The AP-1 Adaptor Complex Binds to Immature Secretory Granules from PC12 Cells, and Is Regulated by ADP-Ribosylation Factor

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Abstract. Immature secretory granules (ISGs) in endocrine and neuroendocrine cells have been shown by morphological techniques to be partially clathrin coated (Orci, L., M. Ravazzola, M. Amherdt, D. Lonvard, A. Perrelet. 1985*a*. *Proc. Natl. Acad. Sci. USA*. 82: 5385–5389; Tooze, J., and S.A. Tooze. 1986. *J. Cell Biol.* 103:839–850). The function, and composition, of this clathrin coat has remained an enigma. Here we demonstrate using three independent techniques that immature secretory granules isolated from the rat neuroendocrine cell line PC12 have clathrin coat components associated with their membrane. To study the nature of the coat association we have developed an assay whereby the binding of the AP-1 subunit γ-adaptin to ISGs was reconstituted by addition of rat or bovine brain cytosol. The amount of γ-adaptin bound to the ISGs was ATP independent and was increased fourfold by the addition of GTPγS. The level of exogenous γ-adaptin recruited to the ISG was similiar to the level of γ-adaptin present on the ISG after isolation. Addition of myristoylated ARF1 peptide stimulated binding. Reconstitution of the assay using AP-1 adaptor complex and recombinant ARF1 provided further evidence that ARF is involved in γ-adaptin binding to ISGs; BFA inhibited this binding. Trypsin treatment and Tris-stripping of the ISGs suggest that additional soluble and membrane-associated components are required for γ-adaptin binding.

In neuroendocrine and endocrine cells, secretory granules bud from the *trans*-Golgi network (TGN) when a dense-core aggregate, containing sorted regulated secretory proteins, is enveloped by a specifc membrane. These newly formed secretory granules have been referred to as immature secretory granules (ISGs),[1] as they have been shown to be an intermediate in the biogenesis of secretory granules. During storage in the cell and vectorial transport to the plasma membrane, the ISG is converted into a mature secretory granule (MSG): immature and mature secretory granules can be distinguished by their morphological (Farquhar et al., 1978; Orci et al., 1985*a*; Tooze and Tooze, 1986) and biochemical properties (Tooze et al., 1991). ISGs are morphologically characterized as those secretory granules that are proximal to the TGN, have a partially condensed dense core, and an irregular, loose membrane. Furthermore, a clathrin coat is present on parts of the ISG membrane as was demonstrated by immunogold labeling of the ISGs with anti-clathrin antibodies (Orci et al., 1985*a*; Tooze and Tooze, 1986). In addition, the processing of pro-hormones, for example pro-insulin, has been shown to begin in the ISG (Orci et al., 1985*b*).

MSGs, on the other hand, occur distal from the TGN and have highly condensed dense cores and a more uniform limiting membrane. Clathrin coats have not been detected on MSGs (Orci et al., 1985*a*; Tooze and Tooze, 1986). Furthermore, MSGs contain predominantly fully processed hormones (Orci et al., 1985*b*). The maturation of the ISG to an MSG involves translocation of the maturing ISG to the periphery of the cell, changes in the structure of the dense core (Michael et al., 1987), and in the size of the ISG (Farquhar et al., 1978; Tooze et al., 1991; Bauerfeind and Huttner, 1993). Concomitant with these changes is the loss of the clathrin coat from the membrane of the maturing ISG.

Several intracellular membranes, for example the plasma membrane and the TGN, have regions which are clathrin coated (for review see Pearse and Robinson, 1990). The clathrin coats on the plasma membrane mediate the clustering of ligand-bound *trans*-membrane receptors and participate in the formation of coated vesicles (for reviews see Pearse and Robinson, 1990; Schmid, 1993). The clathrin coat is comprised of clathrin triskelions and adaptor proteins which are recruited from the cytoplasm (for reviews see Pearse and Robinson, 1990; Pley and Parham, 1993).

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1. *Abbreviations used in this paper*: AP, adaptor proteins; ARF, ADP-ribosylation factor; BFA, brefeldin A; CSV, constitutive secretory vesicle; CgB, chromogranin B; GTPγS, guanosine 5′-*O*-3-thiotriphosphate; hsPG, heparan sulfate proteoglycan; ISG, immature secretory granule; MSG, mature secretory granule; Pool 1, adaptor-enriched pool; PNS, postnuclear supernatant; SgII, secretogranin II.

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So far, two classes of adaptor protein complexes have been described: the AP-2 complex which is found primarily at the plasma membrane, and the AP-1 complex which is localized predominantly to the Golgi complex (for review see Robinson, 1993). The binding of AP-1 to Golgi membranes requires ARF1, a low molecular weight GTP-binding protein (Stamnes and Rothman, 1993; Traub et al., 1993). The amino-terminal region of ARF contains the information essential for ARF function and may be the region of the molecule which interacts with the effector protein, while the association of ARF with the membrane is regulated by GTP hydrolysis and mediated by the myristic acid moiety at the NH_2-terminus (Boman and Kahn, 1995). ARF1 has been identified as a component of Golgi-derived nonclathrin-coated (COP-coated) vesicles (Serafini et al., 1991), clathrin-coated vesicles isolated from brain cytosol (Lenhard et al., 1992), and of post-Golgi vesicles (Nickel et al., 1994).

The AP-1 and AP-2 adaptor complexes are highly homologous heterotetramers comprised of two distinct subunits each with a molecular mass in the range of 110 kD, a 50-kD subunit, and an 18-kD subunit. The 110-kD subunits in the AP-2 complex are referred to as α and β, those in the AP-1 complex are designated γ and β′ (for review see Robinson, 1992). The highly homologus β- and β′-subunits mediate the interaction of the adaptor complex with the clathrin triskelions (Ahle and Ungewickell, 1989). The α- and γ- subunits are believed to confer the required binding specificity of AP-2 and AP-1, and have been postulated to bind to components in the membrane (Wong and Brodsky, 1992; Robinson, 1994). γ-Adaptin binding to Golgi membranes occurs via association with the cytoplasmic domain of the mannose-6-phosphate receptor (M6PR) (Glickman et al., 1989; Le Borgne et al., 1993), and it has been suggested that this association, together with the ARF-mediated binding may be sufficient for clathrin-coat formation (Ludwig et al., 1995). In contrast, Traub and colleagues have recently proposed a model, based on their results and those of others (Wong and Brodsky, 1992; Robinson, 1993; Traub et al., 1993, 1995) in which the initial interaction of γ-adaptin with Golgi membranes requires γ-adaptin receptors or "docking" proteins. Traub et al. (1995) propose that these docking proteins, in addition to ARF, bind to the amino-terminal core domain of γ-adaptin, before the interaction of the carboxy-terminal appendage with the cytoplasmic domain of a transmembrane receptor.

Although a clathrin coat has been seen by electron microscopy on a wide variety of ISGs in different cell types (Orci et al., 1985*a*; Tooze and Tooze, 1986), its function is unknown. One unlikely possibility is that the clathrin coat has no specific function and is simply a remnant of the budding of the ISG from the TGN, and that during the maturation process the clathrin coat slowly dissociates from the ISG. Two lines of evidence argue against this possibility. First, the clathrin coat has never been seen by electron microscopy to encompass the entire surface of the nascent secretory granule budding from the TGN, thus it is unlikely that a clathrin coat physically drives the budding of the ISG. Second, the clathrin coat on the ISG often appears to be enveloping small vesicular structures in the process of budding from the maturing granule (Tooze and Tooze, 1986). These electron micrographs strongly suggest that the clathrin coat is involved in the removal of membrane and content from the ISG by vesicular traffic and is not a functionless vestige.

Regardless of their function, clathrin coats are present on the surface of the ISGs and this raises the question which class of adaptor complex is responsible for the clathrin binding. Since the AP-1 complex is concentrated in the Golgi region of all cell types, and has been shown to bind to Golgi membranes (Robinson and Kreis, 1992; Wong and Brodsky, 1992; Le Borgne et al., 1993, Stamnes and Rothman, 1993; Traub et al., 1993) and mediate the interaction of clathrin with the Golgi (Traub et al., 1995), it is a priori the most likely candidate, however, this has not been previously established experimentally. Elucidation of this question by morphological techniques is not straightfoward because of the low numbers of ISGs in regulated secretory cells, and the limited extent of the clathrin coats on the ISGs. Here we demonstrate that γ-adaptin is present on ISGs from PC12 cells, and to obtain further information regarding the role of the clathrin coat on the ISGs and the components involved in coat association, we have developed an assay to reconstitute binding of γ-adaptin to ISGs.

Materials and Methods

Reagents

Carrier-free [^{35}S]sulfate, ^{125}I-protein A, and [^{3}H]myristic acid were from Amersham (Little Chalfont, UK). Nucleotides, creatine phosphate, and creatine phosphokinase were from Boehringer Mannheim (Mannheim, Germany). Brefeldin A (BFA), myristic acid, *p*-nitrophenyl-α-D-mannoside, soybean trypsin inhibitor and trypsin were from Sigma (Poole, UK). Fine chemicals were from BDH (Lutterworth, UK), Boehringer Mannheim, GIBCO-BRL (Paisley, UK), or Sigma Chem. Co. (St. Louis, MO).

Cells and Antibodies

PC12 cells (clone 251; Heumann et al., 1983), originally obtained from Dr. H. Thoenen (Martinsried/Germany), were maintained as described (Tooze and Huttner, 1990). Monoclonal antibodies against clathrin (TD.1) (Nähtke et al., 1992), bovine γ-adaptin (100/3) (Ahle et al., 1988), and ARF (1D9) were used at a dilution of 1:1,000. Monoclonal antibodies to TGN38 (2F7.1) (Horn and Banting, 1994) and chromogranin B (CgB) (219.6) (Rosa et al., 1989) were used at a dilution of 1:500. We raised a rabbit polyclonal antibody (STO-25) directed against γ-adaptin as follows. The complete mouse γ-adaptin hinge region (Robinson, 1990) was cloned via PCR in frame to the COOH terminus of GST using the pGEX-3X system (Pharmacia, Milton Keynes, UK). The fusion protein was expressed in BL21 cells and purified using glutathione Sepharose 4B (Pharmacia) according to the manufacturer's protocol. The antibody was raised by immunization of a rabbit with the fusion protein and used at a dilution of 1:250. For competition experiments the antibody was preincubated for 16 h at 4°C with 0.1 mg/ml GST/γ-adaptin hinge fusion protein or with GST alone.

Radiolabeling of PC12 Cells

PC12 cells were pulse labeled with [^{35}S]sulfate and chased at 37°C as described (Tooze and Huttner, 1990; see figure legends for details). To label TGN, PC12 cells were incubated for 5 min with 1 mCi [^{35}S]sulfate × ml^{-1}. Labeling of ISGs and constitutive secretory vesicles was achieved by incubation for 5 min with 1 mCi [^{35}S]sulfate × ml^{-1} and a subsequent chase for 15 min, whereas to label MSGs, PC12 cells were incubated for 6 h with 0.2 mCi [^{35}S]sulfate × ml^{-1} and chased for 12 h.

Preparation of PC12 Immature and Mature Secretory Granules

ISGs, CSVs, and MSGs were prepared from PC12 cells by velocity and equilibrium sucrose gradient centrifugation. Six 150-cm^2 dishes of cells, grown to 80% confluency, were used to prepare a postnuclear supernatant (PNS) as previously described (Tooze and Huttner, 1992), which was subjected to sucrose gradient fractionation except that a step gradient (0.8 M–1.6 M sucrose) was used instead of a continuous gradient for the equilibrium gradient centrifugation. The ISGs used for the binding assay (fractions 7–9) contained ~0.5 μg/μl protein. Protein concentrations of the respective fractions were determined using (BioRad, Hemel Hempstead, UK) IgG as a standard.

Preparation and Gel Filtration of Bovine or Rat Brain Cytosol

Bovine brain cytosol was prepared as described (Malhotra et al., 1989). Rat brain cytosol was prepared from three rat brains homogenized in 25 mM Hepes, 25 mM KCl, 2.5 mM MgOAc (pH 7.2). Nuclei, membranes, and other sedimentable components were removed by centrifugation at 30,000 *g* for 30 min at 4°C followed by a centrifugation at 100,000 *g* for 90 min. Bovine and rat brain cytosols were dialyzed against 25 mM Hepes, 25 mM KCl, 2.5 mM MgOAc (pH 7.2). Before use the cytosol was incubated for 30 min at 37°C and was clarified for 1 h at 100,000 *g* at 4°C in a TL-100 table top ultracentrifuge except in Fig. 7 *A*.

For the preparation of a γ-adaptin–enriched pool, the bovine brain cytosol (2 ml of 22.3 mg/ml) was fractionated by size on a preparative grade Superose 6 column (1.6 × 51 cm, 100-ml bed vol, Pharmacia) according to Stamnes and Rothman (1993), using 25 mM Hepes, 25 mM KCl, and 2.5 mM MgOAc (pH 7.2). Each column fraction (1 ml vol) was analyzed by Western blotting for the presence of clathrin, γ-adaptin, and ARF. Fractions containing γ-adaptin but not clathrin or ARF were pooled (Pool I), and concentrated 10-fold in a Centricon microconcentrator (Amicon, Stonehouse, UK).

Preparation of Recombinant Myristoylated ARF1

Recombinant myristoylated ARF1 (mARF1) was purified from *Escherichia coli* (strain BL21[DE3]) coexpressing the ARF1 gene in pET11d and yeast *N*-myristoyltransferase in pBB131 as described (Randazzo et al., 1992) with the following modifications: the bacterial suspension was sonicated for 3 × 30 s at the maximal settings with an Ultrasonic Processor (Jencons Scientific, Leighton Buzzard, UK) on ice before clarification by centrifugation at 100,000 *g* for 60 min at 4°C and the final gel-filtration step was excluded from the purification protocol. The resulting flow-through and wash from the DEAE column were concentrated to 5 ml using a YM10 membrane and dialyzed against 25 mM Hepes, 25 mM KCl, and 2.5 mM MgOAc (pH 7.2) (Amicon, Inc., Beverly, MA). As a control uninduced bacteria were grown and processed in the same way. Each purification step was analyzed for the presence of ARF1 by 15% SDS-PAGE and immunoblotting with mAb 1D9. Myristoylation was followed by the incorporation of [^{3}H]-myristic acid into the newly induced protein (Helms et al., 1993).

Cell-Free Assay to Reconstitute γ-Adaptin Binding to PC12 Secretory Granules

Typically, 125 μl of ISGs were mixed with varying amounts of rat brain cytosol or bovine brain cytosol (0–5.0 μg/ml) (for details see figure legends) in a final volume of 250 μl containing 25 mM Hepes, 25 mM KCl, and 2.5 mM magnesium acetate (pH 7.2) (binding buffer). Where indicated, 8.3 μl of an ATP-regenerating system (Tooze and Huttner, 1990) were added. In all experiments the ATP-regenerating system was used as the source of ATP. The amounts and types of other nucleotides present in the reactions are described in the figure legends.

Alternatively, 125 μl ISGs and crude recombinant mARF1 (100 μg protein) or a noninduced bacterial control sample (100 μg protein), prepared as described above, were incubated with or without 100 μM GTPγS for 10 min at 37°C in binding buffer (1st step). After the addition of the γ-adaptin containing Pool I (50 μg protein), prepared by size fractionation of bovine brain cytosol as described above, and the ATP-regenerating system or other nucleotides, as indicated in the figure legends, a further incubation was performed for 20 min at 37°C (2nd step). When BFA was used, the ISGs were preincubated for 10 min at 37°C with 10 μg/ml BFA before the 1st step. Secretory granules were preincubated for 10 min at 37°C with the $[AlF_4]^-$ (prepared freshly from 50 mM $NH_4Al[SO_4]_2$ and 1 M KF stock solutions) before cytosol was added. A peptide representing the first 14 NH_2-terminal amino acids of human ARF1 (ARF1pep) (Kahn et al., 1992) as well as its N-myristoylated form (mARF1pep) or myristic acid alone were added to the assay at a concentration of 100 μM where indicated. Peptides were added from a 10-mM stock solution in dH_2O (ARF-pep) or DMSO (mARFpep). A 10-mM stock solution of myristic acid was prepared in DMSO.

After incubation for 30 min at 37°C, 750 μl of ice cold binding buffer were added and the secretory granules were pelleted by centrifugation for 60 min at 4°C with 45,000 rpm in a TLA-45 rotor using a TL-100 table top ultracentrifuge. All assays were done at least in duplicate. The pellets were resuspended in 20 μl of SDS sample buffer, analyzed by SDS-polyacrylamide minigels, and subjected to immunoblot analysis followed by incubation with ^{125}I-protein A. After exposure to film the amount of radioactivity bound was quantitated with a phosphorimager system (Molecular Dynamics, Chesham, UK).

Membrane Stripping and Trypsin Pretreatment of ISGs

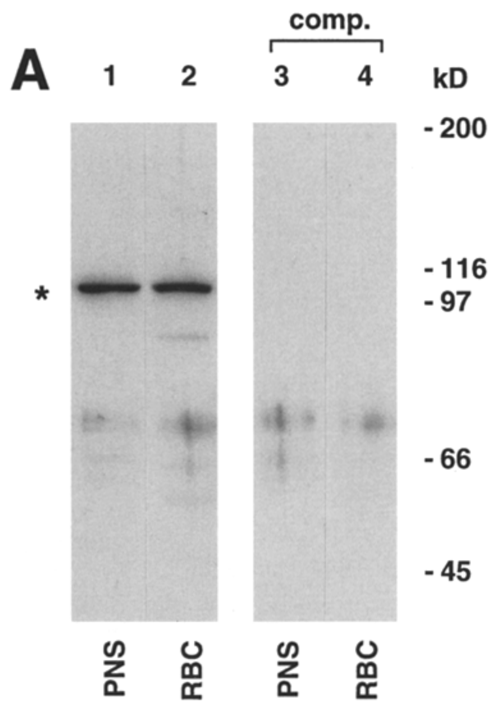
To remove peripheral-associated proteins from the ISG membrane (stripping), the granules were preincubated before the binding assay in 0.5 M KCl, 0.5 M Tris/HCl, pH 7.2, or 10 mM Hepes pH 7.2 (control). After a 15-min incubation on ice, the granules were sedimented through a sucrose cushion (0.5 M sucrose, 10 mM Hepes, pH 7.2) for 1 h with 100,000 *g* at 4°C and were resuspended in 1.2 M sucrose, 10 mM Hepes, pH 7.2. For trypsinization of exposed membrane proteins, the ISGs were incubated for 15 min at 37°C with or without trypsin as indicated. The reaction was terminated by the addition of 1 mg/ml soybean trypsin inhibitor (STI). As a control STI was added before the incubation with trypsin. The subsequent γ-adaptin–binding assay was carried out as described above.

Immunoisolation

Immunoisolation of sulfate-labeled TGN, ISGs, or MSGs using polyclonal antibody STO-25 was carried out as previously described (Urbé et al., 1993) except BSA was omitted during all incubation steps. For competition experiments the bound antibody was saturated before the immunoisolation with 0.1 mg/ml GST/γ-adaptin–hinge fusion protein, and the fusion protein was added at the same concentration throughout the immunoisolation. For the immunoisolation 100 μl of the TGN enriched fraction 9 of the velocity gradient, ISG equilibrium gradient fraction 8, and MSG equilibrium gradient fraction 11 were used, respectively.

Immunoelectron Microscopy

ISGs incubated with rat brain cytosol were fixed in 2% paraformaldehyde in 0.1 M Sørensen's phosphate buffer, pH 7.4, for 2 h and embedded in 10% gelatine, immersed in 2.3 M sucrose/PBS for 4 h, and frozen in liquid nitrogen. Ultrathin cryosections were cut on an UltracutS microtome with FC4E cryo attachment and transferred onto Formvar-coated grids. Sections were quenched for 15 min with 50 mM glycine, blocked for 15 min with 0.5% fish skin gelatin in PBS (FSG/PBS), and then labeled with STO-25, preimmune sera at a 1:5 dilution in 0.05% FSG/PBS, or the immune sera preincubated with the GST-fusion protein as described above. After an overnight incubation at 4°C the sections were washed three times over a 15-min period with 0.05% FSG/PBS, and then were labeled with a 1:50 dilution of protein A–conjugated to 5-nm colloidal gold (Slot and Geuze, 1985) in 0.05% FSG/PBS for 30 min. After three 5-min rinses in PBS, the sections were fixed in 2.5% glutaraldehyde in PBS for 5 min, and then washed three times for 5 min in PBS and dH_2O. After immunolabeling the sections were stained with uranyl acetate in dH_2O and embedded in PVA as described by Tokuyasu (1989). The sections were examined either with a JEOL 1200 FX or Zeiss10C electron microscope. Quantitation of the number of gold particles was performed using fields similar to those in Fig. 5, *a* and *b*. The background (i.e., gold particles over section areas without membranes) observed with each condition was extremely low and therefore ignored. Grids were examined at magnifications of 120,000. For each treatment, number of gold particles bound specifically per ISG was counted on a 60 $μm^2$ surface of random sections on three grids. Measurements were performed directly with a JEOL 1200 EX electron microscope with a video attachment.



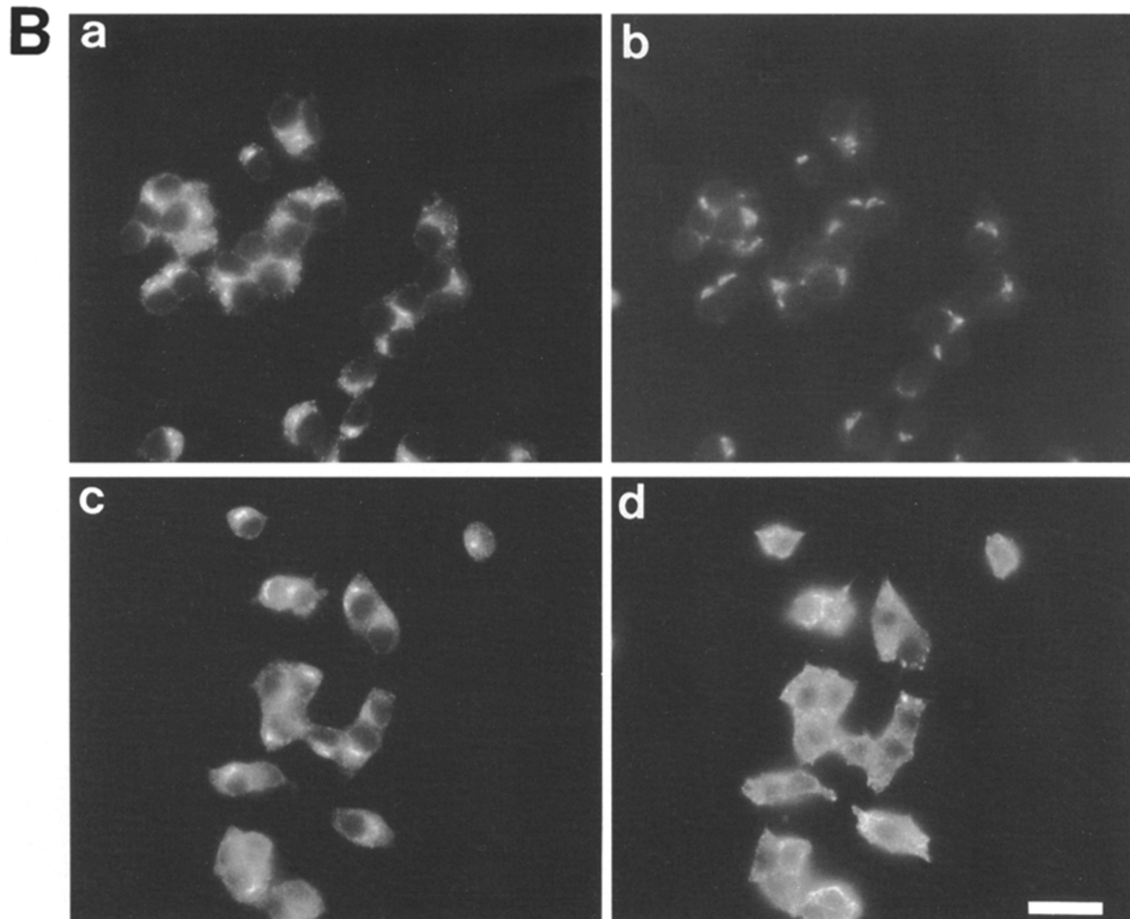


Figure 1. Characterization of the anti–γ-adaptin antibody ST0-25. (*A*) Equal amounts of protein either from a PC12 cell PNS (lanes *1* and *3*) or rat brain cytosol (lanes *2* and *4*) were immunoblotted with ST0-25 (lanes *1* and *2*) or with ST0-25 preincubated with the GST/γ-adaptin hinge fusion protein (*comp.*; lanes *3* and *4*). The asterisk indicates γ-adaptin. (*B*) Double labeling of PC12 cells for γ-adaptin using ST0-25 (*a* and *c*), and monoclonal antibodies to either TGN38 (*b*) or CgB (*d*) by indirect immunofluorescence. ST0-25 is revealed by anti-rabbit antibodies conjugated to Cy3, while TGN38 and CgB are revealed by anti-mouse antibodies conjugated to DTAF. Note, different fixation conditions are used in *a* and *b* and *c* and *d*, which leads to slight differences in the appearance of the ST0-25 labeling. Bar equals 10 μm.

Indirect Immunofluorescence

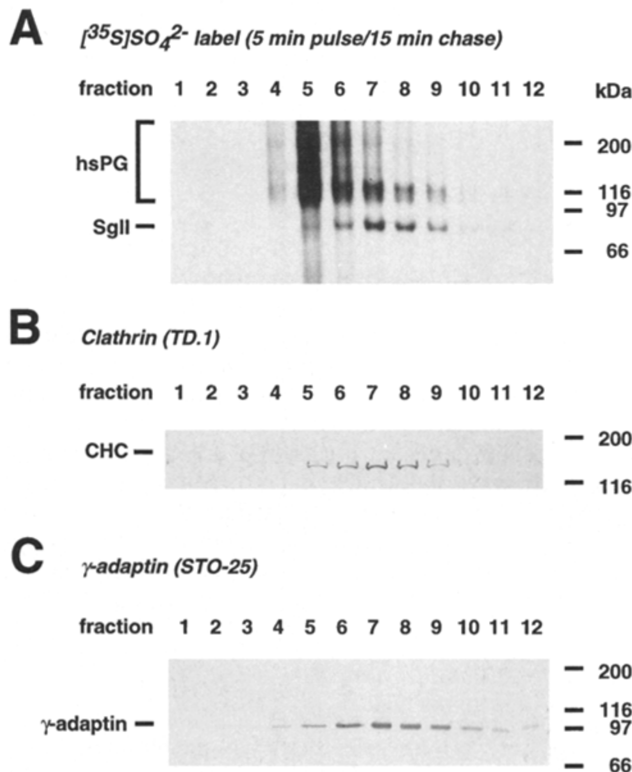
For double labeling with antibodies against γ-adaptin and CgB, the PC12 cells were fixed and processed as previously described (Dittié and Tooze, 1995). For double labeling with antibodies against γ-adaptin and TGN38 the cells were fixed for 10 min at −20°C in methanol and permeabilized for 1 min in acetone. The double labeling was carried out by sequential incubation of the cells with a mixture of the appropriate primary and secondary antibodies diluted in PBS/0.2% gelatin. The primary antibody was detected using Cy3 and DTAF-conjugated secondary antibodies (Jackson Immuno Research, West Grove, CA). The cells were mounted in Moviol 4-88 (Harco, UK), viewed with a Zeiss axiophot, and photographed using standard techniques.

Results

Membrane-associated Clathrin and γ-Adaptin Codistribute with Immature Secretory Granules

To investigate whether secretory granules from PC12 cells have AP-1 coat-protein components we employed antibodies recognizing γ-adaptin and clathrin. The anti–γ-adaptin antibody, ST0-25, was raised to a GST-fusion protein containing the entire hinge region of mouse γ-adaptin. Analysis of immunoblots revealed that ST0-25 recognizes rat γ-adaptin from PC12 cells and rat brain (Fig. 1 *A*, lanes *1* and *2*). The specificity of ST0-25 for rat γ-adaptin was shown by competition experiments with the GST/γ-adaptin–hinge fusion protein, which abolished the specific signal (Fig. 1 *A*, lanes *3* and *4*), whereas the control GST fusion protein alone had no effect (data not shown). ST0-25 was further characterized by indirect immunofluorescent labeling. Using a species-specific monoclonal antibody (100:3), γ-adaptin has been previously localized to the Golgi complex in bovine and human cells (Ahle et al., 1988). Furthermore, colocalization of γ-adaptin with TGN38, a *trans*-Golgi network resident membrane protein (Luzio et al., 1990), has been demonstrated using indirect immunofluorescence labeling (Reaves and Banting, 1994). In PC12 cells in double-labeling experiments the pattern obtained with ST0-25 is predominantly perinuclear, and largely coincident with the labeling for TGN38 (Fig. 1 *B*, panels *a* and *b*). In contrast, double-labeling experiments with ST0-25 and 219.6, a monoclonal antibody specific for a secretory granule core protein CgB (Rosa et al., 1989) show there is little overlap between γ-adaptin with CgB (Fig. 1 *B*, panel *c* and *d*). The mAb 219.6 labels both MSGs and ISGs in the PC12 cells and since the latter are in the minority, only a small overlap in the signals obtained for γ-adaptin and CgB is to be expected.

The distribution of the clathrin coat components was determined by immunoblotting enriched fractions of ISG and MSGs obtained from sucrose gradients (Fig. 2). To remove the soluble, cytoplasmic pool of coat proteins before



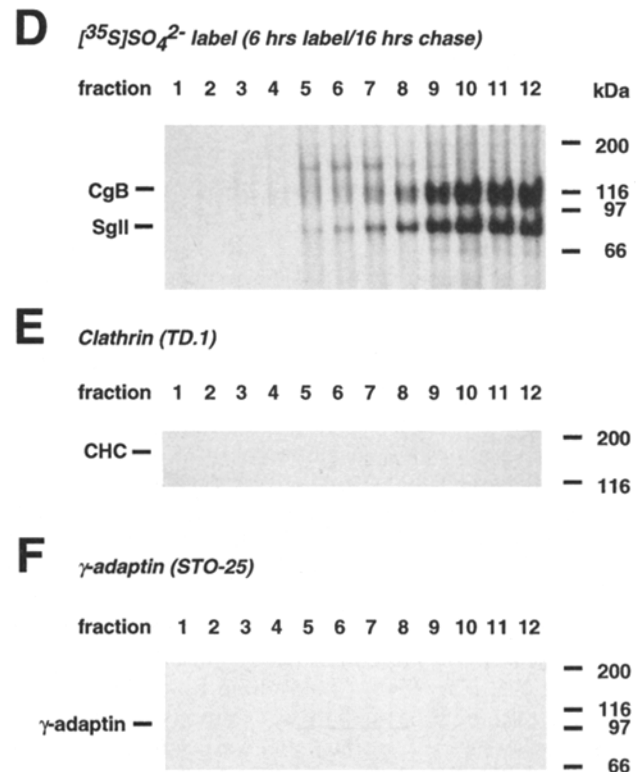


Figure 2. Clathrin coat components codistribute with ISGs on equilibrium sucrose gradients. Membrane pellets prepared from PC12 cells labeled with [^{35}S]sulfate as indicated in *A* and *D*, or unlabeled PC12 cells (*B*, *C*, *E*, and *F*) were fractionated by velocity sucrose gradient centrifugation. The light vesicle pool (fractions *2–4*) or the heavy vesicle pool (fractions *5–7*) were further fractionated by equilibrium sucrose gradient centrifugation (EG) as described in Materials and Methods. In *A* and *D* equal volumes of each fraction were analyzed by SDS-PAGE followed by fluorography. The positions of SgII and the hsPG, or SgII and CgB are indicated in *A* and *D*, respectively. In *B*, *C*, *E*, and *F* equal volumes of each fraction were analyzed by immunoblotting with TD.1 and ST0-25. In *B* and *E* the clathrin heavy chain (*CHC*) is indicated, while in *C* and *F* γ-adaptin is indicated.

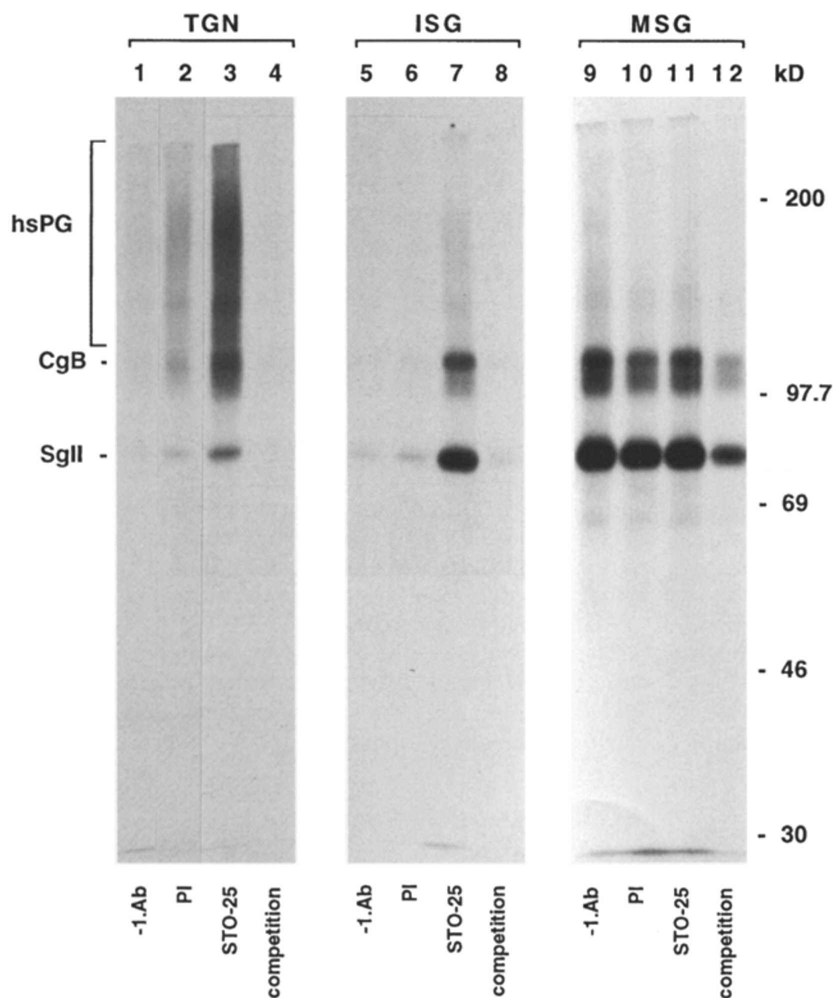


Figure 3. Immunoisolation of membranes with the anti–γ-adaptin antibody ST0-25. [^{35}S]Sulfate-labeled TGN (5 min pulse, no chase), ISG (5 min pulse, 15 min chase), and MSG fractions (6 h label, O/N chase) from PC12 cells were prepared as described in Materials and Methods. For each immunoisolation an equal volume of starting material was incubated with Staph A alone (−1.Ab), with Staph A precoated with preimmune sera (*PI*), Staph A precoated with ST0-25 (*ST0-25*), or with Staph A precoated with ST0-25 preincubated with GST/γ-adaptin–hinge fusion protein (*competition*). The immunoisolated material was analyzed by 7.5% SDS-PAGE and subsequent fluorography. Positions of the sulfate-labeled molecules, hsPG, CgB, and SgII are indicated.

fractionation, a membrane pellet was prepared from the postnuclear supernatant, and then subjected to velocity controlled centrifugation. From this gradient a light vesicle pool containing post-Golgi vesicles (CSVs and ISGs) and a heavy vesicle pool containing MSGs, were obtained and subjected to equilibrium centrifugation. Equilibrium centrifugation of the light vesicle pool containing the post-Golgi vesicles allows the resolution of those vesicles which contain a heparan sulfate proteoglycan (hsPG), the marker for the CSVs, from those vesicles which contain secretogranin II (SgII), the marker for both ISGs and MSGs (Tooze and Huttner, 1990; Rosa et al., 1985). As both the hsPG and SgII are sulfated in the TGN the distribution of these post-Golgi vesicles on the equilibrium gradient (Tooze et al., 1991) was confirmed by pulse-labeling PC12 cells with a 5-min pulse of [^{35}S]sulfate followed by a 15-min chase: the bulk of the SgII was found in fractions 7–9 in ISGs, whereas most of the hsPG was found in fractions 5 and 6 in CSVs (Fig. 2 *A*).

The fractions from the gradient of the light vesicle pool containing post-Golgi vesicles were immunoblotted with TD.1 to reveal the distribution of clathrin. As seen in Fig. 2 *B*, most of the membrane-associated clathrin heavy chain was detected in fractions 5–9. The distribution of γ-adaptin, detected by immunoblotting the same fractions with ST0-25 (Fig. 2 *C*) coincided with the distribution of clathrin (Fig. 2 *B*). The peak of immunoreactivity for both clathrin and γ-adaptin was found in fractions 7 and 8, the fractions which contain the peak of [^{35}S]sulfate-labeled SgII. These results demonstrate that both clathrin and γ-adaptin cosediment with ISGs during equilibrium gradient centrifugation.

After equilibrium centrifugation of a heavy vesicle pool from PC12 cells, the MSGs, identified by sulfate-labeled SgII (Fig. 2 *D*), were found in fractions 9–12 as expected (Tooze et al., 1991). Immunoblotting of the fractions obtained from the heavy vesicle pool revealed no clathrin (Fig. 2 *E*) and only very low amounts of γ-adaptin (Fig. 2 *F*).

Immunoisolation of ISGs with Anti–γ-Adaptin Antibody

As a control we tested whether TGN membranes, which are known to have membrane-associated γ-adaptin (for review see Robinson, 1992), could be immunoisolated with anti–γ-adaptin antiserum ST0-25. After a 5-min pulse the [^{35}S]sulfate-labeled molecules, SgII and the hsPG, present in the TGN fraction from a velocity gradient (fraction 9, see Fig. 2, Tooze and Huttner, 1990) were both immunoisolated with ST0-25 (Fig. 3, lane *3*). Having thus es-

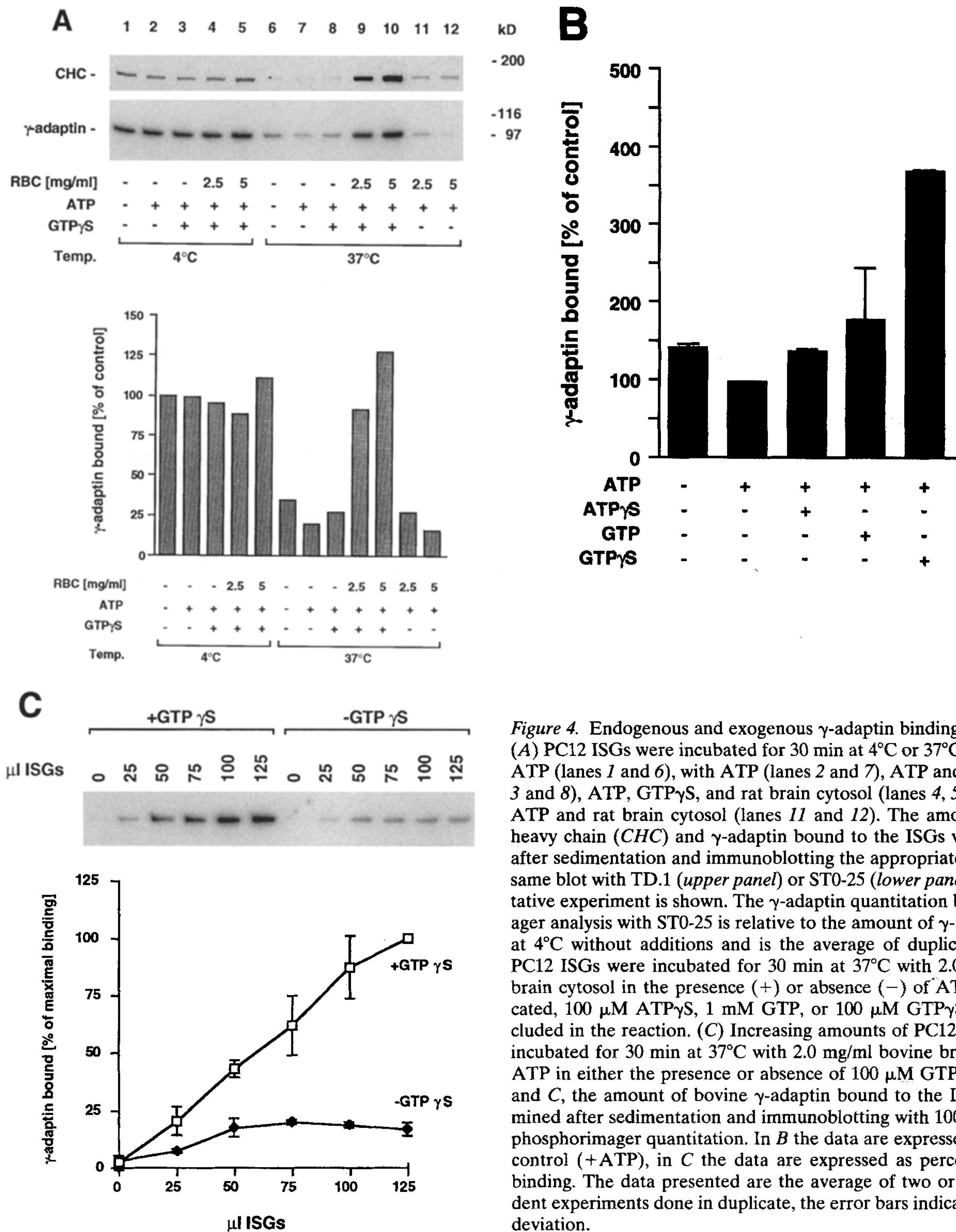


Figure 4. Endogenous and exogenous γ-adaptin binding to PC12 ISGs. (*A*) PC12 ISGs were incubated for 30 min at 4°C or 37°C either without ATP (lanes *1* and *6*), with ATP (lanes *2* and *7*), ATP and GTPγS (lanes *3* and *8*), ATP, GTPγS, and rat brain cytosol (lanes *4*, *5*, and *9*, *10*), or ATP and rat brain cytosol (lanes *11* and *12*). The amount of clathrin heavy chain (*CHC*) and γ-adaptin bound to the ISGs was determined after sedimentation and immunoblotting the appropriate regions of the same blot with TD.1 (*upper panel*) or ST0-25 (*lower panel*). A representative experiment is shown. The γ-adaptin quantitation by phosphorimager analysis with ST0-25 is relative to the amount of γ-adaptin present at 4°C without additions and is the average of duplicate values. (*B*) PC12 ISGs were incubated for 30 min at 37°C with 2.0 mg/ml bovine brain cytosol in the presence (+) or absence (−) of ATP. Where indicated, 100 μM ATPγS, 1 mM GTP, or 100 μM GTPγS were also included in the reaction. (*C*) Increasing amounts of PC12 cell ISGs were incubated for 30 min at 37°C with 2.0 mg/ml bovine brain cytosol and ATP in either the presence or absence of 100 μM GTPγS. For both *B* and *C*, the amount of bovine γ-adaptin bound to the ISGs was determined after sedimentation and immunoblotting with 100/3, followed by phosphorimager quantitation. In *B* the data are expressed as percent of control (+ATP), in *C* the data are expressed as percent of maximal binding. The data presented are the average of two or more independent experiments done in duplicate, the error bars indicate the standard deviation.

tablished that ST0-25 can be used to immunoisolate vesicles with γ-adaptin on their membrane, ISGs, containing SgII pulse labeled with [^{35}S]sulfate for 5 min and chased for 15 min, were immunoisolated with ST0-25 (Fig. 3, lane *7*). Neither the TGN fractions nor the ISGs were immunoisolated in the absence of the primary antibody or with the preimmune serum (Fig. 3, lanes *1* and *2*, and *5* and *6*) and competition with the GST/γ-adaptin–hinge fusion protein, abolished the specific signal (Fig. 3, lanes *4* and *8*). This demonstrates directly that the γ-adaptin detected in the ISG fractions is present on ISGs.

MSGs, containing [^{35}S]sulfate-labeled SgII, were not specifically immunoisolated with ST0-25 (Fig. 3, lane *11*). The nonspecific binding was however much greater in

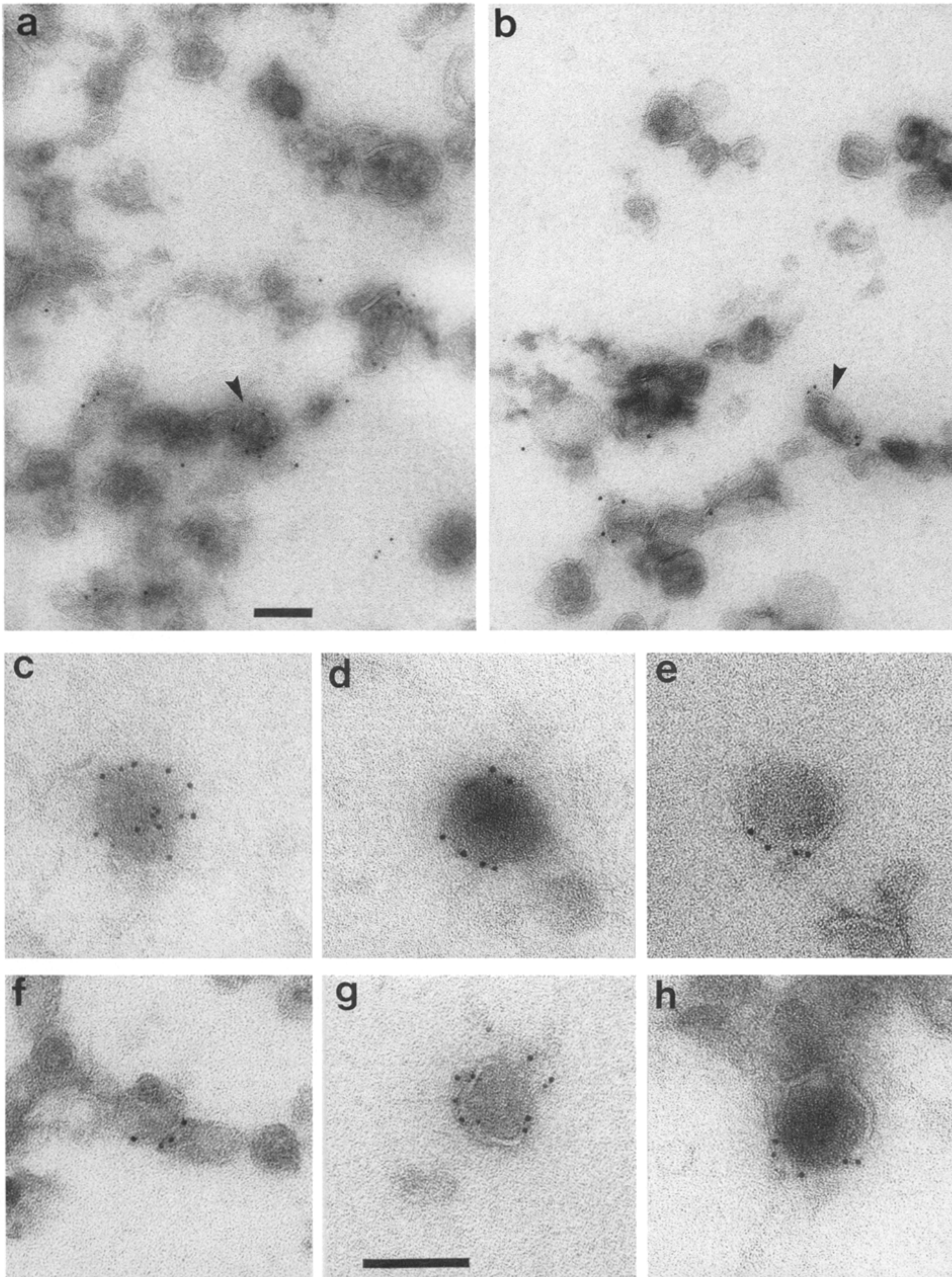


Figure 5. Ultrastructural localization of γ-adaptin on PC12 ISGs. PC12 ISGs were incubated for 30 min at 37°C with 4.0 mg/ml rat brain cytosol in the presence of GTPγS. After sedimentation the granules were prepared for cryosectioning as described in Materials and Methods. Bound γ-adaptin was detected by incubation of the sections with ST0-25 followed by incubation with 5-nm gold-conjugated protein A. *a* and *b* are representative overviews of the results, *c–h* are selected fields. The arrowheads in *a* and *b* indicate ISGs which are labeled with ST0-25. Bar equals 100 nm.

these fractions (Fig. 3, lanes *9–12*) and was observed even without the primary antibody (Fig. 3, lane *9*). Similar immunoisolation experiments (data not shown) performed with TGN, ISG, and MSG fractions and the anti-clathrin antibody X22 coupled to Sepharose (obtained from F. Brodsky), confirmed the result with ST0-25, namely that the ISGs have clathrin coat components associated with their membrane.

Reconstitution of γ-Adaptin Binding to Immature Secretory Granules

Golgi membranes have a saturable pool of endogenous γ-adaptin which remains associated during their isolation at 4°C (Traub et al., 1993). This pool of endogenous γ-adaptin has been shown to dissociate from the membranes at 37°C, exposing free binding sites which can be occupied by exogenous γ-adaptin in the presence of GTPγS. To determine if the γ-adaptin bound to isolated ISGs showed a similar behavior, ISGs were incubated at 37°C in the presence of rat brain cytosol and GTPγS, and the γ-adaptin bound to the ISG was quantitated using ST0-25.

A significant amount of γ-adaptin was detected on ISGs incubated at 4°C either without or with ATP, or with ATP and GTPγS (Fig. 4 *A*, lanes *1–3*). Addition of 2.5 or 5.0 mg/ml rat brain cytosol, in the presence of ATP and GTPγS at 4°C did not increase the level of rat γ-adaptin bound (Fig. 4 *A*, lanes *4* and *5*). Upon incubation of ISGs at 37°C without cytosol either in the absence or presence of ATP, or ATP and GTPγS the amount of γ-adaptin bound was reduced (Fig. 4 *A*, lanes *6–8*) to ~25% of that bound at 4°C. Addition of 2.5 or 5.0 mg/ml rat brain cytosol in the presence of ATP and GTPγS resulted in a saturable increase in the amount of γ-adaptin bound to the ISGs to a level comparable to that detected at 4°C (Fig. 4 *A*, lanes *9* and *10*). Furthermore, the amount of γ-adaptin bound after incubation with rat brain cytosol in the absence of GTPγS (Fig. 4 *A*, lanes *11* and *12*) was similar to that detected at 37°C with ATP and GTPγS alone (Fig. 4 *A*, lane *8*). These results demonstrate that a discrete number of γ-adaptin–binding sites are present on the ISGs, and that at 37°C there is dissociation of the endogenous γ-adaptin from the ISG membrane and saturable rebinding of exogenous γ-adaptin from cytosol, dependent upon GTPγS. In these experiments the behavior of clathrin was similar to that of the γ-adaptin (Fig. 4 *A*). Incubation of ISGs at 37°C without cytosol and GTPγS resulted in the dissociation of endogenous bound clathrin from the membrane. Upon addition of cytosol and GTPγS clathrin was rebound to the membrane (Fig. 4 *A*).

To analyze further the nature and requirements for clathrin coat assembly on the ISG, and in particular the binding of γ-adaptin, we employed an in vitro assay previously described which exploits the monoclonal antibody 100/3 to study the association of exogenous bovine γ-adaptin with isolated Golgi membranes of other species (Stamnes and Rothman, 1993). We incubated ISGs with bovine brain cytosol in the absence or presence of ATP, and the exogenous bovine γ-adaptin bound was quantitated by immunoblotting with 100/3. As seen in Fig. 4 *B*, γ-adaptin binding to ISGs was detected both with and without ATP. These results support those of Traub and co-workers (Traub et al., 1993) who did not observe an ATP-dependent binding of AP-1 to Golgi membranes. To determine if other nucleotides were required, ATPγS, GTP, and GTPγS were added. Addition of GTPγS resulted in maximum bovine γ-adaptin binding to ISGs, on average fourfold over the control without GTPγS. Note the fourfold increase in γ-adaptin binding observed with bovine brain cytosol after addition of GTPγS is comparable to the increase obtained using rat brain cytosol and GTPγS (cf Fig. 4 *A*, lanes *9* and *11*, and 4 *B*). Addition of GTP resulted in at most a twofold increase in the binding of γ-adaptin (Fig. 4 *B*). A similar stimulatory effect of GTPγS has been previously described for γ-adaptin recruitment to isolated Golgi membranes (Traub et al., 1993; Stamnes and Rothman, 1993) and permeabilized cells (Robinson and Kreis, 1992; Wong and Brodsky, 1992; Le Borgne et al., 1993).

Using bovine brain cytosol at a protein concentration of 2 mg/ml in the presence or absence of GTPγS, the amount of exogenous bovine γ-adaptin bound was proportional to the amount of ISGs added. As expected from Fig. 4 *B*, γ-adaptin binding to ISGs was significantly higher in the presence of GTPγS. Only a minor amount of γ-adaptin was detected in the pellet in the absence of ISGs, demonstrating that the signal obtained is not a result of nonspecific aggregation and sedimentation of the bovine γ-adaptin (Fig. 4 *C*).

γ-Adaptin Can be Labeled with ST0-25 on the Membrane of ISGs Using Immunoelectron Microscopy

Although no morphologically intact stacked Golgi membranes were seen in epon sections of the ISG fraction, and no sialyltransferase and mannosidase II activities were found in the ISG fraction (Tooze and Huttner, 1990, and data not shown), it remained possible that the γ-adaptin binding we measured in the ISG fraction was to contaminating fragmented Golgi membranes or other vesicles rather than to ISGs. To demonstrate directly that γ-adaptin is bound to ISGs after incubation with rat brain cytosol immunoelectron microscopy was performed using ST0-25. Two representative overview sections and a gallery of selected micrographs are shown in Fig. 5. After incubation of ISGs at 37°C with rat brain cytosol and GTPγS, immunogold labeling demonstrated that γ-adaptin was detected on the membrane of ISGs. Quantitation of the number of gold particles present on the ISG membrane was performed on ISGs incubated at 37°C with rat brain cytosol in the presence of GTPγS after labeling with either ST0-25, or preimmune sera, or ST0-25 preincubated with the GST-

Table I. Quantitation of the Immunogold Labeling of ISGs

	No. of ISGs counted	No. of ISGs positive	No. of gold particles/ISG			
			1	2	3	≥4
ST0-25	1190	248	151	60	22	15
ST0-25 pi	997	9	6	3	0	0
ST0-25 comp	1125	53	37	15	1	0

Sections were incubated with either ST0-25, the preimmune sera for ST0-25 (ST0-25 pi), or ST0-25 preincubated with the GST/γ-adaptin hinge fusion protein (ST0-25 comp), and processed as described in Materials and Methods. Quantitation of the number of gold particles was performed using fields similar to those in Fig. 5, *a* and *b* as described in Materials and Methods.

hinge fusion protein (Table I). Using ST0-25, of the 1190 ISGs counted, 21% were labeled, and of these, ~15% were labeled with three or more gold particles.

MSGs incubated under identical conditions with rat brain cytosol followed by immunogold labeling with ST0-25 showed no specific labeling. Approximately 3% of the MSGs had one or two gold particles on their membrane. Finally, no other morphologically identifiable structures were labeled above background with ST0-25 in the cryosections, confirming that the signal obtained by immunoblotting is a result of γ-adaptin binding specifically to ISGs.

γ-Adaptin Binding to ISGs Is Mediated by ARF

The stimulatory effect of GTPγS on the binding of γ-adaptin to ISGs, and the previous results obtained with Golgi membranes (Stamnes and Rothman, 1993; Traub et al., 1993) suggested that ARF might be involved. To test this, we added a myristoylated peptide corresponding to the NH_2-terminal sequence of ARF1 to the reaction mixture while increasing the concentration of the bovine brain cytosol (Fig. 6 *A*). At low cytosol concentrations the addition of 100 μM myristoylated ARF1 peptide (mARF1pep) stimulated the γ-adaptin binding ~4–5-fold over the γ-adaptin binding observed with the nonmyristoylated ARF1 peptide (ARF1pep). At high concentrations of cytosol (2–4 mg/ml), the myristoylated ARF1 peptide increased the γ-adaptin binding ~2–3-fold (Fig. 6 *A*). The addition of an equivalent volume of the solvent or 100 μM myristic acid in DMSO (data not shown), caused no significant change in the γ-adaptin binding.

To show directly that ARF is involved in γ-adaptin binding, the binding assay was reconstituted in a two-step protocol using recombinant myristoylated ARF1 (mARF1) and an adaptor-enriched pool (Pool I) obtained from bovine brain cytosol after gel-filtration (Fig. 6 *B*). Maximum γ-adaptin binding was observed when the ISGs were preincubated with recombinant mARF1 and GTPγS followed by the addition of Pool I and ATP, compared to the con-

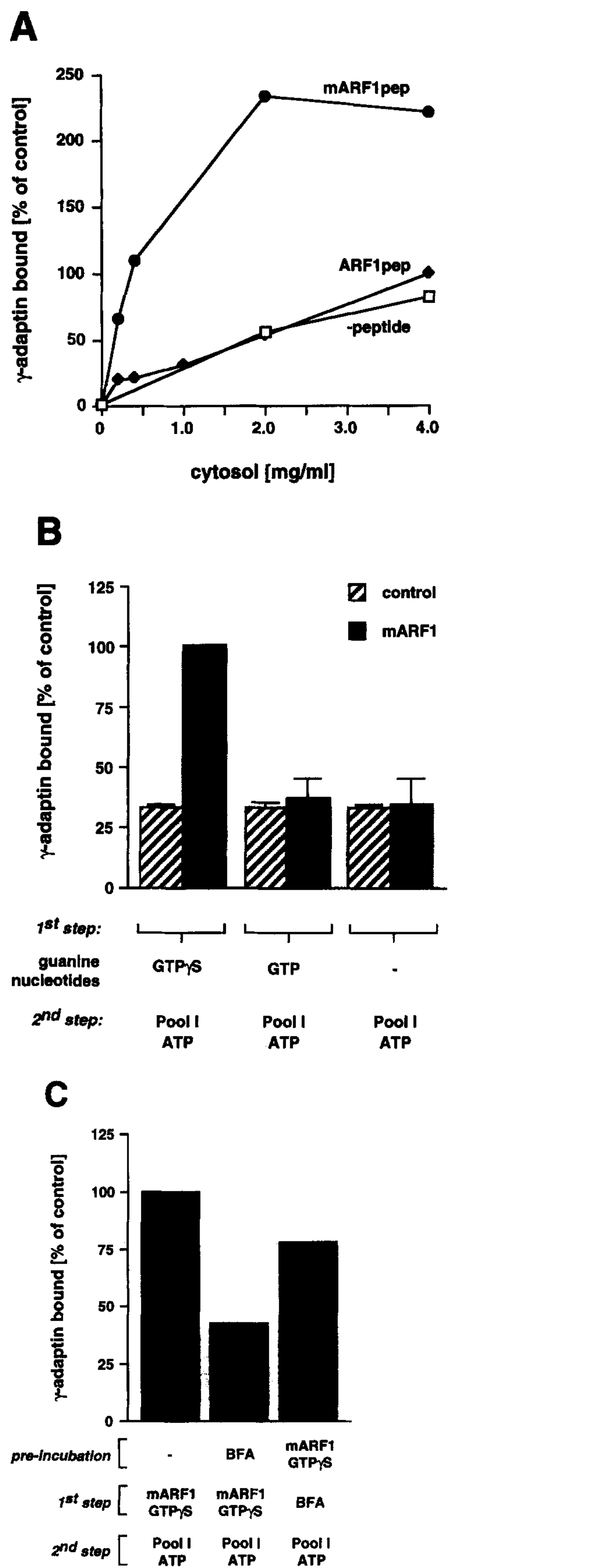


Figure 6. Reconstitution γ-adaptin binding to PC12 ISGs is dependent upon mARF1. (*A*) PC12 ISGs were incubated for 30 min at 37°C with ATP and increasing amounts of bovine brain cytosol. Synthetic NH_2-terminal ARF1 peptides, myristoylated (*mARF1pep*) or nonmyristoylated (*ARF1pep*), were added to final concentration of 100 μM. The amount of bovine γ-adaptin bound was determined after sedimentation and immunoblotting with 100/3, followed by phosphorimager quantitation. The data shown are the average of duplicate values and are normalized to the amount of γ-adaptin bound in presence of the ARF1 peptide and 4.0 mg/ml cytosol. (*B*) PC12 ISGs were incubated for 10 min at 37°C with recombinant mARF1 or a noninduced bacterial control sample (*control*) in the absence or presence of either 100 μM GTPγS or 1 mM GTP. Then, a γ-adaptin–enriched fraction (*Pool I*) and ATP were added and the incubation was continued for an additional 20 min at 37°C. The γ-adaptin bound was determined as in *A* and the data are expressed as percent of maximal binding (*mARF1* and *GTPγS*) and represent the average of three or more independent experiments done in duplicate. The error bars indicate the standard deviation. (*C*) PC12 ISGs were preincubated at 37°C with no additions, with 10 μg/ml BFA, or recombinant mARF1 and 100 μM GTPγS. After 10 min, recombinant mARF1 and GTPγS, or BFA were added as indicated. After an additional 10 min at 37°C, Pool I and ATP were added, and incubated for an additional 20 min at 37°C. The γ-adaptin bound was determined as in *A* and the data are expressed as percent of control (−BFA) and represent the average of duplicate values.

trol. The binding of γ-adaptin to ISGs using Pool I and recombinant mARF1 was not influenced by the addition of $[AlF_4]^-$, a known activator of heterotrimeric G-proteins (Finazzi et al., 1994), either in the presence or absence of ATP and GTP (data not shown). These data demonstrate that mARF1 is involved in the binding of γ-adaptin to ISGs.

Additional data to support the involvement of ARF in the γ-adaptin binding to ISGs was obtained by testing the sensitivity of this binding process to BFA. BFA causes dissociation of both γ-adaptin and β-COP, a subunit of COP-coats, from Golgi membranes (Klausner et al., 1992). The target of BFA is thought to be the guanine nucleotide exchange factor responsible for the exchange of GDP for GTP bound to ARF (Donaldson et al., 1991; Helms et al., 1993; Donaldson and Klausner, 1994). Pretreatment of the ISGs with 10 μg/ml BFA before the addition of mARF1 and GTPγS resulted in an ~60% decrease in the amount of γ-adaptin bound from Pool I. This decrease in γ-adaptin binding to ISGs could be prevented by incubation of the ISGs with mARF1 and GTPγS before the addition of BFA (Fig. 6 *C*).

Binding of γ-Adaptin Requires Components on the ISGs Membrane

To test if the binding of γ-adaptin to ISGs requires membrane proteins, or membrane-associated proteins, ISGs were pretreated with increasing amounts of trypsin for 10 min at 37°C, incubated with bovine brain cytosol, and the exogenous γ-adaptin binding was determined. As shown in Fig. 7 *A*, pretreatment of the ISGs with 0.5 mg/ml trypsin decreased by ~50% the γ-adaptin binding to the ISGs, and binding was completely abolished by pretreatment with 1 mg/ml trypsin. These results indicate that a membrane-associated protein (or proteins) is required which is susceptible to trypsin cleavage.

To investigate an additional requirement for membrane-associated proteins ISGs were preincubated with 0.5 M Tris/HCl at pH 7.2, to remove the clathrin and associated adaptor complexes from the ISG membrane (Chang et al., 1993; Anderson, 1993). After Tris-stripping, virtually all of the endogenous γ-adaptin, revealed by ST0-25, and clathrin present on the ISG membrane were removed (Fig. 7 *B*, compare lanes *1* and *6* and *2* and *7*) together with the small amount or ARF present (Fig. 7 *B*, lanes *2* and *7*). Upon addition of bovine brain cytosol to unstripped and stripped ISGs, translocation of γ-adaptin from the cytosol to the ISGs was detected (Fig. 7 *B*, lanes *3* and *8*). High-Tris-stripping reduced the efficiency with which clathrin rebound to the ISG membrane (Fig. 7 *B*, lanes *3* and *8*) as previously reported using Tris-stripped plasma membranes (Moore et al., 1987).

Interestingly, the amount of γ-adaptin bound was similar for both unstripped and stripped ISGs, suggesting that the recruitment of the exogenous γ-adaptin from cytosol cannot be increased by prior removal of the endogenous coat components. A similar observation was made after treatment of the ISGs with 0.5 M KCl instead of Tris, i.e., no increase in the amount of exogenous γ-adaptin bound to the salt-treated ISGs was observed in comparison to the untreated controls (data not shown).

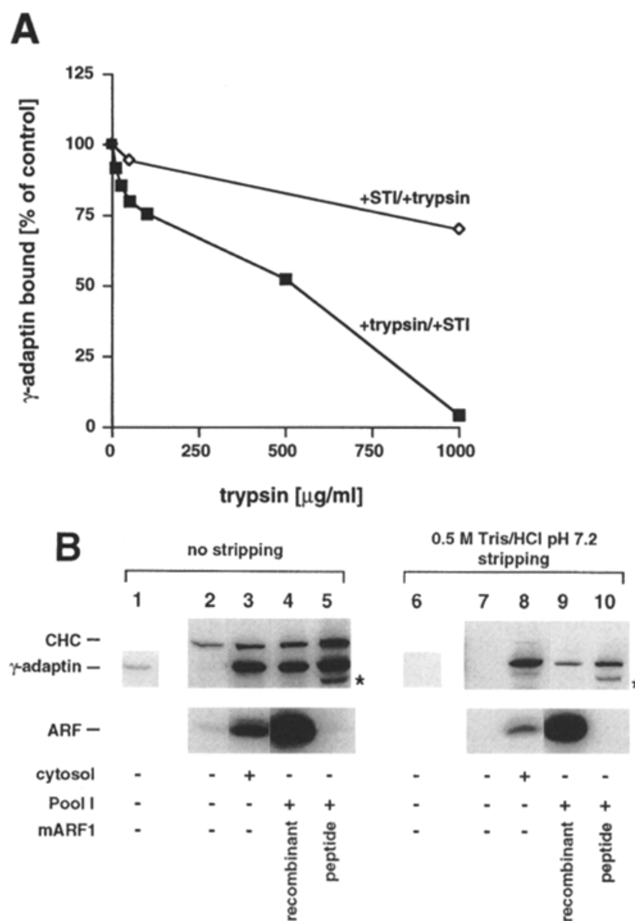


Figure 7. Pertubation of endogenous ISG membrane components required for γ-adaptin-binding by trypsin treatment and stripping with high concentrations of Tris. (*A*) Before the γ-adaptin binding assay, PC12 ISGs were preincubated for 10 min at 37°C with increasing concentrations of trypsin (0–1,000 μg/ml). Soybean trypsin inhibitor was added either before the trypsin (+STI/+trypsin) or at the end of the preincubation period (+trypsin/+STI). The ISGs were then incubated for 30 min at 37°C with 2.0 mg/ml bovine brain cytosol, ATP, and 100 μM GTPγS. γ-Adaptin was quantitated by phosphorimager analysis after immunoblotting with 100/3. The data are expressed as percent of the control without trypsin and are the average of duplicate values. (*B*) The PC12 ISGs were incubated for 15 min at 4°C without (lanes *1–5*) or with (lanes *6–10*) 0.5 M Tris/HCl, pH 7.2, and then pelleted. The resuspended ISGs were incubated for 10 min at 37°C with 100 μM GTPγS (lanes *1*, *2*, *6*, and *7*), recombinant mARF1 (100 μg) and 100 μM GTPγS (lanes *4* and *9*), or 100 μM mARF1 peptide (lanes *5* and *10*). Finally, 4.0 mg/ml bovine brain cytosol, 100 μM GTPγS and ATP (lanes *3* and *8*), or Pool I and ATP (lanes *4*, *5*, *9*, and *10*), or ATP (lanes *1*, *2*, *6*, and *7*) were added, and the ISGs were incubated for an additional 20 min at 37°C. After sedimentation the ISGs were analyzed by immunoblotting. To assess the endogenous level of γ-adaptin with or without stripping STO-25 (lanes *1* and *6*) was used. In lanes *2–5* and *7–10*, the clathrin heavy chain (CHC), γ-adaptin, and ARF bound to the ISGs was revealed with antibodies TD.1, 100/3, or 1D9, respectively. Incubations to detect the clathrin heavy chain and bovine γ-adaptin on the same blot were performed in a mixture of TD.1 and 100/3. A representative experiment is shown. Note that upon addition of Pool I and the mARF1 peptide a band (*) with a molecular weight which is lower than γ-adaptin appears (lanes *5* and *10*). This band cross-reacts with 100/3 and might be a degradation product of γ-adaptin.

Surprisingly, however, using recombinant mARF1 and Pool I, γ-adaptin binding to stripped ISGs was drastically reduced compared with unstripped ISGs (Fig. 7 *B*, lanes *4* and *9*). A 3–4-fold reduction in the γ-adaptin binding was also observed after incubation of the Tris-stripped ISGs with the Pool I and the mARF1 peptide compared to the unstripped ISGs (Fig. 7 *B*, lanes *5* and *10*). These results are in contrast to the data obtained using complete cytosol and suggest that a component, or components, required for maximum γ-adaptin binding are removed from the ISG membrane by the Tris-stripping. These components would be predicted to be present in the cytosol, but not in Pool I or the recombinant mARF1 preparation.

As shown above the binding of the γ-adaptin to ISGs is mediated by mARF1, therefore the component removed by Tris-stripping may be directly involved in the ARF binding, e.g., the nucleotide exchange factor, or the putative ARF receptor. To test this hypothesis we measured the amount of ARF present on the ISGs both before and after Tris-stripping, and after incubation with either cytosol or Pool I and recombinant mARF1. The level of endogenous ARF on the ISGs (see Fig. 7 *B*, lane *2*) was comparable to that detected on TGN membranes and significantly higher than that on MSGs (data not shown). Incubation of ISGs in the presence of GTPγS and either bovine brain cytosol or recombinant mARF and Pool I resulted in a large increase in the level of ARF on the ISG membrane (Fig. 7 *B*, compare lane *2* with lanes *3* and *4*). It is likely, however, that some of the bound mARF1 we measure after incubation is only loosely associated with the lipid bilayer and not stabilized by interaction with a membrane component (Helms et al., 1993). Control experiments, done by incubating Pool I and recombinant mARF1 at 37°C with GTPγS without ISGs, confirmed that the recombinant mARF1 was not precipitating during the incubation (data not shown). The low level of endogenous ARF measured on the ISGs was not changed by incubation with Pool I and the mARF1 peptide (Fig. 7 *B*, compare lane *2* with *5*). After Tris-stripping the efficiency of ARF recruitment to the ISGs, using either cytosol or Pool I and recombinant mARF1 was not significantly affected (Fig. 7 *B*, cf lanes *3* and *8* and *4* and *9*). The level of ARF bound to the stripped ISGs was not correlated with the amount of γ-adaptin bound. These results suggest that although mARF1 is necessary, it is not sufficient for the binding of γ-adaptin to the ISG membrane, and other components sensitive to Tris-stripping are required.

Discussion

In this study we show that the association of the clathrin coat on PC12 cell ISGs is mediated by γ-adaptin. These data, together with the requirements for ARF, imply that it is the AP-1 complex (γβ′-adaptins) which is involved in the clathrin binding to ISGs. The recruitment of exogenous γ-adaptin to ISGs is a result of exchange with the endogenous pool: no significant increase in the total amount of γ-adaptin bound to the ISG membrane compared to ISGs kept at 4°C can be observed after addition of cytosol and GTPγS and incubation at 37°C. These results demonstrate that the endogenous pool of γ-adaptin dissociates from the ISG membrane to expose a limited number of γ-adaptin–binding sites, which are then occupied by the added exogenous γ-adaptin. These results are consonant with those obtained using Golgi membranes (Traub et al., 1993).

The level of both the endogenous γ-adaptin and clathrin on the ISGs decreases upon incubation at 37°C in the absence of added cytosolic factors and nucleotides. It is unlikely that this decrease is due to the formation of clathrin-coated vesicles during the incubation because the requirements for clathrin-coated vesicle budding include ATP and cytosol (for review see Schmid, 1993). It remains a possibility that when the incubation conditions are favorable there may be clathrin-coated vesicle formation in the binding assay. We have not yet addressed this point and assume any newly formed clathrin-coated vesicles would sediment during the assay and contribute to the signal we measure; our assay will have to be modified to allow separation of putative newly formed clathrin-coated vesicles from the ISGs.

The data reported here extend our knowledge of the clathrin coat on the ISG, and the composition of ISG. The ISG is an intermediate in the biogenesis of mature dense core secretory granules and has been predicted to have a distinct composition compared to the MSG (for review see Tooze, 1991), although to date the composition of the ISG has not been elucidated by biochemical methods. We have shown that γ-adaptin binding to ISGs requires mARF1 in a GTP-bound form, and therefore additional components of the ISG might be the guanine nucleotide exchange factor, and an ARF receptor. Furthermore, these components may be either inactive or not present on MSGs since MSGs do not have clathrin coats, are unable to bind γ-adaptin, and the MSG fraction contains significantly less ARF than the ISG fraction (data not shown). In addition, the results obtained with high Tris-stripping suggest that membrane-associated proteins required for γ-adaptin binding, and present also in cytosol, may be additional unidentified components of the ISG.

Removal of membrane from the ISGs is a prerequisite of their maturation which involves changes in the ratio of volume to the surface area (Salpeter and Farquhar, 1981) as a result of ISG–ISG fusion (Tooze et al., 1991) or a decrease in size of the ISG (Bauerfeind and Huttner, 1993). Using sedimentation analyses the change in the size of the ISG has been quantitated: it is estimated that the homotypic fusion of three to five ISGs occurs during maturation, resulting in a size increase of the secretory granule core diameter from 80 to 120 nm (Tooze et al., 1991). Our hypothesis is that the clathrin coat on the ISG mediates the removal of the excess membrane, present as a result of ISG–ISG fusion, through the formation of clathrin-coated vesicles, and that budding of clathrin-coated vesicles will prove to be correlated with the homotypic fusion of ISGs. We have calculated based on the known size of the ISG core diameter (80 nm) and MSG core diameter (120 nm) and clathrin-coated vesicle diameter (50 nm) that approximately three ISGs would have to fuse during maturation to provide sufficient excess membrane to form one coated vesicle.

While serving to remove membrane from the surface of maturing ISGs, clathrin-coated vesicles will remove mem-

brane-bound proteins, and, in their lumen, soluble proteins destined for vesicular transport. This raises the following questions, (*a*) which proteins are removed and why, and (*b*) are these proteins responsible for binding the γ-adaptin to the membrane? The purpose of this vesicular transport step could conceivably be to recycle, or sort proteins from the maturing ISG. There are three possible destinations for these vesicles; (*a*) the TGN, (*b*) the endosome/lysosome, and (*c*) the plasma membrane. An example of a protein recycled from the ISG back to the TGN might be the putative sorting receptor which has been postulated to mediate binding either of individual molecules, or aggregates of regulated secretory proteins in the TGN to initiate budding (for review see Burgess and Kelly, 1987; Chanat et al., 1992). Examples of proteins sorted from the ISG to the endosome could be the M6PR or TGN38. Finally, the ISG-derived coated vesicle may be removing components destined for constitutive or constitutive-like (Kuliawat and Arvan, 1994) secretion from the cell. We do not however believe that the ISG-derived clathrin-coated vesicles are removing missorted proteins destined for constitutive secretion: our result obtained by immunoisolation of ISGs with ST0-25 shows that there was essentially no hsPG, the marker for the constitutive secretory pathway in PC12 cells, in the γ-adaptin positive ISG population. It has also previously been demonstrated that the hsPG is sorted from the ISG in the TGN (Tooze and Huttner, 1990).

In the absence of data which conclusively demonstrate which proteins initially mediate the binding of γ-adaptin to the membrane, and which are transported by clathrin-coated vesicles, we favor the hypothesis that in PC12 cells it is M6PR and lysosomal enzymes which are being removed from the maturing ISG, and that this receptor and these lysosomal enzymes are partially missorted into the ISG during formation in the TGN. Two independent pieces of evidence support this proposal. First, it has been shown that AP-1 binds to the M6PR in the Golgi complex: γ-adaptin binding to the cytoplasmic domain of the M6PR is specific for sequences containing a phosphorylated serine residue (Glickman et al., 1989; Le Borgne et al., 1993). Secondly, lysosomal enzymes have been detected in the immature secretory granules isolated from the β-cells of the endocrine pancreas, and although it has not been shown that M6PR is present in these immature secretory granules, the removal of the lysosomal enzymes from the ISG is sensitive to tunicamycin which blocks the formation of the M6P-recognition sequence (Kuliawat and Arvan, 1994).

This assumption does not however mean that the M6PR is the γ-adaptin docking receptor in the ISGs. It remains a possibility that other transmembrane receptor molecules, or membrane-associated proteins which are analogous to the putative docking protein (Traub et al., 1995), may bind γ-adaptin in a secretory granule specific mechanism. Potential candidates could include secretory granule membrane constituents which are only required for maturation. It also cannot be excluded that several distinct components, for example a separate receptor for both γ-adaptin and ARF, are required: stable association of γ-adaptin leading to clathrin coat formation might require regulation of these binding sites or even cooperation between the two receptors to form an active-binding complex on the ISG membrane which could then interact with the protein(s) destined for vesicular transport. During secretory granule maturation one, or all of these proteins might be removed from the ISG membrane by budding of coated vesicles, and targeted to another membrane. The trypsin sensitive protein(s) and the soluble components removed by Tris-stripping remain to be characterized; the identification of these components should help elucidate the function of the clathrin coat on the ISG.

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