Targeting and Mistargeting of Plasma Membrane Adaptors In Vitro

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Abstract. Targeting and recruitment of the plasma membrane (PM) clathrin-coated vesicle adaptor complexes has been studied using an in vitro system based on permeabilized acceptor cells and donor cytosol. Through the use of species- and/or tissue-specific antibodies, only newly recruited exogenous PM adaptors are visualized. Targeting of PM adaptors can be switched from the plasma membrane to a perinuclear compartment by GTPγS or excess calcium. Prior treatment with brefeldin A prevents GTPγS-induced mistargeting. Double-labeling immunofluorescence and immunogold EM indicate that the perinuclear PM adaptor binding compartment is late endosomal. We propose that receptors for PM adaptors cycle between the plasma membrane and an endosomal storage compartment. Normally the receptors would be switched on only at the plasma membrane, but both GTPγS and calcium are capable of reversing this switch. Intracellular sequestration of PM adaptor receptors may provide the cell with a mechanism for up-regulating endocytosis following a burst of exocytosis.

In the eukaryotic cell, vesicular carriers mediate membrane traffic between the organelles of the secretory and endocytic pathways. The first event in membrane traffic is the recruitment of cytosolic coat proteins onto a particular membrane to facilitate the budding of a coated transport vesicle (Rothman and Orci, 1992). Thus, this process must be tightly regulated to ensure that the coat proteins are recruited onto the appropriate membrane at the correct time.

So far, three types of coated transport vesicles have been purified and characterized: clathrin-coated vesicles that bud from the plasma membrane, clathrin-coated vesicles that bud from the TGN, and non-clathrin-coated, or COP-coated vesicles, which bud from the Golgi stack. The clathrin-coated vesicles that bud from the plasma membrane carry out the selective endocytosis of ligands such as LDL and transferrin (Pearse and Robinson, 1990; Schmid, 1992). Clathrin provides the scaffolding required to build the vesicle, while protein complexes known as adaptors (or APs) attach the clathrin to the membrane and may also trap specific receptors within the coated pit (Pearse, 1988). The clathrin-coated vesicles that bud from the TGN have been implicated in the sorting of newly synthesized lysosomal enzymes bound to the mannose-6-phosphate receptor (Glickman et al., 1989). Although the clathrin is the same on the two types of coated vesicles, immunolocalization studies have shown that different adaptors are associated with the plasma membrane and the TGN (Robinson, 1987; Ahle et al., 1988). The two adaptors are highly homologous: both are heterotetramers comprising two 90–110-kd adaptins (α and β for the plasma membrane, γ and β′ for the TGN), complexed with two smaller proteins of ~50 and ~20 kd (Robinson, 1992). COP-coated vesicles, which are believed to be responsible for traffic through the Golgi stack, are also coated with protein complexes that cycle on and off the membrane, known as coatomers (Waters et al., 1991). One of the subunits of the coatomer complex, β-COP, shows weak homology with β-adaptin (Serafini et al., 1991; Duden et al., 1991).

Support for the idea that coat proteins mediate vesicle formation has been provided through the use of the drug brefeldin A (BFA)[1] (Pelham, 1991; Klausner et al., 1992). Brief exposure of cells to this drug causes rapid redistribution to the cytoplasm of both coatomers and TGN adaptors, and subsequently the secretory pathway is blocked and profound changes occur in the morphology of the Golgi complex (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Robinson and Kreis, 1992; Wong and Brodsky, 1992). Effects of BFA can be blocked by pretreating intact cells with AlF_4- or by pretreating permeabilized cells with GTPγS (Donaldson et al., 1991*a*; Lippincott-Schwartz et al., 1991). However, BFA has no effect on the distribution of plasma membrane adaptors or on receptor-mediated endocytosis.

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1. *Abbreviations used in this paper:* BFA, brefeldin A; CF, cationized ferritin; M6PR, mannose-6-phosphate receptor; PM, plasma membrane; rhod-WGA, rhodamine-wheat germ agglutinin.

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Thus, although the coats on the clathrin-coated vesicles that bud from the plasma membrane and those that bud from the TGN are very similar, the regulation of coat protein recruitment appears to be more similar between TGN clathrin-coated vesicles and COP-coated vesicles.

Each of the three types of coat protein complexes is recruited onto a precisely defined membrane compartment. It is not yet understood how the proteins are targeted, but in vitro systems have been developed for studying the binding of coat proteins to membranes. The recruitment of adaptors onto the TGN can be reconstituted using permeabilized acceptor cells and donor cytosol, and assayed by immunofluorescence or Western blotting using a species-specific antibody against γ-adaptin. These studies have shown that it is possible to maintain the specificity of adaptor targeting in vitro (Robinson and Kreis, 1992; Wong and Brodsky, 1992). Recruitment of cytosolic β-COP onto enriched Golgi membranes has also been demonstrated (Donaldson et al., 1991*b*; Ktistakis et al., 1992). The two processes are similar in that both are blocked by BFA and enhanced by GTPγS. Different approaches have been used to study the binding of adaptors to the plasma membrane. In one system, cells attached to a substrate are sonicated to remove all but their adherent plasma membranes, endogenous adaptors are stripped with 0.5 M Tris or 0.6 M NaCl, and either cytosol or purified plasma membrane (PM) adaptors are added (Moore et al., 1987; Mahaffey et al., 1990). These studies indicate that an integral membrane protein(s) may act as a receptor for PM adaptors. A different assay, based on the use of electron microscopy and broken A431 cells, has revealed that the addition of cytosol plus energy induces the formation of new PM-coated pits (Smythe et al., 1989). Although this result does not necessarily imply that PM adaptors are recruited from the cytosol, the system has subsequently been modified to assay for the sequestration of receptor-bound transferrin into deeply invaginated coated pits and coated vesicles, and purified PM adaptors have been shown to promote this process when added to dilute cytosol (Smythe et al., 1992; Carter et al., 1993).

To address the specific question of PM adaptor targeting, we have developed an in vitro system, which allows newly recruited PM adaptors to be detected directly, and which does not require the removal of endogenous adaptors first. The assay is similar to the assay for TGN adaptor recruitment in that donor cytosol is added to permeabilized acceptor cells and newly recruited adaptors are visualized using species- or tissue-specific antibodies. We find that cytosolic PM adaptors are able to associate specifically with the plasma membranes of acceptor cells. However, under certain conditions, the adaptors are mistargeted to a different compartment, indicating that it is possible to interfere with the targeting machinery.

Materials and Methods

Cell Culture

NRK cells and Vero cells were maintained in DME medium supplemented with 10% FBS, 4 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Stably transfected 3T3 cells expressing a construct containing the GLUT-4 localization signal (Hudson et al., 1992) were kindly provided by M. Birnbaum (Harvard Medical School, Boston, MA). These cells were cultured in the above medium supplemented with 500 μg/ml G418.

Preparation of Cytosol

Cytosol was prepared from pieces of pig or rat brain that had been stored in liquid nitrogen. The tissue was allowed to thaw slowly and was then homogenized in ~2.5 vol of cytosol buffer (25 mM Hepes-KOH [pH 7.0], 125 mM potassium acetate, 25 μM magnesium acetate, 1 mg/ml glucose, and 1 mM DTT) in a 15-ml glass homogenizor using about 20 strokes of the bulb. The homogenate was spun at 4°C for 15 min at 50,000 rpm in a Beckman TL ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA), using a TL100.3 rotor, and the supernatant was collected and stored in aliquots in liquid nitrogen. The protein concentration was found to be approximately 15 mg/ml.

Free calcium concentration was measured using a fluorometric assay based on the calcium-binding dye FLUO 3. Measurements were made of three separate preparations of pig brain cytosol, and were found to be 38, 48, and 93 μM.

Reagents and Antibodies

Most reagents were obtained from Sigma Immunochemicals (St. Louis, MO). The fluorescently labeled anti-mouse and anti-rabbit antibodies were obtained from Amersham (Amersham, England). Anti-rat transferrin receptor mAb was obtained from Serotec (Oxford, England). DiI-LDL was purchased from Molecular Probes (Eugene, OR).

The antibodies used to detect newly recruited α-adaptin were the species-specific mouse mAb AC2-M15 (Robinson, 1987); C4, an affinity-purified rabbit antiserum raised against a fusion protein containing a 22-amino acid insert found only in brain α-adaptin (Ball, C. L., and M. S. Robinson, manuscript in preparation); and MC4, a polyclonal mouse version of the above. Additional antibodies were generously provided by others. X22, a mouse mAb against clathrin heavy chain (Brodsky, 1985), was a gift from Frances Brodsky (UCSF, San Francisco, CA); mAb 100/3, a species-specific mouse mAb against γ-adaptin (Ahle et al., 1988), was a gift from Ernst Ungewickell (Washington University, St. Louis, MO); the rabbit polyclonal antiserum against TGN38 (Luzio et al., 1990) was a gift from Paul Luzio (University of Cambridge, Cambridge, U.K.); anti-EAGE, a rabbit anti-peptide antiserum that recognizes β-COP (Duden et al., 1991), was a gift from Thomas Kreis (University of Geneva, Geneva, Switzerland); the rabbit polyclonal antiserum against the mannose-6-phosphate receptor was a gift from Suzanne Pfeffer (Stanford University, Stanford, CA); GM10, a mouse mAb against secretory granule membranes (Grimaldi et al., 1987) which recognizes LGP120, was a gift from John Hutton (University of Cambridge, Cambridge, U.K.); and the rabbit antiserum against GLUT-4 was a gift from Gwyn Gould (University of Glasgow, Glasgow, U.K.) (Brant et al., 1992).

Adaptor Targeting In Vitro

Immunofluorescence localization of newly recruited PM adaptors was carried out essentially as previously described for TGN adaptors. Briefly, cells growing on multi-well test slides were washed in cytosol buffer, frozen by placing the slide on a metal block embedded in dry ice, thawed, and incubated with freshly thawed brain cytosol. ATP was added to the cytosol at a concentration of 1 mM from a 10× stock solution in TE buffer (10 mM Tris, 1 mM EDTA), together with a regenerating system (5 mM creatine phosphate and 80 μg/ml creatine phosphokinase). For some experiments, GTPγS was also added at a concentration of 100 μM, although 10 μM produced a similar but less pronounced effect.

After incubating the cells with cytosol for 10 min at 37°C, the slides were washed and fixed, usually in methanol for 5 min at −20°C followed by acetone for 30 s at −20°C. However, to preserve the antigenicity of the transferrin receptor, it was necessary to fix the cells in 2% paraformaldehyde in cytosol buffer for 30 min at room temperature. Cells were then permeabilized with 0.1% (vol/vol) NP-40 in PBS for 10 min and further washed with PBS before labeling for immunofluorescence.

This method was adapted and scaled up for immunoelectron microscopy. Tissue culture cells were grown in 6-cm dishes until ~75% confluent. After washing with cytosol buffer, the cells were permeabilized by rapid freezing in liquid nitrogen, thawed, quickly washed again with cytosol buffer, and incubated with 2 ml of freshly prepared pig brain cytosol for 10 min at 37°C. The cytosol was then aspirated off and replaced very briefly with fixative (cytosol buffer [with no DTT] containing 2% paraformaldehyde and 0.1% glutaraldehyde), and the cells were then incubated with fresh fixative for 30 min at room temperature. After fixation, the free aldehyde groups were quenched by a series of washing steps: cytosol buffer with DTT; TBS, pH

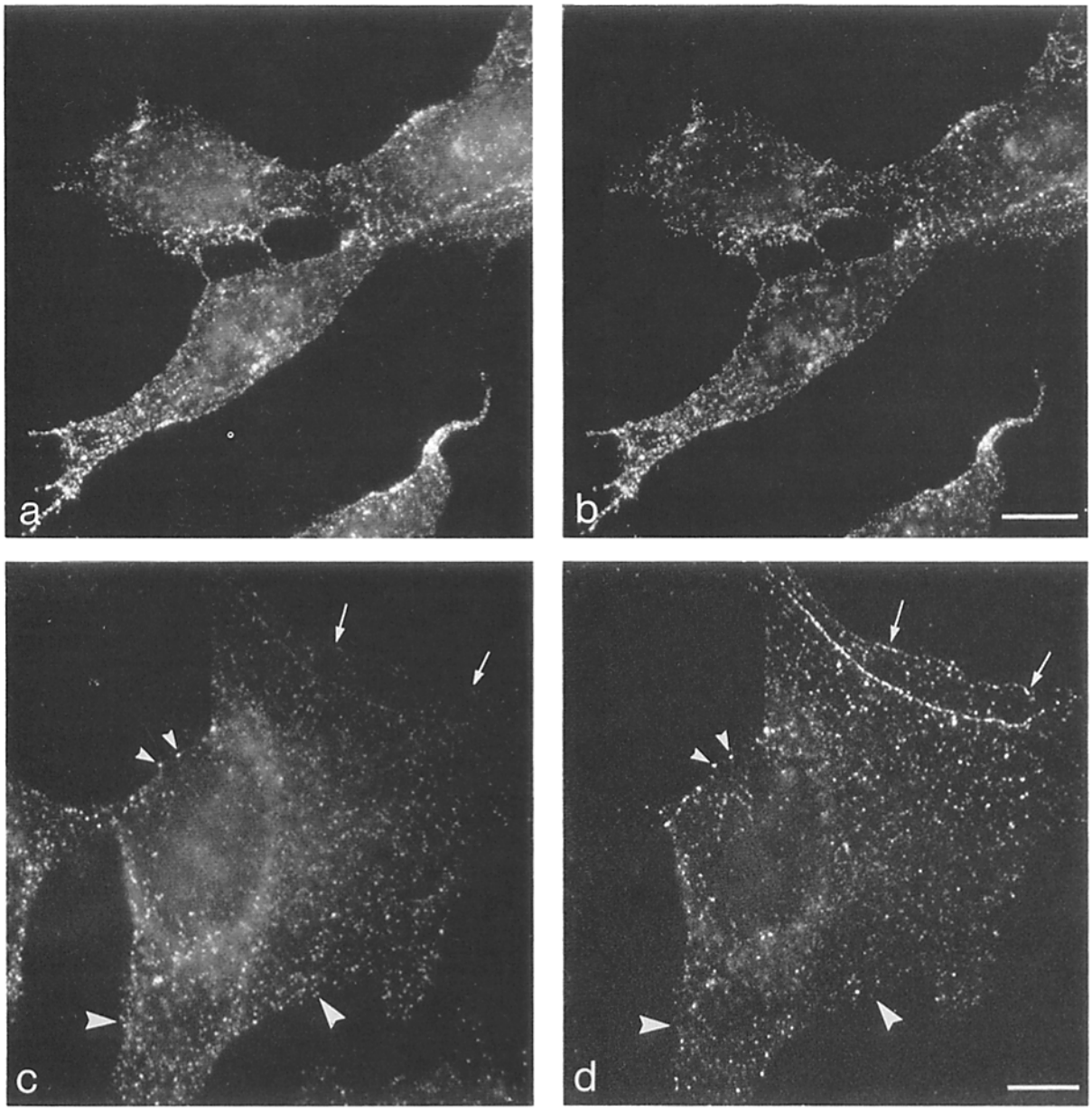

Figure 1. Localization of newly recruited PM adaptors and endogenous PM adaptors. (*a* and *b*) NRK cells were permeabilized and incubated with pig brain cytosol, fixed using methanol/acetone, and then double labeled using AC2M15, a species-specific mAb against α-adaptin (*a*), and C4, a polyclonal antiserum against a brain-specific insert in α-adaptin (*b*). Both antibodies are specific for newly recruited PM adaptors. Bar, 12 μm. (*c* and *d*) Permeabilized Vero cells incubated with rat brain cytosol allow localization of the endogenous (*c*) and exogenous (*d*) PM adaptors using AC2M15 and C4, respectively. The arrows indicate areas where the exogenous PM adaptors have displaced the endogenous, the small arrowheads indicate coated pits that contain both, and the large arrowheads indicate coated pits containing only endogenous PM adaptors. The holes in the cell where the plasma membrane has peeled back can often be visualized as bright lines or rings positive for newly recruited PM adaptors (e.g., just below the *arrows* in *d*). Bar, 8 μm.

8.0; PBS, pH 7.4; PBS with 1 mg/ml $NaBH_4$ for 5 min; PBS; PBS with 5 mg/ml BSA for 5 min; and finally PBS with 1 mg/ml BSA (PBS/BSA). The cells were then incubated with the primary antibody at the appropriate dilution in PBS/BSA for 1 h at room temperature, washed for 15 min in PBS/BSA, and incubated for 1 h at room temperature with the secondary gold-labeled antibodies. For double-labeling experiments, the gold antibodies had been preabsorbed with either mouse or rabbit IgG as appropriate coupled to CNBr-activated Sepharose. After the antibody incubations, the cells were washed for 5 min with PBS/BSA, followed by PBS, and then by 0.1 M cacodylate, pH 7.4. The cells were fixed again with 2.5% glutaraldehyde, 0.2% tannic acid in 0.1 M cacodylate for 30 min at room temperature, washed with 0.1 M cacodylate for 15 min, postfixed with 1% OsO_4 in 0.1 M cacodylate for 30 min at room temperature, washed with 0.1 M cacodylate and then with water, and stained en bloc with 1% uranyl acetate. The cells were then serially dehydrated, scraped off the dish, and embedded in Spurr's resin. Silver sections were collected on 75-mesh Formvar-coated grids and examined in a Phillips CM10 microscope operated at 60 kV.

The immunogold double labeling for PM and TGN adaptors was quantified using the following criteria. Membranes with at least two gold particles of either 5 or 10 nm were scored and the numbers of gold particles of each size per labeled membrane were counted. To determine the extent of coincidence of the two types of gold particles, the numbers of gold particles of one size which were present on a membrane with two or more gold particles of the other size were counted.

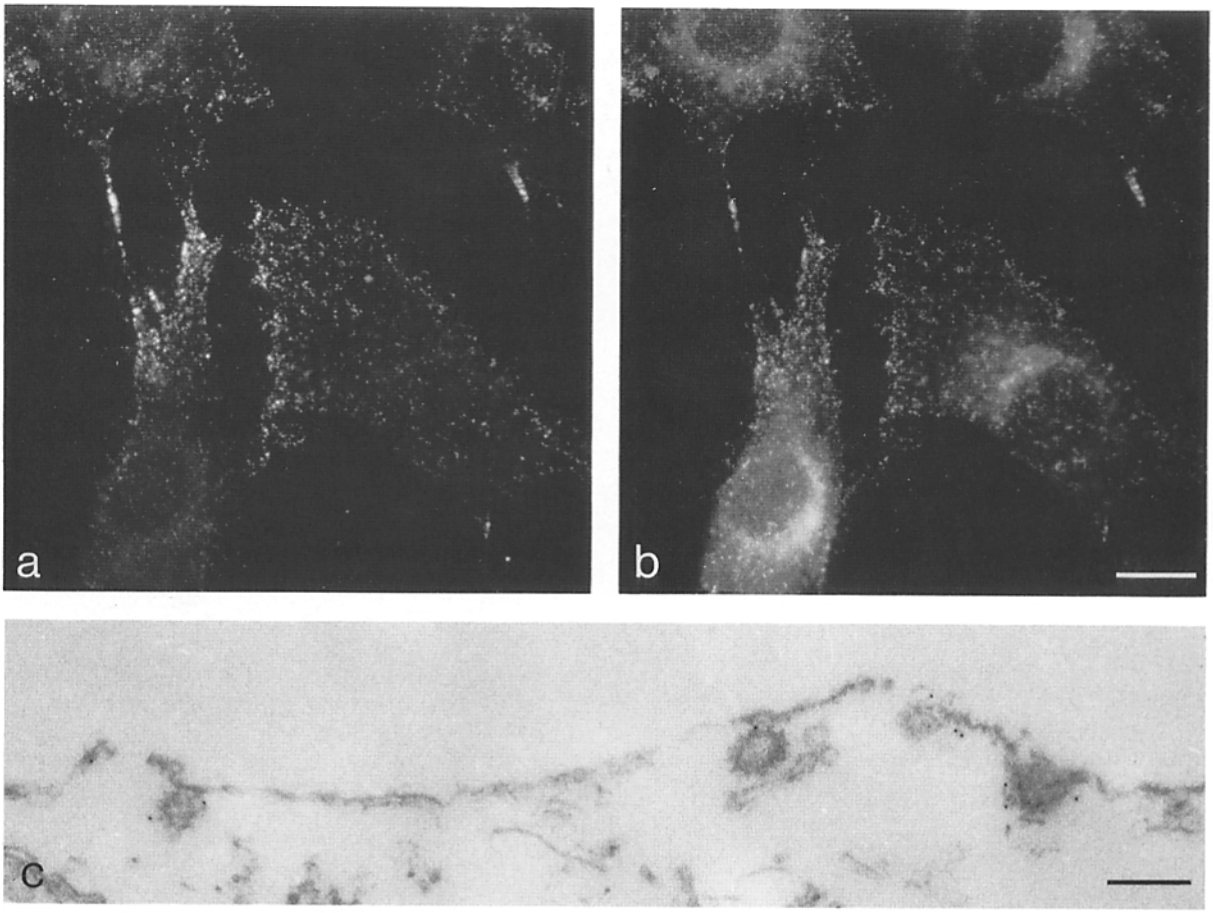

Figure 2. Newly recruited PM adaptors are incorporated into coated pits. (*a* and *b*) NRK cells incubated with pig brain cytosol were fixed and double labeled using C4 (*a*) and X22 (*b*), a mAb against the clathrin heavy chain. The colocalization of the punctate structures indicates that newly recruited PM adaptors are incorporated into clathrin-coated pits. The nonuniform labeling with C4 is probably a result of nonuniform permeabilization, preventing the adaptors in the donor cytosol from gaining access to the entire cell. The clathrin labeling is perinuclear as well as punctate because it is associated with the TGN as well as with the plasma membrane. (*c*) PM adaptors recruited from the cytosol were labeled for EM with C4 followed by anti-rabbit IgG coupled to 5-nm gold. The label is associated with plasma membrane coated pits. Bars: (*a* and *b*) 12 μm; and (*c*) 150 nm.

Ligand Uptake

For studies on the localization of endocytosed rhodamine-labeled wheat germ agglutinin (rhod-WGA), NRK cells were washed with serum-free medium and incubated for 1 h in serum-free medium containing 10 μg/ml rhod-WGA at 4°C to allow the lectin to bind. The cells were then transferred to a 37°C incubator for a further hour, after which lectin still present on the plasma membrane was removed with 50 mM N-acetyl glucosamine in serum-free medium. The cells were then washed with cytosol buffer and permeabilized as described above.

WGA conjugated to 10-nm gold was used as an endocytic marker in immunoelectron microscopy experiments. NRK cells grown to 75% confluency in 6-cm dishes were washed with serum-free medium, incubated in serum-free medium containing WGA-gold diluted 1:4 (supplied concentration: A_{520} = 5.0) for 1 h at 37°C, and washed with cytosol buffer before permeabilization. Cationized ferritin (CF) at a concentration of 5 mg/ml was also used as an endocytic marker for immunoelectron microscopy experiments, essentially as described above except the cells were incubated with the ligand for 2 h at 37°C.

Results

Recruitment of Adaptors onto the Plasma Membrane

Recruitment of PM adaptors onto the plasma membrane can be reconstituted in vitro using a system essentially the same as the one used to study TGN adaptor recruitment (Robinson and Kreis, 1992). NRK acceptor cells (a rat cell line) are permeabilized by freezing and thawing, and then incubated for 10 min at 37°C with donor cytosol (prepared from pig brain) containing ATP and an ATP regenerating system. Two different antibodies can be used to visualize PM adaptors recruited from the donor cytosol without any background from endogenous adaptors on the acceptor cell plasma membrane. AC2-M15 is a species-specific mouse mAb against α-adaptin which recognizes the protein from most mammals but not from rat (Robinson, 1987), while C4 is a rabbit polyclonal antibody that recognizes a brain-specific insert in α-adaptin.

Fig. 1 (*a* and *b*) shows newly recruited PM adaptors in NRK cells double labeled with AC2-M15 (*a*) and C4 (*b*). The labeling looks very similar to that seen in untreated cells stained for endogenous PM adaptors (Robinson, 1987). Omission of the ATP and regenerating system resulted in only trace amounts of labeling at the plasma membrane (data not shown). Using the same combination of antibodies to label Vero cells (a monkey cell line) which had been incubated with donor cytosol prepared from rat brain, it is possible to label the endogenous PM adaptors with AC2M15 (Fig. 1 *c*)

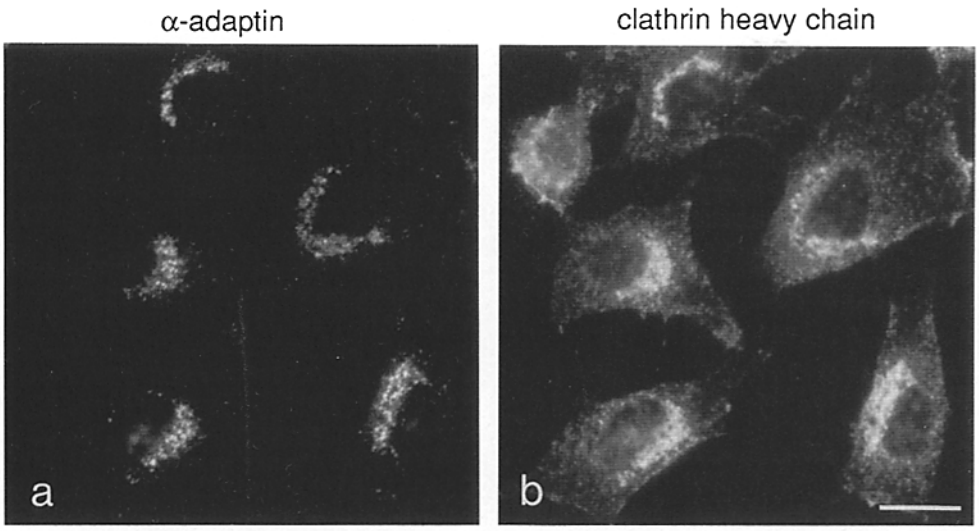

Figure 3. PM adaptors mistargeted to a perinuclear compartment. NRK cells were permeabilized and then incubated with cytosol containing 100 μM GTPγS. Labeling with C4 (*a*) shows that newly recruited PM adaptors are localized to a perinuclear compartment. Double labeling with X22 reveals that there are still clathrin-coated pits remaining at the plasma membrane (*b*). Bar, 15 μm.

and distinguish them from the exogenous PM adaptors labeled with C4 (Fig. 1 *d*). In such cells, it appears that the exogenous PM adaptors can displace the endogenous ones. However, this displacement requires complete permeabilization, so that cells that are not uniformly permeabilized may have regions that contain almost exclusively exogenous PM adaptors (Fig. 1, *arrows*), regions that contain both endogenous and exogenous PM adaptors (*small arrowheads*), and also regions that contain mostly endogenous PM adaptors (*large arrowheads*).

The punctate nature of the labeling pattern suggests that the newly recruited PM adaptors are incorporated into clathrin-coated pits. This was confirmed by double labeling with C4 (Fig. 2 *a*) and a mAb against clathrin heavy chain, X22 (Fig. 2 *b*). The dots that can be resolved at the cell periphery, presumably representing individual coated pits, are virtually identical with the two antibodies. Further evidence that adaptors recruited from the donor cytosol are incorporated into coated pits was obtained by immunogold EM. Fig. 2 *c* shows cells labeled before embedding with C4 followed by anti-rabbit IgG coupled to 5-nm gold particles. Labeling is almost exclusively associated with plasma membrane coated pits, although frequently the gold particles are adjacent to the pits rather than directly over them, possibly because the clathrin lattice acts as a barrier to the antibody conjugates.

GTPγS Mistargets the Adaptors to a Perinuclear Compartment

Because GTPγS enhances the recruitment of both TGN adaptors and β-COP onto the appropriate membrane (Robinson and Kreis, 1992; Wong and Brodsky, 1992; Donaldson et al., 1991*b*), the effect of GTPγS on the targeting and recruitment of PM adaptors was investigated. Fig. 3 *a* shows NRK cells which were incubated with cytosol plus 100 μM GTPγS, fixed, and then labeled with C4 antibodies to localize the newly recruited PM adaptors. Surprisingly, the adaptors have not been recruited to the plasma membrane, but instead are associated with a perinuclear compartment. This does not reflect a loss of coated pits from the plasma membrane: double labeling with anti-clathrin (Fig. 3 *b*) indicates that there are abundant clathrin-coated pits remaining. Thus, it seems likely that the GTPγS is preventing PM adaptor recruitment onto the plasma membrane and causing mistargeting to a perinuclear compartment.

Effects of BFA and Calcium

Previous studies on coat protein recruitment have shown that GTPγS and BFA can act antagonistically (Lippincott-Schwartz et al., 1991; Robinson and Kreis, 1992). Thus, the effect of BFA on PM adaptor recruitment was investigated. Addition of 80 μg/ml BFA alone to the donor cytosol does not affect the recruitment of PM adaptors onto the plasma membrane in vitro (Fig. 4 *a*), consistent with in vivo studies which did not show any effect of the drug on the distribution of PM adaptors (Robinson and Kreis, 1992; Wong and Brodsky, 1992). However, preincubation with cytosol containing BFA followed by cytosol containing BFA plus GTPγS prevents GTPγS-induced mistargeting and causes PM adaptors to be recruited onto the plasma membrane (Fig. 4 *b*). In contrast, preincubation with cytosol containing GTPγS followed by cytosol containing BFA causes adaptors to be recruited onto the perinuclear compartment (Fig. 4 *c*). Thus, BFA can prevent GTPγS-induced mistargeting, but only if it is added first.

GTPγS is not the only reagent that causes PM adaptors to be mistargeted to a perinuclear compartment: calcium also has an effect. Normally the donor cytosol was prepared in a buffer containing 100 μM EDTA. When the EDTA was omitted, the concentration of free calcium in our cytosol preparations was found to be in the region of 60 μM, which is ~10^3-fold higher than physiological concentrations (Alberts et al., 1989). Presumably this is because the cytosol was prepared by homogenizing pieces of frozen brain, which could cause release of calcium from intracellular stores as well as some contamination with extracellular fluid. Addition of nonchelated donor cytosol to the acceptor cells results in variable amounts of perinuclear labeling and relatively little plasma membrane labeling (Fig. 5 *a*). Addition of 100 μM EGTA, which preferentially chelates calcium, can restore targeting of the adaptors to the plasma membrane (Fig. 5 *b*), while addition of 100 μM EGTA plus 200 μM $CaCl_2$ causes mistargeting of adaptors to the perinuclear compartment (Fig. 4 *c*). Thus, calcium has a similar

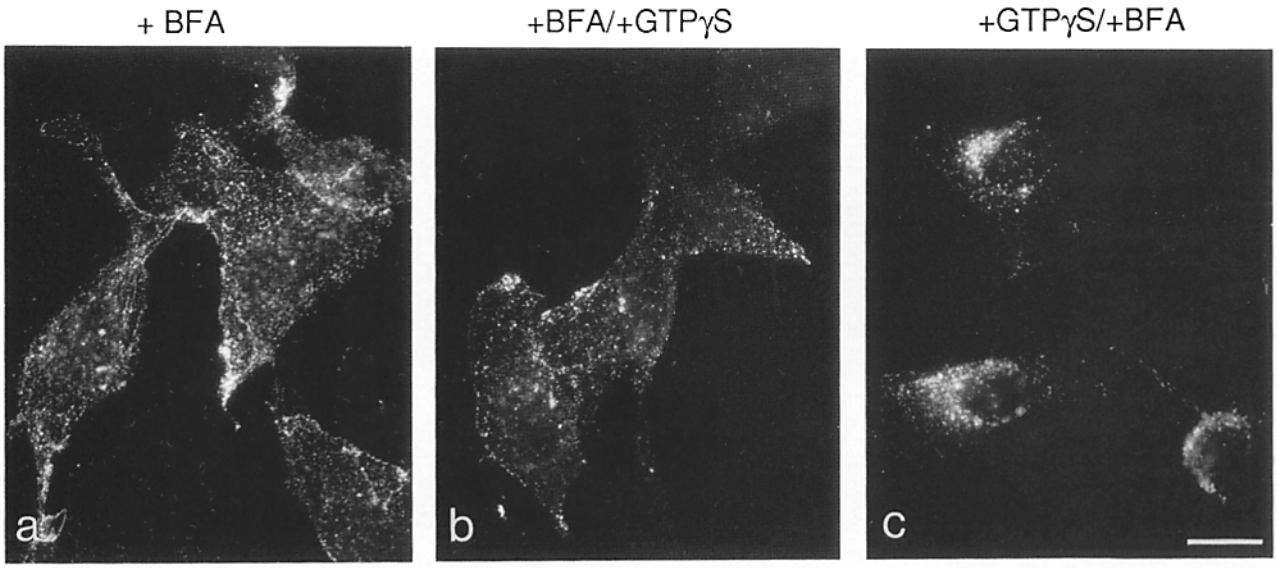

Figure 4. Effect of BFA on GTPγS-induced mistargeting. (*a*) Permeabilized NRK cells were incubated with cytosol plus 80 μg/ml BFA. Newly recruited PM adaptors (labeled with AC2-M15) are targeted to the plasma membrane. (*b*) Permeabilized NRK cells were incubated for 10 min with cytosol plus 80 μg/ml BFA, followed by cytosol containing 80 μg/ml BFA plus 100 μM GTPγS for a further 10 min. Newly recruited PM adaptors (labeled with C4) are targeted to the plasma membrane. (*c*) Permeabilized NRK cells were incubated with cytosol plus 100 μM GTPγS for 10 min, followed by cytosol containing 80 μg/ml BFA for a further 10 min. Under these conditions, the PM adaptors (labeled with C4) are mistargeted to the perinuclear compartment. Bar, 20 μm.

effect to that of GTPγS in preventing plasma membrane recruitment and causing mistargeting.

Identification of the Perinuclear Compartment

Immunofluorescence Microscopy. To determine the identity of the compartment to which the PM adaptors are mistargeted, a series of double-labeling immunofluorescence experiments was carried out on cells that had been incubated with cytosol containing 100 μM GTPγS. Fig. 6 (*a* and *d*) shows cells double labeled with the two antibodies that detect newly recruited α-adaptin: AC2M15 (*a*) and C4 (*d*). The complete colocalization seen with these two antibodies, which recognize the same antigen but different epitopes, provides a benchmark with which the other immunofluorescence figures can be compared.

To test whether the PM adaptors are being promiscuously

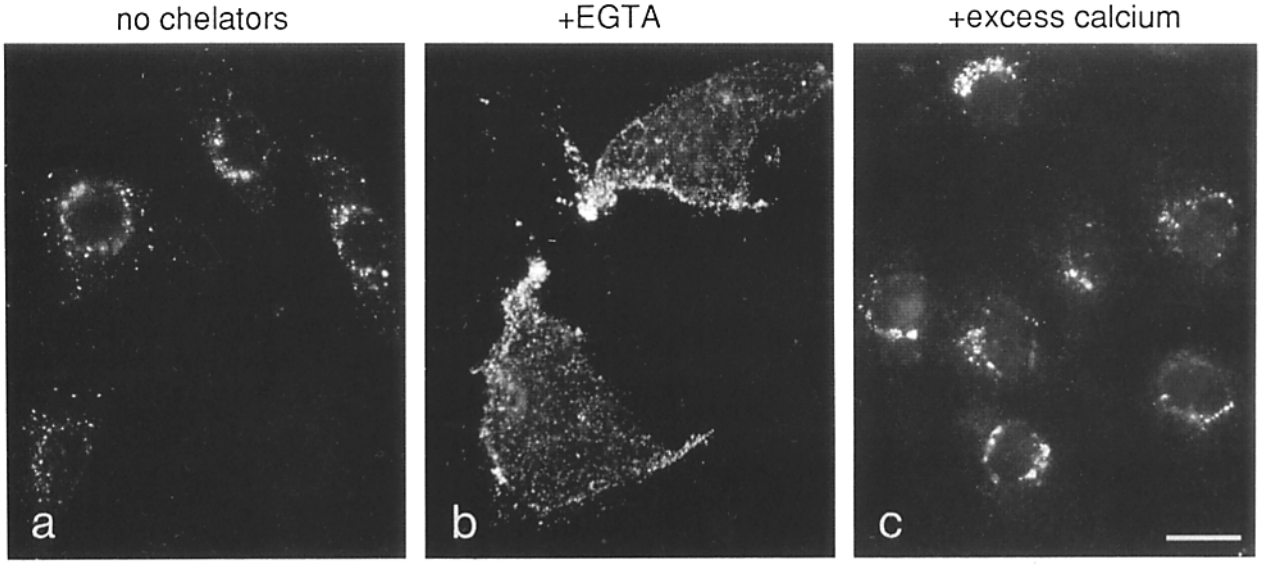

Figure 5. Effect of calcium on the targeting of PM adaptors. (*a*) Permeabilized NRK cells were incubated with cytosol without any chelators added. The newly recruited PM adaptors (labeled with C4) are localized to the perinuclear region. (*b*) Permeabilized NRK cells were incubated with cytosol containing 100 μM EGTA. Newly recruited PM adaptors (labeled with C4) are associated with the plasma membrane. (*c*) Permeabilized NRK cells were incubated with donor cytosol containing 100 μM EGTA and 200 μM $CaCl_2$. The perinuclear location of the PM adaptors (labeled with C4) confirms that calcium is able to induce the mistargeting of the adaptors. Bar, 20 μm.

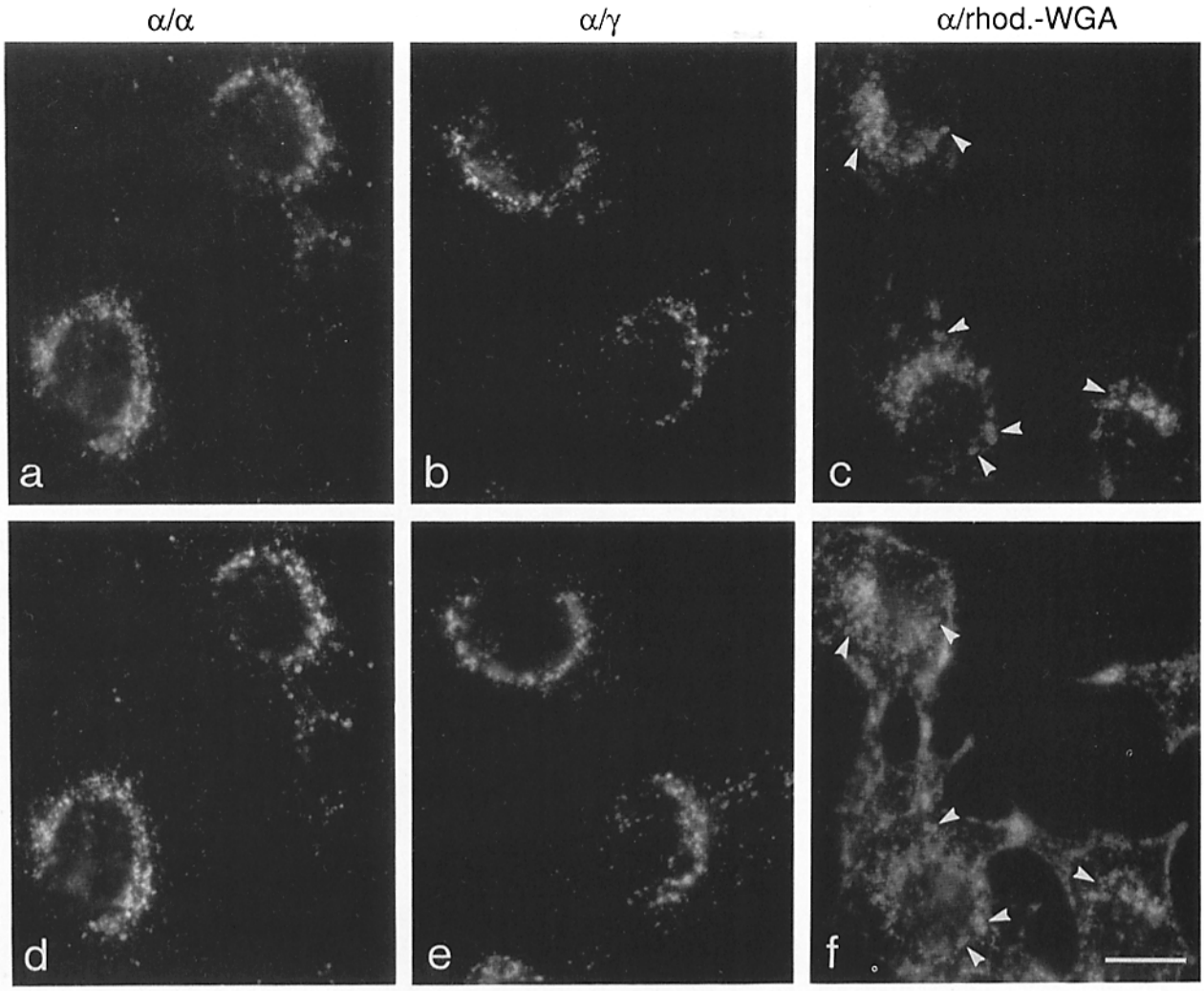

Figure 6. Double labeling of mistargeted PM adaptors. Permeabilized NRK cells were incubated with cytosol plus 100 μM GTPγS before fixation and double labeling. (*a* and *d*) Cells were double labeled with the two antibodies that recognize newly recruited PM adaptors: AC2-M15 (*a*) and C4 (*d*), resulting in complete coincidence. (*b* and *e*) Cells were double labeled for newly recruited PM adaptors with C4 (*b*) and newly recruited TGN adaptors with the species-specific antibody against γ-adaptin, mAb 100/3 (*e*). Although both antibodies label the perinuclear region of the cell, many of the fine details are different. (*c* and *f*) Cells were allowed to endocytose 10 μg/ml rhodamine-WGA for 1 h at 37°C before permeabilization (*f*), and double labeled for newly recruited PM adaptors with C4 (*c*). There is marked overlap between the two labels. Bar, 10 μm.

recruited onto the TGN along with TGN adaptors, cells were labeled for newly recruited PM adaptors (Fig. 6 *b*) and newly recruited TGN adaptors (Fig. 6 *e*), visualized with the species-specific antibody mAb 100/3, which recognizes γ-adaptin from pig but not rat. Although the antibodies both label compartments in the perinuclear region of the cell, careful examination of the micrographs reveals that there are differences in the two labeling patterns. This result suggests that PM and TGN adaptors are being recruited onto different compartments, although it does not rule out the possibility that the adaptors are incorporated into different coated regions of the same compartment. Double labeling with antibodies against other Golgi proteins, residing both in the TGN (TGN38) and on the Golgi stack (β-COP), also produce somewhat different patterns (data not shown). Thus, the compartment to which PM adaptors are mistargeted does not appear to be part of the Golgi apparatus, although it is in the same general region of the cell.

The endosomal system also has a perinuclear distribution in the cell. To label endocytic compartments, cells were allowed to endocytose rhodamine-conjugated WGA for 1 h before permeabilization (Fig. 6 *f*). Double labeling for newly recruited PM adaptors (Fig. 6 *c*) shows that many of the fluorescent structures coincide. A high degree of coincidence was also seen with endocytosed fluorescent LDL (data not shown). However, the lack of complete colocalization suggests that the adaptors may be binding to a subcompartment of the endosomal system.

Fig. 7 shows cells double labeled for mistargeted PM adaptors (*a*, *b*, *e*, and *f*) and markers for different endosomal subcompartments (*c*, *d*, *g*, and *h*). The transferrin receptor (Fig. 7 *c*), which is found in early recycling endosomes (Trowbridge, 1991), shows little if any clear colocalization with the PM adaptors (*a*). Both the mannose-6-phosphate receptor (M6PR) and LGP120 are membrane proteins found in the late endosome/pre-lysosome compartment; in addi-

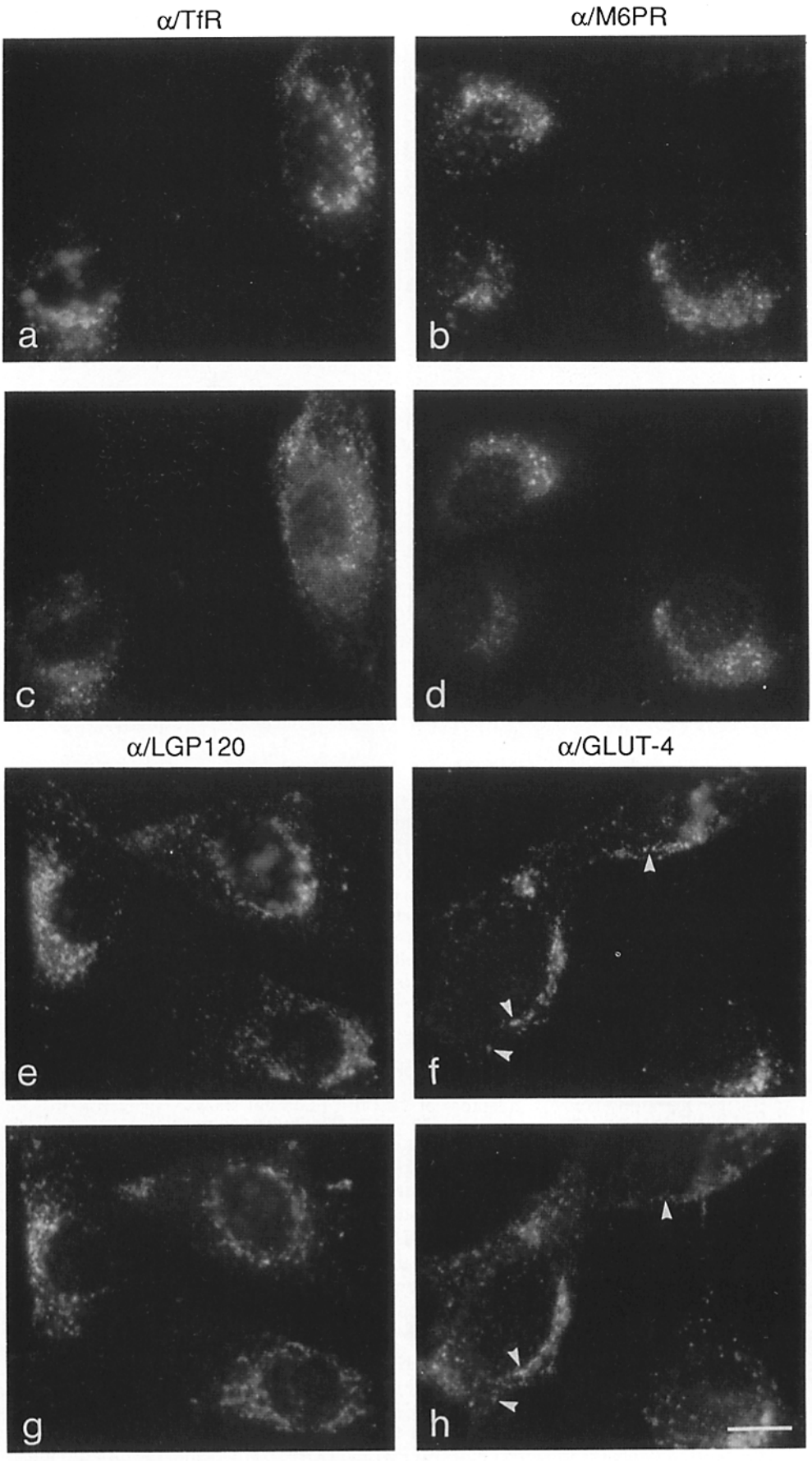

Figure 7. Double labeling for endosomal subcompartments. Permeabilized cells were incubated with cytosol containing 100 μM GTPγS before fixation and double labeling with antibodies against newly recruited PM adaptors (*a*, *b*, *e*, and *f*) and markers for endosomal subcompartments. (*a* and *c*) NRK cells were double labeled with C4 (*a*) and a mAb against the transferrin receptor (*c*). (*b* and *d*) NRK cells were double labeled with AC2-M15 (*b*) and an antiserum against the mannose-6-phosphate receptor (*d*). (*e* and *g*) NRK cells were double labeled with C4 (*e*) and a monoclonal antibody against LGP120 (*g*). (*f* and *h*) 3T3 cells stably transfected with GLUT-4 were double labeled with AC2M15 (*f*) and an antiserum against GLUT-4 (*h*). The best colocalization is seen with GLUT-4, although it is still less than that seen with endocytosed WGA (see Fig. 6, *c* and *f*) Bar, 10 μm.

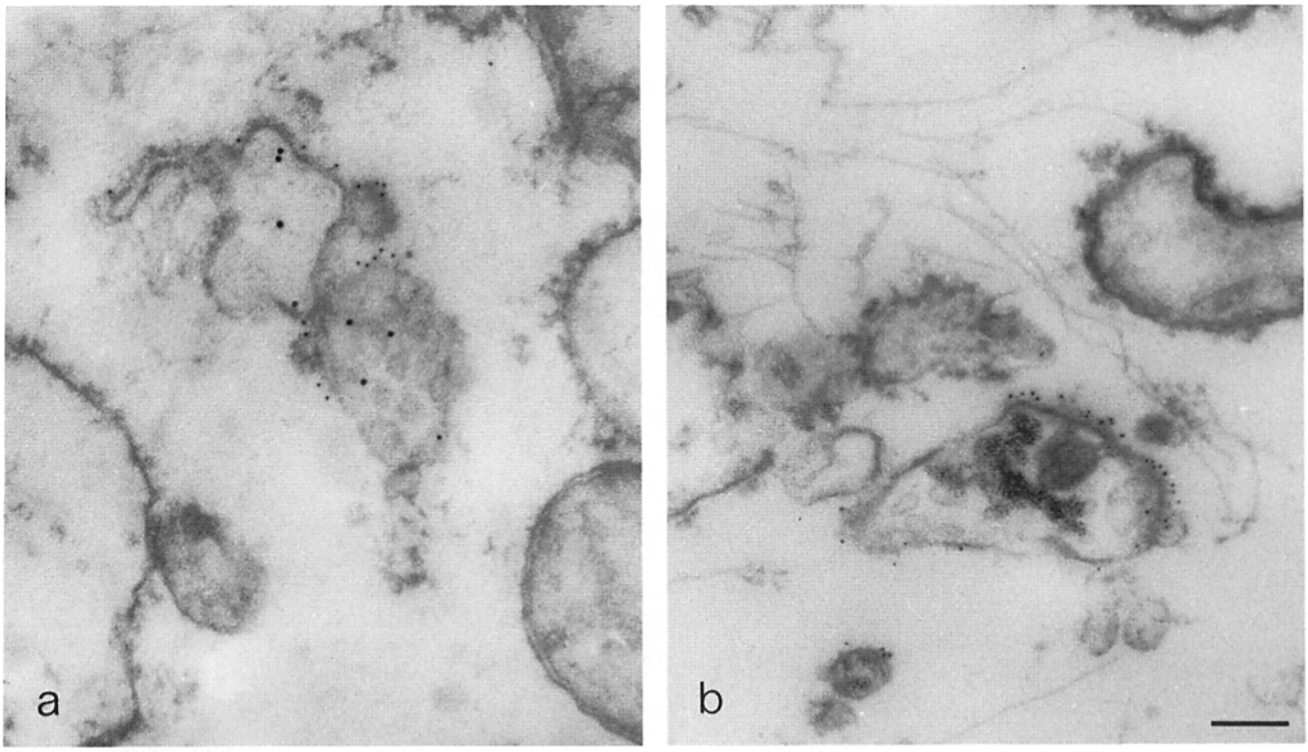

Figure 8. EM labeling of endosomal compartments. NRK cells were incubated either with WGA coupled to 10-nm colloidal gold for 1 h (*a*) or with cationized ferritin for 2 h (*b*) before permeabilization and incubation with cytosol containing 100 μM GTPγS. Newly recruited PM adaptors were labeled with C4 followed by 5-nm anti-rabbit gold. Labeling can be seen on structures containing both endocytic tracers, and although patchy, its density is higher than that found on the plasma membrane (see Fig. 2 *c*). Clathrin-like coats can often be seen on vesicles budding from the endosomes. Bar, 130 nm.

tion, some of the M6PR is localized in the TGN (Griffiths and Simons, 1986), while most of the LGP120 is concentrated in lysosomes (Harter and Mellman, 1992). Both proteins have significantly different patterns from PM adaptors (Fig. 7, compare *b* with *d* and *e* with *g*), although there may be some overlap. Another marker for an endosomal subcompartment is the glucose transporter GLUT-4, which is thought to be sequestered into a specialized storage compartment (Hudson et al., 1992; Piper et al., 1992). Stably transfected 3T3 cells expressing GLUT-4 were double labeled for newly recruited PM adaptors (Fig. 7 *f*) and GLUT-4 (*h*). Although there is less overlap than with endocytosed WGA, the two antigens appear to show partial coincidence. These results suggest that the PM adaptor recruiting compartment is probably a late endosomal subcompartment, which shows some overlap with the GLUT-4 compartment, but is not particularly rich in either the M6PR or LGP120. However, to determine the nature of this compartment more precisely, it was necessary to use immunoelectron microscopy.

Immunoelectron Microscopy. Immmunogold labeling of mistargeted PM adaptors revealed that the compartment to which they bind consists mainly of multivesicular bodies and tubulovesicular elements, frequently coated with clathrin lattices. To confirm that the compartment is endosomal, cells were allowed to internalize either WGA coupled to 10-nm gold, or CF, then permeabilized, incubated with cytosol containing 100 μM GTPγS, and labeled with C4 followed by anti-rabbit IgG coupled to 5-nm gold. Fig. 8 *a* shows endosomes containing WGA, while Fig. 8 *b* shows an endosome containing CF. In both cases, the endosomes are covered with newly recruited α-adaptin, confirming that the PM adaptor binding compartment is part of the endosomal system.

The partial colocalization of mistargeted PM adaptors and GLUT-4 was also investigated at the EM level. Fig. 9 shows four serial sections of a transfected 3T3 cell double labeled for newly recruited α-adaptin (10-nm gold) and GLUT-4 (5-nm gold). A tubular network studded with clathrin-coated buds can be seen running through the sections, and it is labeled with both antibodies. However, there were also compartments which were labeled exclusively for either PM adaptors or GLUT-4 (data not shown), indicating that the two antigens do not completely colocalize.

Double labeling was also used to investigate the possibility that in the presence of GTPγS, PM adaptors and TGN adaptors might be recruited onto the same compartment. In Fig. 10, newly recruited TGN adaptors are labeled with 10-nm gold, while newly recruited PM adaptors are labeled with 5-nm gold. The TGN adaptors are localized on a tubular, clathrin-coated compartment, with a morphology characteristic of the TGN. The PM adaptors are concentrated on a different compartment, but one that is in close proximity to the TGN. Thus, in this figure the two adaptor binding compartments appear to be distinct, although they are sufficiently close together to make resolution at the light microscope level very difficult (e.g., see Fig. 6, *b* and *e*). To investigate the possibility that there may be partial coincidence

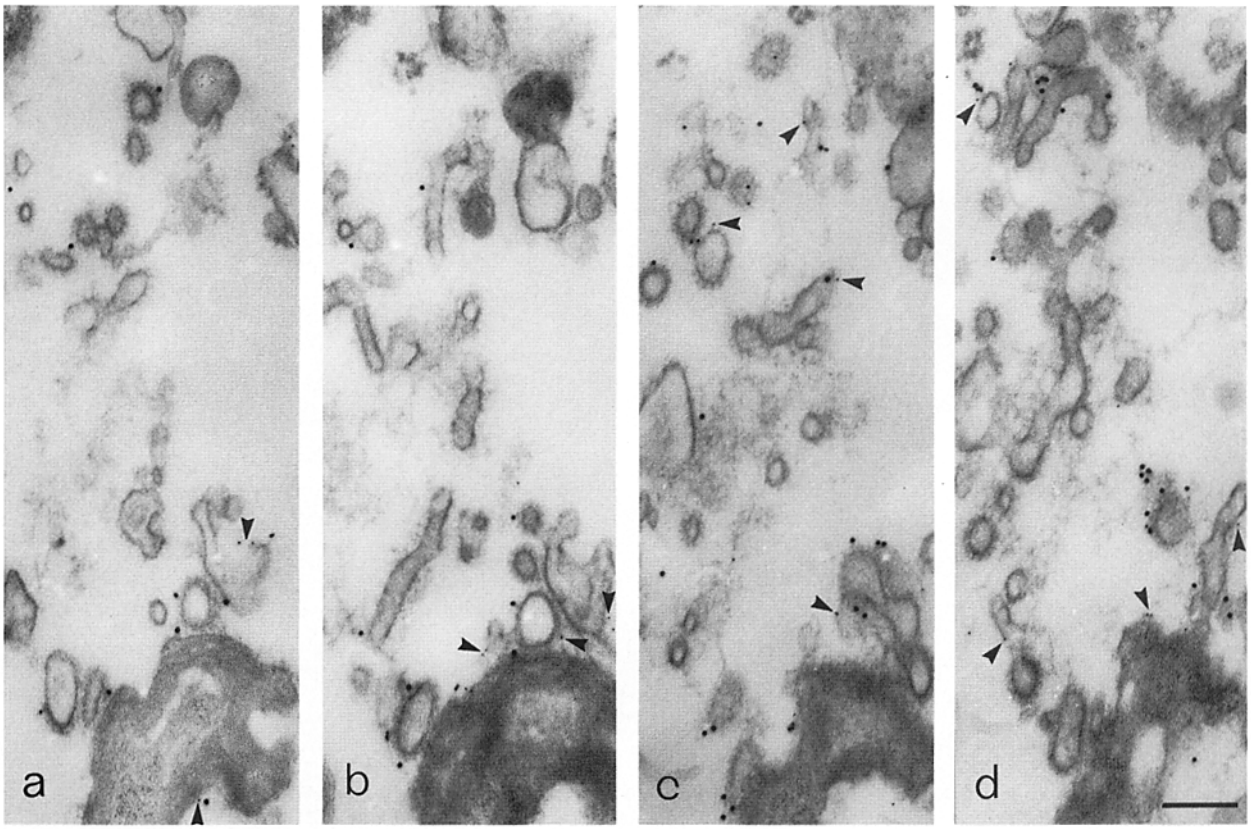

Figure 9. Immunoelectron microscopy double labeling for GLUT-4. 3T3 cells stably transfected with GLUT-4 were permeabilized and incubated with cytosol plus 100 μM GTPγS, and then double labeled for newly recruited PM adaptors with MC4 (a mouse antiserum against the same antigen as C4) and GLUT-4. These were followed by 10-nm anti-mouse and 5-nm anti-rabbit gold-conjugated antibodies. Views of four serial sections are shown in *a* to *d*. The arrowheads point to some of the 5-nm gold particles, which are on structures also labeled with 10-nm gold particles. Clathrin-coated buds are also associated with some of the structures. Bar, 190 nm.

between PM and TGN adaptors, additional micrographs were examined and the labeled membranes were scored for gold particles of both sizes (Fig. 11). Approximately 80% of membranes scored were labeled exclusively with either 5- or 10-nm gold. Where coincident labeling was observed, it was generally restricted to the level of just one or two gold particles. Thus, although there may be a limited amount of colocalization of PM and TGN adaptors, for the most part they appear to be recruited onto different membranes.

Discussion

We have demonstrated that PM adaptor complexes can be recruited onto the plasma membrane in vitro without prior removal of endogenous PM adaptors. The addition of cytosol plus energy to permeabilized cells may activate the coated vesicle cycle, freeing adaptor-binding sites that were previously occupied and allowing new adaptors to bind. These adaptors are able to displace the endogenous PM adaptors and to become incorporated into coated pits. Thus, our system provides a direct assay for PM adaptor recruitment under physiological conditions.

Because our antibodies enable newly recruited PM adaptors to be visualized specifically, it is possible to determine whether the adaptors are binding to the plasma membrane or to a different compartment. We have found that addition of GTPγS to the system causes PM adaptors to associate with an endocytic compartment. Although one possible explanation for this finding might be that in the presence of GTPγS, coated vesicles bud from the plasma membrane, fail to uncoat, and fuse with endosomes, we feel that this is unlikely for two reasons. First, under these conditions there are still clathrin-coated pits on the plasma membrane but they are not labeled with antibodies against newly recruited PM adaptors; thus, one would have to propose that the newly recruited PM adaptors are incorporated into coated pits that preferentially pinch off. Second, stage-specific assays for endocytosis indicate that GTPγS completely blocks coated vesicle budding in vitro, possibly by acting on dynamin or a related protein (Carter et al., 1993). The stage-specific assays also indicate that GTPγS inhibits another very early event associated with the formation of plasma membrane coated pits. It seems likely that in their system as well as in ours, GTPγS has at least two effects: it prevents preexisting coated pits from pinching off, while at the same time preventing new coated pits from forming by causing PM adaptors to be recruited onto the wrong compartment.

Although we cannot formally rule out the possibility that in the normal sequence of events, PM adaptors are recruited first onto endosomes but then quickly follow the recycling pathway to the plasma membrane, we feel that this too is unlikely. Examination of cells incubated with control cytosol did not reveal any transient association of the PM adaptors with endosomes; and in cells in which the PM adap-

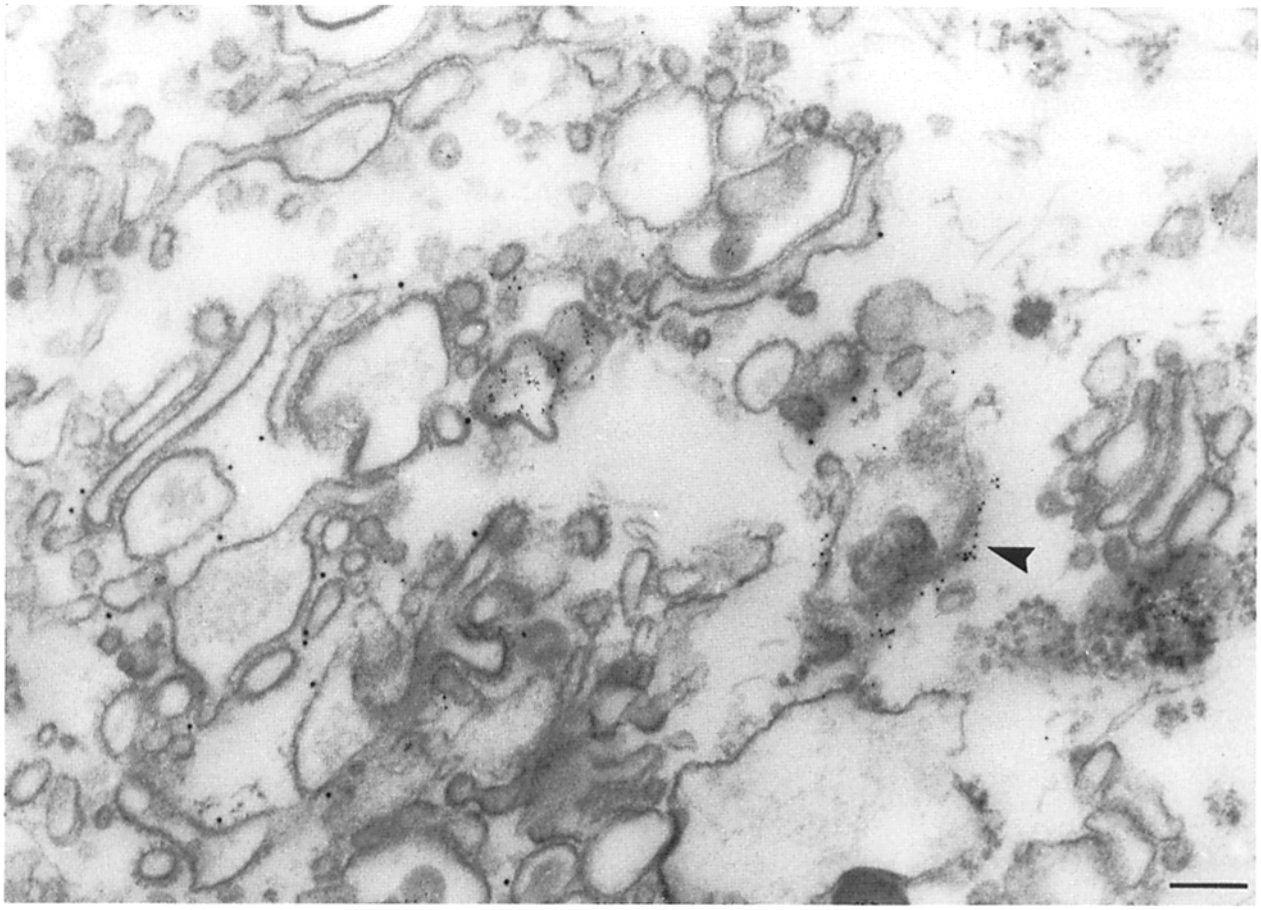

Figure 10. Immunoelectron microscopy double labeling for newly recruited PM and TGN adaptors. Permeabilized NRK cells were incubated with cytosol plus 100 μM GTPγS. After fixation the cells were labeled with C4 (5-nm gold) to localize newly recruited PM adaptors and mAb100/3 (10-nm gold) to localize newly recruited TGN adaptors. The 10-nm gold particles label a clathrin-coated tubular compartment, presumably the TGN. The arrowhead points to a structure heavily labeled with 5-nm gold particles. The fine particulate matter in the small paired structure in the center of the micrograph is not gold but a precipitate. Bar, 150 nm.

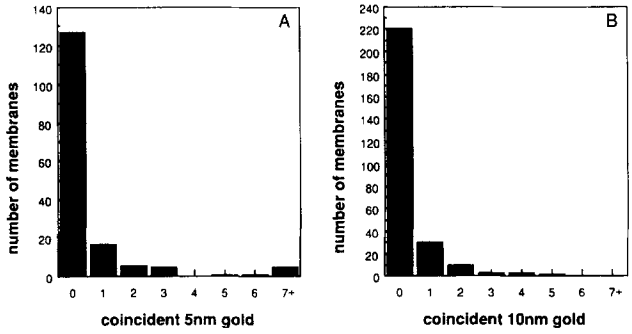

Figure 11. Quantification of the immunogold EM double labeling of PM and TGN adaptors. 434 membranes from 47 micrographs were scored for the presence of 5- and 10-nm gold particles. A total of 1,741 5-nm gold particles and 544 10-nm gold particles were counted. 221 membranes had exclusively 5-nm gold labeling, 127 membranes had only 10-nm gold labeling, and 86 had both 5- and 10-nm gold labeling. *A* shows the numbers of coincident 5-nm gold particles on membranes with two or more 10-nm gold particles. Most of the coincident labeling was restricted to just one or two 5-nm gold particles per membrane. *B* shows the numbers of coincident 10-nm gold particles on membranes with two or more 5-nm gold particles.

tors had been mistargeted, there was little if any coincidence with the transferrin receptor, a marker for recycling endosomes. Moreover, treatment with BFA completely blocks the GTPγS-induced recruitment of PM adaptors onto endosomes but does not prevent recruitment onto the plasma membrane, either in our in vitro system or in vivo (Robinson and Kreis, 1992; Wong and Brodsky, 1992).

Recent reports have suggested that the effects of BFA may be mediated by the small GTP-binding protein ARF, which has been implicated in the recruitment of coatomers onto Golgi membranes (Donaldson et al., 1992; Helms and Rothman, 1992). It is possible that ARF may also play a role in the recruitment of TGN adaptors and in the mistargeting of PM adaptors, both of which are also sensitive to BFA. However, because the three types of coat proteins are all recruited onto different membranes, there must be other factors that specify the target compartment. Moreover, we have investigated the effects of some other potential activators of GTP-binding proteins and find that mastoparan, which is thought to be specific for heterotrimeric GTP-binding proteins (Bomsel and Mostov, 1992), also causes PM adaptors to be mistargeted to a perinuclear compartment, although one with a somewhat more disperse morphology (data not

shown). In contrast, another non-hydrolysable GTP analogue, GMP-PNP, has no detectable effect on PM adaptor targeting, even when used at a concentration (1 mM) which promotes TGN adaptor recruitment (data not shown), possibly because the relevant GTP-binding protein(s) bind this analogue with low affinity.

Elevated calcium levels also cause mistargeting of PM adaptors. Calcium has been implicated in the pinching off of endocytic-coated vesicles (Lin et al., 1992), but our results suggest a possible role for calcium further upstream in the process of coat protein recruitment. Interestingly, Wang et al. (1993) have shown that calmodulin inhibitors can produce a similar mistargeting of PM adaptors in vivo. Calcium/calmodulin-dependent protein kinases regulate many intracellular events; however, preliminary experiments have failed to show any effects of either kinase inhibitors or phosphatase inhibitors in our system (data not shown). At present, therefore, the most likely explanation for our observations is that activation of several different pathways may lead to PM adaptor mistargeting.

The perinuclear compartment to which PM adaptors are mistargeted has been defined as endosomal because it can be filled with endocytosed ligands. The small amount of colocalization with newly recruited TGN adaptors at the EM level can probably be explained by the trafficking known to occur between the TGN and the endosomal system. The PM adaptor-binding compartment appears to be a late endosomal one, both because it consists mainly of multivesicular bodies, which is the typical morphology of late endosomes, and because of its lack of coincidence by immunofluorescence with the transferrin receptor. However, immunofluorescence indicates that the compartment is not particularly enriched in either the M6PR or LGP120, both of which have been shown to be present in late endosomes (Griffiths and Simons, 1986; Harter and Mellman, 1992). The dynamic nature of the endocytic system makes precise definition of the endocytic compartments and their intermediates very difficult, but taken together these observations suggest that the PM adaptors may be recruited onto a specialized subcompartment of the late endosomal system. Clathrin is frequently associated with the compartment as well, indicating that once adaptors have bound to a membrane, even if it is not one with which they are normally associated, cytosolic clathrin can then bind to the adaptors, leading to the assembly of coated vesicles.

The ligand receptors that are concentrated in coated pits at the plasma membrane are also found in endosomes, and one possibility is that PM adaptors are recruited onto these membranes by binding to the cytoplasmic tails of such proteins. However, ligand receptors generally cycle between the plasma membrane and an early endosomal compartment, while the adaptor binding compartment appears to be a late endosomal one. Therefore, we propose that PM adaptors are initially recruited onto the membrane by binding to a specific adaptor receptor, which may correspond to the elastase-sensitive protein(s) identified in the PM adaptor binding assay of Mahaffey et al. (1990). Adaptor binding need not be stoichiometric: transient binding might occur, after which the association of adaptors with the membrane could be stabilized by interactions with lipids or with the cytoplasmic tails of ligand receptors. Thus, although endocytosis of the adaptor receptor is likely to occur, it need not be highly concentrated in coated vesicles. The adaptor receptor could reside not only on the plasma membrane, but also in an endosomal storage compartment from which it could be mobilized to allow the cell to recruit more coat protein onto the plasma membrane, leading to an increased level of endocytosis. For instance, in adipocytes and muscle cells, insulin stimulation results in the exocytosis of vesicles derived from a specialized endosomal subcompartment highly enriched in the glucose transporter GLUT-4 (Slot et al., 1991*a*, *b*). This is accompanied by a threefold increase in the amount of clathrin associated with the plasma membrane (Corvera, 1990), facilitating the rapid recycling of GLUT-4 back to the storage compartment. The partial colocalization of GLUT-4 and PM adaptors in GTPγS-treated transfected cells suggests that the cell may be able to insert GLUT-4 and PM adaptor receptors into the plasma membrane simultaneously.

Because PM adaptors are not normally recruited onto endosomes, the cell must have a way of switching on the adaptor receptor when it is on the plasma membrane and switching it off in the endosomal compartment. Both GTPγS and calcium appear to be capable of reversing the switch. Although we do not yet know the precise details of how the switch might operate, it is likely to involve other proteins residing in the plasma membrane and endosomal compartments (e.g., GTP-binding proteins), and possibly signal transduction across the membrane as well.

We would like to thank Morrie Birnbaum for his generous gift of the stably transfected GLUT-4 3T3 cell line. We also gratefully acknowledge the following people for their gifts of antibodies: Frances Brodsky, Ernst Ungewickell, Paul Luzio, Thomas Kreis, Suzanne Pfeffer, John Hutton, and Gwyn Gould. Ian Marshall and Colin Taylor (Department of Pharmacology, Cambridge) kindly helped with the measurements of free calcium concentrations in the cytosol. We also thank Paul Luzio, Liz Conibear, Sean Munro, John Kilmartin, and Richard Anderson for helpful comments on the manuscript, and Li-Hsien Wang and Richard Anderson for communicating unpublished results.

This work was supported by grants from the Wellcome Trust and the Medical Research Council.

Received for publication 15 May 1993 and in revised form 20 August 1993.

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