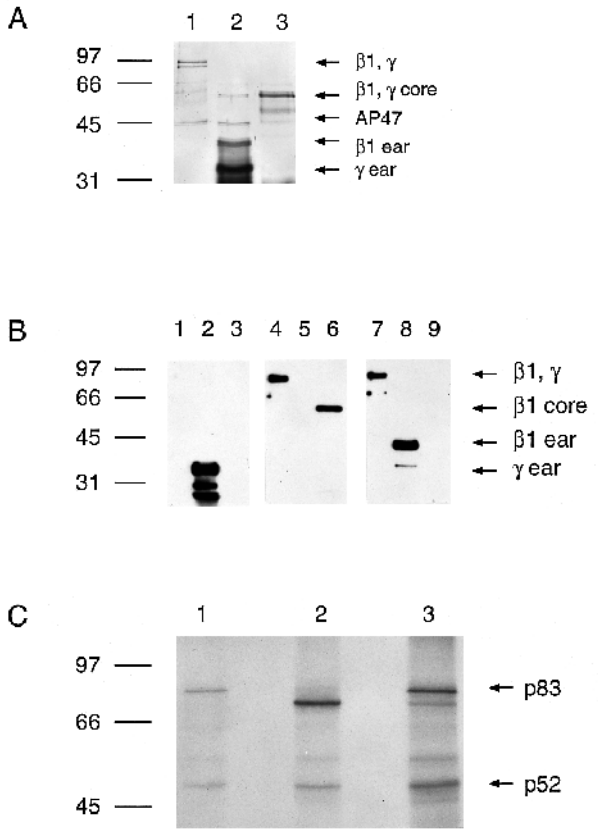

Fig. 2. AP1-binding proteins interact with the proteolytically-derived AP1 core domain. To determine what domain of AP1 associates with the 83 kDa and 52 kDa proteins, AP1 was proteolytically digested with trypsin and the fragments separated by size fractionation. Generation of appendage and core domains from intact AP1 was determined by SDS-PAGE and (A) silver staining or (B) western blotting and autofluorography. (A) Intact AP1 (lane 1), separated AP1 appendages ('ears') (lane 2), and separated AP1 cores (lane 3). (B) To confirm the identities of the fragments in A, western blots were probed with antibodies recognizing the γ appendage ('γ ear') (100/3, lanes 1-3), the β1 core fragment (100/1, lanes 4-6), and the β1 appendage ('β1 ear') (GD/1, lanes 7-9). Intact AP1 is in lanes 1, 4, and 7, separated appendages are in lanes 2, 5, and 8, and separated cores are in lanes 3, 6, and 9. No antibody to the γ core fragment was available. A longer exposure was required to detect intact AP1 with 100/3 (lane 1), consistent with the observations of Ungewickell and coworkers (Schröder and Ungewickell, 1991); signal was still absent from the core domains. Assigned positions of AP1 subunits and fragments are indicated. (C) AP1 proteolytic fragments separated by size fractionation were coupled to AffiGel-10 at equimolar concentrations. MDBK membrane proteins from biosynthetically labeled cells were incubated with affinity resins of intact AP1 (lane 1), AP1 appendages (lane 2), and AP1 cores (lane 3). Bound proteins were eluted and analyed by SDS-PAGE and autoradiography. Positions of 83 kDa and 52 kDa proteins are indicated. Migration positions of molecular mass marker proteins are indicated on the left in kDa.

Binding of membrane proteins to the AP1 core domain

AP1 association with TGN membranes is mediated primarily through targeting signals in the AP1 core domain (Page and Robinson, 1995; Traub et al., 1995). To determine which protein domain of AP1 was binding the 83 kDa and 52 kDa proteins, AP1 was digested with trypsin (Schröder and Ungewickell, 1991) and the domains separated using a CentriCon filtration device. Production of the appendage and core fragments from the β1 and γ subunits, and their separation by size fractionation was verified by silver staining (Fig. 2A) and western blotting (Fig. 2B). Appendage-specific antibodies 100/3 and GD/1 recognized intact AP1 and the separated γ and β1 appendages, respectively. The β1 core-specific antibody 100/1 recognized intact AP1 and the separated core fragment. The separated appendages and the core (containing the γ and β1 core fragments plus the 47 and 19 kDa subunits) were coupled to AffiGel-10 at approximately equimolar levels. This was ensured by determination of the protein concentration of the coupled species (correcting for the presence of proteases and inhibitors) and by confirmation that coupling to the resin was 80-90% complete based on SDS-PAGE and silver staining (not shown).

Upon incubation of labeled membrane proteins with the immobilized AP1 fragments, the 83 kDa and 52 kDa proteins bound primarily to the core domain (Fig. 2C). This result is most evident for the 83 kDa polypeptide (which, as discussed

below, is the AP1-binding component). The 83 kDa and 52 kDa membrane proteins appeared to interact more strongly with AP1 cores than with intact AP1. This may reflect an enhanced accessibility of binding sites on the cores when the appendages are removed. Such an effect of proteolytic cleavage is also observed in the enhancement of binding of the 100/3 antibody to the core domain as compared to its binding to the intact AP1 γ-subunit. This is clearly seen by the difference between protein staining and antibody reactivity in Fig. 2.

Interestingly, the appendage affinity resin efficiently bound proteins of 75 and 50 kDa which displayed only weak association with immobilized core domains and intact AP1. These distinct protein interactions for the separated AP1 domains are consistent with the reported membrane-binding behavior of chimeric adaptor proteins, which display primary membrane specificity through the core domain and a weaker specificity through the appendage domains (Page and Robinson, 1995).

Membrane association of AP1-binding proteins

Many proteins involved in clathrin-mediated vesicular transport exist in cytosolic and membrane-associated pools, including the coat proteins (Goud et al., 1985; Robinson and Kreis, 1992; Wong and Brodsky, 1992) and ARF (Donaldson et al., 1991). To determine the distribution of the 83 kDa and 52 kDa AP1-binding proteins, biosynthetically labeled MDBK cells were fractionated into cytosol, membrane-associated proteins extracted with Tris, or detergent-solubilized membrane proteins resistant to Tris extraction. These fractions were tested for the presence of the AP1-binding proteins by incubation with immobilized AP1. The 83 kDa and 52 kDa proteins were detected solely in the Tris-resistant membrane protein pool (Fig. 3A, lane 6). The membrane proteins removed by Tris extraction also included several proteins which bound to AP1 affinity resin. These are presumably clathrin coat-associated proteins, which would be expected to bind to AP1. To establish more conclusively that the AP1-binding proteins are resistant to Tris extraction, membranes were washed with increasing concentrations of Tris at pH 7.0, and the washed membranes were solubilized and incubated with AP1 affinity resin (Fig. 3B). Under wash conditions which removed AP1 itself from membranes but left behind an integral membrane protein, the heavy chain of the class I histocompatibility molecule (MHC I), the AP1-binding proteins remained membrane-associated. Only 0.8 M Tris, which partially released MHC I from membranes, caused a reduction

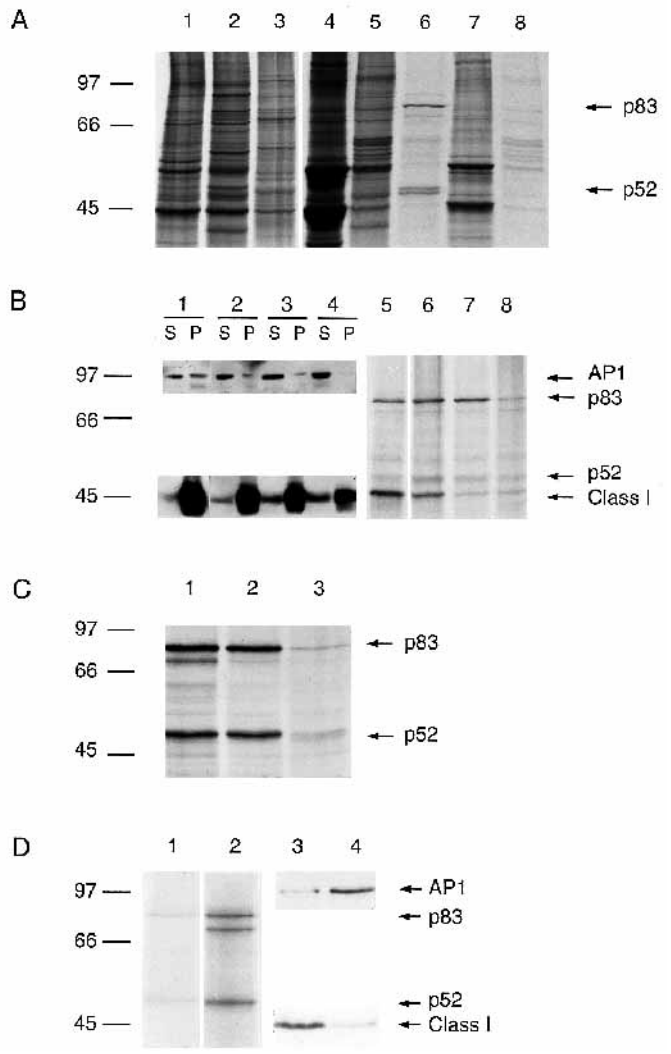

Fig. 3. AP1-binding proteins are tightly membrane-asssociated but are not integral membrane proteins. (A) Biosynthetically labeled MDBK cells were mechanically homogenized and subfractionated by ultracentrifugation and Tris extraction of membranes into coat (Tris-extracted) protein (lane 1), cytosol (lane 2), and membrane-associated (Tris-resistant) protein (lane 3) fractions. AP1 affinity resin was incubated with coat proteins (lane 4), cytosol (lane 5), membrane-associated proteins (lane 6), coat proteins diluted tenfold (lane 7), and cytosol diluted tenfold (lane 8). Proteins bound to the resin were eluted and detected by SDS-PAGE and autoradiography. (B) MDBK membranes prepared by freeze-thaw rupture of biosynthetically labeled cells were washed with Buffer E (samples 1 and 5) or with 0.4 M (samples 2 and 6), 0.6 M (samples 3 and 7), or 0.8 M (samples 4 and 8) Tris-HCl, pH 7.0. In samples 1-4, presence of AP1 and MHC I in buffer washes (S) and on washed membranes (P) was determined by western blotting and autofluorography. In samples 5-8, membranes were solubilized after buffer washes, and membrane proteins were incubated with AP1 affinity resin. AP1-binding proteins were eluted from the resin and detected by autoradiography. The prominent 45 kDa band which appears in lanes 5 and 6 is not the Class I MHC heavy chain. It is released from membranes by increasing concentrations of Tris, and it may be actin or a component of the clathrin coat. This protein was not observed in all experiments. (C) Membranes from biosynthetically labeled, freeze-thaw ruptured MDBK cells were washed with Buffer E (lane 1), 0.6 M Tris-HCl, pH 7.0 (lane 2), or 0.1 M carbonate, pH 11.2 (lane 3). Membrane-associated proteins then were incubated with AP1 affinity resin. AP1-binding proteins were detected by SDS-PAGE and autoradiography. (D) Biosynthetically labeled, affinity-purified AP1-binding proteins (lanes 1 and 2) and unlabeled MDBK membranes solubilized in Buffer E with 0.2% polidocanol (lanes 3 and 4) were phase-partitioned against Triton X-114. Detergent (lanes 1 and 3) and aqueous (lanes 2 and 4) phases then were separated and analyzed by SDS-PAGE and autoradiography to detect AP1-binding proteins (lanes 1 and 2), or by western blotting and autofluorography (lanes 3 and 4) to detect AP1 (using 100/3) and MHC I heavy chain (using HC10). The band below p83 in lane 2 is a contaminating protein. It may be a protein which was inefficiently extracted from membranes by 0.6 M Tris in this experiment, or it may be the 75 kDa protein which binds to AP1 appendages (Fig. 2C). Migration positions of molecular mass marker proteins are indicated on the left in kDa.

in AP1-binding proteins detected (Fig. 3B, lanes 4 and 8). The low level of the 52 kDa protein observed in this and some other experiments was likely due to partial dissociation from the 83 kDa protein under these washing conditions (see Fig. 6). Membranes washed in 1 M NaCl at pH 7.0 also retained AP1-binding proteins (data not shown).

Detection of the AP1-binding proteins was, however, sensitive to washing membranes with 0.1 M carbonate at pH 11.2 (Fig. 3C), a more stringent treatment to remove peripherally-associated membrane proteins (Fujiki et al., 1982). Under these conditions, AP1 was completely removed from the membranes, while MHC I remained membrane-associated (data not shown). Loss of AP1-binding protein activity in carbonate-washed membranes suggested that the proteins might be peripheral membrane proteins. To test this possibility, the 83 kDa and 52 kDa proteins were eluted from AP1 affinity resin and incubated with Triton X-114. This nonionic detergent selectively sequesters integral membrane proteins when allowed to partition away from aqueous buffers at elevated temperatures (Bordier, 1981), an activity that was confirmed by the appropriate distribution of control proteins AP1 and MHC I in the aqueous and detergent phases, respectively (Fig. 3D). Both of the AP1-binding proteins partitioned into the aqueous phase when incubated with Triton X-114 (Fig. 3D). Based on these analyses, the AP1-binding proteins behaved like tightly membrane-associated but not integral membrane proteins.

Membrane localization of AP1-binding proteins

Membrane proteins may interact with AP1 at the TGN, in clathrin-coated vesicles, or at both sites, depending on the nature of the interaction. To analyze clathrin-coated vesicles for the presence of the 83 kDa and 52 kDa proteins, MDBK cells were biosynthetically labeled, homogenized and coated vesicles were separated from other cellular membranes by velocity sedimentation over Ficoll-sucrose (Manfredi and Bazari, 1987). Vesicle-enriched and vesicle-depleted membrane proteins were solubilized and incubated with AP1 affinity resin. The 83 kDa and 52 kDa AP1-binding proteins were detected in vesicle-depleted membranes but not in coated vesicles (Fig. 4B). Their distribution corresponded to that of SRβ, a resident protein of the endoplasmic reticulum, that is not transported by clathrin-coated vesicles. In contrast, the coated vesicle fraction was enriched in clathrin and AP1 and contained significant levels of the cation-independent mannose-6-phosphate receptor (M6PR) (Fig. 4A), though some of the M6PR pool was also in vesicle-depleted membranes. Therefore, the AP1-binding proteins have a distinct distribution compared to clathrin coat proteins and coated vesicle cargo receptors.

Cellular membranes were also fractionated by differential centrifugation (Table 1) and the location of the AP1-binding proteins analyzed by binding solubilized fractions to AP1 affinity resin (Fig. 5). Fractionation of different organelles and membranes was assessed by measuring enzyme activity and by blotting for resident or associated membrane proteins. Fraction I contained all of the AP1-binding proteins and essentially all of the TGN membrane, as measured by furin activity. Fraction I also contained a number of other cellular membranes, but the distribution of TGN membranes was the only one that corresponded to the distribution of the AP1-binding proteins. Fraction III, which contained most of the clathrin-coated vesicle markers, showed the same absence of

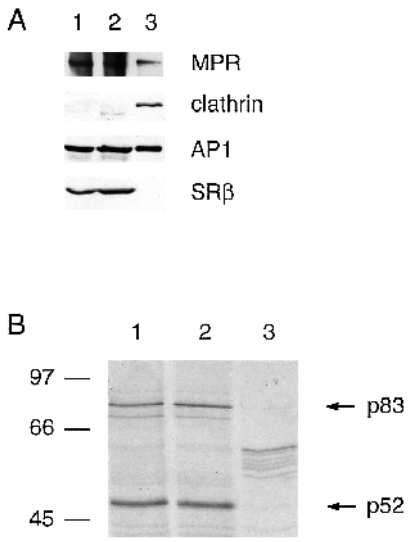

Fig. 4. AP1-binding proteins are not detected in clathrin-coated vesicles. To determine if AP1-binding proteins can be found in clathrin-coated vesicles, biosynthetically labeled MDBK cells were separated into clathrin-coated vesicles and vesicle-depleted membranes by velocity sedimentation. (A) To characterize the separation of vesicles from other membranes, unfractionated MDBK membranes (lane 1), vesicle-depleted membranes (lane 2), and coated vesicles (lane 3) were analyzed by western blotting and autofluorography for the presence of the mannose-6-phosphate receptor (MPR), clathrin, AP1, and SRβ. Note that AP1 is typically found in clathrin-coated vesicles and associated with uncoated membranes, whereas, clathrin is exclusively localized to vesicles. (B) Unfractionated membranes (lane 1), vesicle-depleted membranes (lane 2), and coated vesicles (lane 3) were washed with 0.6 M Tris, solubilized, and incubated with AP1 affinity resin. Bound proteins were eluted and detected by SDS-PAGE and autoradiography. Migration positions of molecular mass marker proteins are indicated on the left in kDa.

Table 1. Differential centrifugation of MDBK membrane fractions

	Membrane fraction*		
Marker proteins†	I	II	III
Group A			
α- Mannosidase II	42	12	46
Furin	88	0	12
β-Hexosaminidase	46	7	47
5′-Nucleotidase	56	16	28
Group B			
M6PR	+++	++	–
Clathrin	+	++	+++++
AP1	++	++	++
AP2	++	+	++
TGN38	+++	+	–
Cathepsin D	–	–	++

*Membrane fractions were produced by differential centrifugation of MDBK membranes at 10,000 ***g*** for 45 minutes (fraction I), 40,000 ***g*** for 60 minutes (fraction II), then 300,000 ***g*** for 90 minutes (fraction III).

†For Group A values indicate percentage of total cell-associated enzyme activity recovered in the respective fractions. For Group B, relative levels of proteins as detected by immunoblotting are indicated; –, no protein detected.

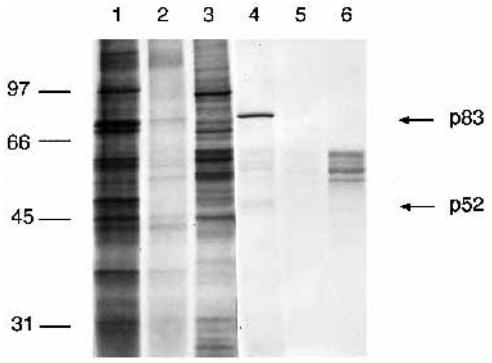

Fig. 5. AP1 binding proteins co-fractionate with TGN membranes. Biosynthetically labeled MDBK membranes were prepared by freeze-thaw lysis and fractionated by differential centrifugation, producing fractions I (lanes 1 and 4), II (lanes 2 and 5) and III (lanes 3 and 6), characterized in Table 1. Membrane fractions were then washed with 0.7 M Tris, solubilized and incubated with AP1 affinity resin. Fractions applied to the affinity resin are shown in lanes 1-3 and eluate from the resin is shown in lanes 4-6, analyzed by SDS-PAGE and detected by autoradiography. Migration positions of molecular mass marker proteins are indicated on the left in kDa. The locations of the 83 kDa and 52 kDa AP1-binding proteins are indicated at the right.

the 83 kDa and 52 kDa AP1-binding proteins but also the presence of another group of AP1-binding proteins detected in Ficoll-sucrose purified clathrin-coated vesicles (Fig. 4b, lane 3), with unknown identity.

Gel filtration of AP1-binding proteins

To establish whether the 83 kDa and 52 kDa proteins might be part of a complex with each other and/or with other AP1-binding proteins, MDBK membrane proteins were solubilized in 0.2% polidocanol and fractionated by gel filtration (Fig. 6). Fractions collected from a Superose 6 column were applied to AP1 affinity resin to determine the migration position of the 83 kDa and 52 kDa proteins. Following detergent-solubilization in Buffer E, the 83 kDa and 52 kDa proteins co-migrated on the column, and their peak detection was in the size range between markers of 270 and 630 kDa (Fig. 6C, fractions 6-8). This suggested that the 83 kDa and 52 kDa proteins were both part of a larger complex, and quite likely complexed with each other. When detergent-solubilization of membranes was carried out in 0.6 M Tris-HCl, pH 7.0, instead of the mild Buffer E, the 83 kDa and 52 kDa proteins migrated between protein markers of 99 and 190 kDa, indicating that the larger complex in which they could be found was disrupted by harsher solubilization conditions (Fig. 6D). In keeping with this observation, 83 kDa and 52 kDa proteins, that were eluted from an AP1 affinity column using 0.7 M Tris, also migrated between the 55 and 190 kDa marker proteins upon gel filtration chromatography (Fig. 6E). It should be noted that the gel filtration column experiments analyzed in Fig. 6 were all run in Buffer E with detergent and that only the conditions for membrane solubilization or protein isolation varied. Furthermore, after the different solubilization conditions, the migration of total protein profiles was not dramatically altered (Fig. 6A vs C). These data suggest that the 83 kDa and 52 kDa proteins are part of a larger protein complex that is labile upon gel filtration after detergent solubilization in 0.6-0.7 M Tris-HCl, pH 7.0.

Fractions containing the 83 kDa and 52 kDa proteins, migrating in the lower molecular mass range after elution from an AP1 affinity column, were re-applied to AP1 affinity resin to determine whether the 83 kDa and 52 kDa proteins still bound AP1 when dissociated from a larger complex. From fractions containing either the 83 kDa protein alone or both the 83 kDa and 52 kDa proteins, only the 83 kDa component bound to AP1 affinity resin (Fig. 6F). This result indicates that after exposure to high Tris and gel filtration chromatography, the 83 kDa and 52 kDa proteins were dissociated and that, once dissociated, only the 83 kDa component binds AP1. The binding of the separated 83 kDa protein to AP1 resin was less efficient than binding of the intact complex containing both the 83 kDa and 52 kDa components (Fig. 6F, and data not shown), suggesting a reduction in binding affinity after separation from the complex. The labile nature of the 83 kDa/52 kDa complex and the inability of the 52 kDa protein to bind directly to AP1 may explain its poor recovery in certain experiments (Figs 3B, 6D).

Interestingly, exposure to a high concentration of Tris caused destabilization only if the 83 kDa and 52 kDa complex was subsequently subjected to gel filtration chromatography. If the 83 kDa and 52 kDa proteins were eluted from an AP1 column with high Tris and then rebound in Buffer E, without further chromatography, binding of both the 83 kDa and 52 kDa components was detected (Fig. 6F, S). This latter observation correlates with the fact that simply washing membranes with 0.6 M Tris leaves the 83 kDa/52 kDa complex intact, in association with the membranes.

Quantitation of AP1-binding proteins in MDBK cells

In spite of a concerted effort, it has not yet been possible to purify the quantities of the 83 kDa and 52 kDa proteins which are required for their identification by protein sequencing. Our experience suggests that such affinity methods as described here are perhaps more useful for qualitative identification of binding proteins than for large-scale purification. It may be that the affinity purification, as performed, lacks a co-factor or requires different ionic conditions to optimize isolation of the AP1-binding proteins. Nonetheless, attempts at scaling up affinity purification of the AP1-binding proteins have at least made it possible to estimate the level of the 83 kDa/52 kDa AP1-binding proteins in MDBK cells. The yields from a preliminary purification procedure are summarized in Table 2.

Briefly, the procedure involves preparing an extract from Tris-washed MDBK membranes and clearing the extract over a Q-Sepharose anion exchange column prior to applying it to a large-scale AP1 affinity column. Trial experiments with radioactively labeled cell lysates indicated that 50% of the AP1-binding proteins were lost in the Q-Sepharose purification step, which removed 90% of total cellular protein, and that the AP1 affinity column bound about 50% of the applied AP1-binding proteins. Approximately equimolar amounts of the 83 kDa and 52 kDa proteins were purified by this procedure, suggesting that they form a 1:1 complex. The yields of 83 kDa and 52 kDa proteins from 7×10^7 MDBK cells, reported in Table 2, are adjusted for the fourfold loss from the two columns.

Quantitative immunoblotting was used to estimate the amount of AP1, AP2 and clathrin in 7×10^7 MDBK cells, to determine the molar ratio of AP1-binding proteins to clathrin

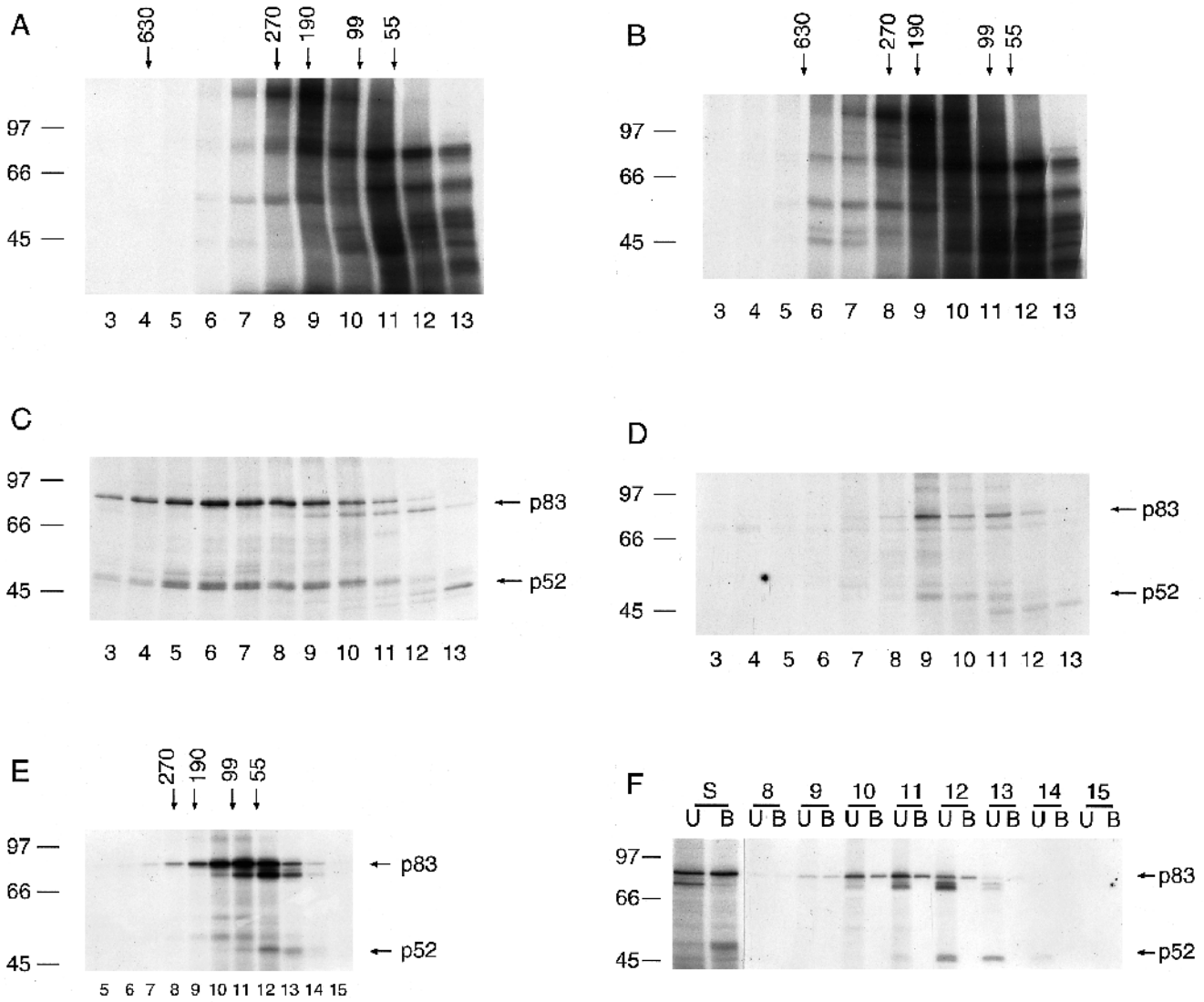

Fig. 6. Gel filtration of AP1-binding proteins. To determine if the AP1-binding proteins are complexed to each other or to other proteins, biosynthetically labeled membrane proteins prepared from freeze-thaw ruptured MDBK cells were solubilized either in Buffer E with 0.2% polidocanol (A and C) or in 0.6 M Tris-HCl, pH 7.0, with 0.2% polidocanol (B and D). Detergent-solubilized proteins were resolved by gel filtration on Sepharose 6. Fractions 3 to 13 were analyzed by SDS-PAGE and autoradiography (A and B). The void volume (2,000 kDa dextran; Pharmacia Biotech, Inc.) eluted in fraction 1. Elution positions of membrane protein markers, as determined by western blotting and autofluorography, are indicated above the figures: 630 kDa, clathrin triskelion; 270 kDa, M6PR; 190 kDa, transferrin receptor dimer; 99 kDa, SRα/SRβ (blotted for SRβ); 55 kDa, MHC I (blotted for heavy chain). The corrresponding gel filtration fractions were incubated with AP1 affinity resin, and bound proteins were eluted and detected by SDS-PAGE and autoradiography (C and D). Fraction numbers are indicated below the lanes. Amounts of protein analyzed in the two experiments were different, accounting for the different levels of proteins in C and D. (E) Biosynthetically labeled AP1-binding proteins were prepared by affinity chromatography using AP1 resin and 0.7 M Tris elution, then were subjected to gel filtration in Buffer E with 0.2% polidocanol using the same column as in A-D. Fractions 5 to 15 were analyzed by SDS-PAGE and autoradiography. Elution positions of membrane proteins (as for A and B) are indicated for reference above (these proteins were not detected among the AP1-binding proteins). Fraction numbers are indicated below the lanes. (F) Unfractionated AP1-binding proteins (eluted from AP1 resin with 0.7 M Tris, sample S) and gel filtration fractions 8 to 15 of affinity-purified AP1-binding proteins (E) were applied to AP1 affinity resin. Unbound (U) and bound (B) proteins were detected by SDS-PAGE and autoradiography. Migration positions of molecular mass marker proteins are indicated on the left in kDa. The positions of the 83 and 54 kDa proteins are indicated at the right and in C refer to the bands in the central lanes and not the distorted side lanes.

coat proteins. Clathrin was estimated to be 0.3% of total protein, in agreement with the values obtained by Goud et al. (1985) from several different cell types. AP2 was approximately equimolar to clathrin and there was half as much AP1 as AP2, also consistent with the expected stoichiometry. Based on these calculations, the 83 kDa/52 kDa complex is present in a 1:10 ratio relative to AP1.

DISCUSSION

The association of AP1 with the *trans*-Golgi network and AP2 with the plasma membrane is thought to be critical for determining when and where clathrin-coated pits form. To understand this process, it is necessary to characterize how adaptors interact with membranes. This study identifies membrane-

Table 2. Quantitation of proteins in 7×10^7 MDBK cells

Protein quantitated	Amount*	Moles
Total protein†	19 mg (B)	
Membrane-associated protein‡	1.0 mg (B)	
Anion exchange flow-through§	0.12 mg (B)	
AP1 binding proteins¶: 83 kDa	400 ng (S)	4.8×10^{-12} moles
52 kDa	200 ng (S)	3.8×10^{-12} moles
Total AP1	14 μg (I)	5.4×10^{-11} moles
Total AP2	28 μg (I)	1.1×10^{-10} moles
Total clathrin triskelions	54 μg (I)	8.6×10^{-11} moles

*Amounts of proteins were determined by Bradford assay (B), silver staining and comparison with standards (S), or immunoblotting and comparison with standards (I) as indicated.

†Total protein was obtained by freeze-thaw lysis and homogenization of 7×10^7 MDBK cells, followed by centrifugation at 500 ***g*** to remove intact cells and nuclei.

‡Total protein sample was centrifuged to pellet membranes, then extracted with 0.6 M Tris-HCl, pH 7.0, to obtain membrane-associated (Tris-resistant) protein, which was solubilized in Buffer E with 0.2% polidocanol.

§Membrane-associated protein was applied to Q Sepharose anion exchange resin, and flow-through (unbound protein) was collected.

¶Anion exchange flow-through was applied to AP1 affinity resin. Bound proteins were eluted with 0.7 M Tris-HCl, pH 7.0, with 0.2% polidocanol. The only bands visible by silver staining were the 83 kDa and 52 kDa proteins previously identified by autoradiography.

associated proteins of 83 and 52 kDa which interact with AP1 in vitro and are the predominant AP1-binding proteins in MDBK membrane preparations whose endogenous coat proteins have been removed. These polypeptides bind specifically to AP1 but not to AP2 or other clathrin coat proteins and they interact with the AP1 core domain which contains target signals for intracellular localization (Page and Robinson, 1995; Traub et al., 1995). Both proteins are tightly membrane-associated but are not integral membrane proteins, and they are excluded from clathrin-coated vesicles and co-fractionate with TGN membranes. Gel filtration data suggest that the 83 kDa and 52 kDa components are complexed with each other. The 83 kDa component mediates binding to AP1, either directly or through an undetected (unlabeled) associated component, while the 52 kDa component must bind AP1 indirectly. The 83 kDa and 52 kDa proteins are present in a 1:10 molar ratio with AP1 in MDBK cells. The overall properties of these AP1-binding proteins suggest a specific function in AP1-membrane interactions. It should be noted that several additional AP1-binding proteins were identified in this study but as they either bound at a low level, bound only AP1 fragments or were not membrane-associated, they were not selected as a focus for this investigation. Concurrent with the studies reported here, Robinson's laboratory (Seaman et al., 1996) used a cross-linking approach to identify AP1-binding proteins and have found an 80 kDa rat protein that interacts with the β1-subunit, that may be the same as the 83 kDa component of the complex we have identified, in bovine cells.

In certain respects, the 83/52 kDa AP1-binding proteins have characteristics which might be expected of proteins involved in AP1 localization to the TGN. These include their binding to the AP1 core domain and the fact that they do not interact with AP2. AP2-membrane binding is clearly regulated by different factors from those which mediate AP1 binding to the TGN (Robinson and Kreis, 1992; Wong and Brodsky, 1992; Zhang et al., 1994; Li et al., 1995). In addition the 83/52 kDa proteins have membrane association properties, an intracellular distribution and relative abundance that are consistent with a role in AP1 localization. It is conceivable that peripheral membrane proteins, such as the 83/52 kDa proteins, could form an AP1 binding site in combination with an integral membrane protein of the TGN or directly recognizing the lipid bilayer. Different intracellular membranes have characteristically distinct lipid compositions (van Meer, 1993) which could themselves be recognized by localization proteins. Interestingly, AP2 binds to liposomes having the approximate composition of the plasma membrane bilayer (Beck et al., 1992), and has an affinity for inositol phosphates.

Analysis of MDBK membranes which were subfractionated by differential centrifugation indicated that the 83/52 kDa AP1-binding proteins co-fractionated with TGN membranes, although did not conclusively demonstrate that the binding complex is restricted to the TGN. Furthermore, the 83/52 kDa proteins were not detected in clathrin-coated vesicles. This latter observation rules out the possibility that they are coated vesicle cargo proteins. The predicted function of an AP1 localization protein may necessitate its exclusion from clathrin-coated vesicles. AP1 would be targeted to TGN membranes through high-affinity binding to a localization protein, but then be 'handed off' to interact with the cytoplasmic domains of receptors with lower affinity, as a coated pit polymerizes into a vesicle, leaving the localization protein behind in the membrane. Through this mechanism, the localization protein would remain localized, and its absence from coated vesicles would allow AP1 to dissociate easily from vesicle membranes during uncoating. This model also is consistent with the low abundance of the 83/52 kDa complex in MDBK cells, with about ten copies of AP1 for each 83 kDa protein. Such an excess of AP1 over a localization complex might be expected, if the localization complex is required only to initiate clathrin coat assembly by recruiting AP1 onto the TGN membrane but does not form a long-lived interaction with AP1.

The property of the 83/52 kDa complex that is least consistent with what might be predicted for an AP1 localization protein is that its interaction with AP1 is apparently insensitive to regulation by GTP-binding proteins. In in vitro membrane-binding assays, binding of AP1 depends on activated ARF (Robinson and Kreis, 1992; Wong and Brodsky, 1992; Stamnes and Rothman, 1993; Traub et al., 1993). However, binding of the 83 and 52 kDa proteins to AP1 affinity resin was insensitive to treatments which alter ARF activity. When brefeldin A and GTPγS were applied to MDBK cells in culture or to solubilized membrane proteins prior to incubation with AP1 affinity resin, binding of the 83/52 kDa proteins was neither consistently inhibited nor enhanced (not shown). In preliminary experiments, adding recombinant ARF1 (gift of W. Cieplak) and GTPγS to solubilized membrane proteins also failed to influence the binding of the 83 and 52 kDa proteins to AP1 affinity resin. However, these findings do not necessarily exclude the 83/52 kDa proteins from consideration as AP1 localization proteins. If the function of ARF is simply to activate localization proteins for AP1 binding (Traub et al., 1993; De Camilli et al., 1996), then AP1 binding by the 83/52 kDa proteins may reflect the behavior of pre-activated localization proteins. The inability to detect an influence of brefeldin A and GTPγS on their binding could also be due to a loss of effector proteins under the binding conditions used.

The studies described here identify membrane-associated AP1-binding proteins that represent novel participants in adaptor-membrane interactions, irrespective of whether they function in AP1 localization. The affinity approach used has not been amenable to large-scale purification but the qualititative analysis that it has made possible has established biochemical properties of the AP1-binding proteins and demonstrates their reproducible and specific interaction with AP1 in vitro. Further characterization of these proteins, using conventional biochemical methods or genetic approaches to isolating larger quantities, will provide insight into how adaptors interact with cellular membranes. Understanding the complexities of this interaction has long been a challenge in the field and this work represents an initial step in defining components involved.

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