Journal of Cell Science 106, 1239-1248 (1993)
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Binding of the cytosolic p200 protein to Golgi membranes is regulated by heterotrimeric G proteins

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SUMMARY

The formation of vesicles for protein trafficking requires the dynamic binding of cytosolic coat proteins onto Golgi membranes and this binding is regulated by a variety of GTPases, including heterotrimeric G proteins. We have previously shown the presence of the pertussis toxin-sensitive $G\alpha_{i\text{-}3}$ protein on Golgi membranes and demonstrated a functional role for $G\alpha_{i\text{-}3}$ in the trafficking of secretory proteins through the Golgi complex. We have also described a brefeldin A-sensitive phosphoprotein, p200, which is found in the cytoplasm and on Golgi membranes. The present study investigates the role of heterotrimeric G proteins in the regulation of p200 binding to Golgi membranes. An in vitro binding assay was used to measure the binding of cytosolic p200 to LLC-PK_1 cell microsomal membranes and to purified rat liver Golgi membranes in the presence of specific activators of G proteins. The binding of p200 to Golgi membranes was compared to that of the coatomer protein β-COP, for which G protein-dependent membrane binding has previously been established. Membrane binding of both p200 and β-COP was induced maximally by activation of all G proteins in the presence of GTPγS. More selective activation of the heterotrimeric G proteins, with AlFn or mastoparan, also induced membrane binding of p200 and β-COP. Pertussis toxin pretreatment of Golgi membranes, to selectively inactivate $G\alpha_{i\text{-}3}$, reduced the AlFn and mastoparan-induced binding of p200 to Golgi membranes, whereas no significant effect of pertussis toxin on β-COP binding was found in this assay. The effect of pertussis toxin thus implicates $G\alpha_{i\text{-}3}$, as one component of a regulatory pathway, in the binding of cytosolic p200 to Golgi membranes. The effects of AlFn and pertussis toxin on p200 membrane binding were also shown in intact cells by immunofluorescence staining. AlFn treatment of cells induced translocation of p200 from the cytoplasm onto the Golgi complex, resulting in a conformational change in some Golgi membranes. The translocation of p200 was blocked by pretreatment of intact NRK cells with pertussis toxin. The data presented here support the conclusion that the binding of the p200 protein to Golgi membranes involves regulation by the pertussis toxin-sensitive heterotrimeric G proteins, specifically the $G\alpha_{i\text{-}3}$ protein.

Key words: Golgi, G protein, p200

INTRODUCTION

Intracellular transport of proteins through the secretory pathway involves vesicular transport between membrane-bound compartments (Palade, 1975; Rothman and Orci, 1992). Several steps in the budding, trafficking and fusion of carrier vesicles in the secretory pathway are now known to be regulated by a variety of GTPases or GTP-binding proteins (G proteins). Several families of monomeric G proteins including the SEC proteins, the rab proteins and the ADP-ribosylation factors (ARFs) have been shown to be involved in various stages of vesicle formation or fusion in eukaryotic cells (Balch, 1990)

A variety of heterotrimeric G proteins, including pertussis toxin-sensitive and cholera toxin-sensitive subunits, are also involved in vesicle trafficking in the secretory pathway (Leyte et al., 1992; Barr, et al., 1991, 1992; Stow et al., 1991; Pimplikar and Simons, 1993). We have previously shown that the pertussis toxin-sensitive heterotrimeric G protein, $G\alpha_{i\text{-}3}$, is localized on Golgi cisternal membranes (Ercolani et al., 1990; Stow et al., 1991). Overexpression or activation of $G\alpha_{i\text{-}3}$ on Golgi membranes retards the Golgi trafficking of a basolaterally secreted proteoglycan and conversely, uncoupling of the $G\alpha_{i\text{-}3}$ subunit by ADP-ribosylation with pertussis toxin, stimulates its Golgi trafficking (Stow et al., 1991). $G\alpha_{i\text{-}3}$ may also regulate trafficking between the rough ER and Golgi, since it has recently been shown that antibodies to $G\alpha_{i\text{-}3}$ inhibit traffic between these compartments in permeabilized cells (Wilson et al., 1993). Pertussis toxin-sensitive heterotrimeric G proteins, including $G\alpha_{i\text{-}3}$, have also been shown to be involved in the cell-free formation of secretory vesicles and granules on *trans*-Golgi network (TGN) membranes (Barr et al., 1991, 1992). More recently, effects of cholera toxin have also implicated

$G\alpha_s$ in the regulation of vesicle formation on TGN membranes (Leyte et al., 1992) and in the apical targetting of viral proteins (Pimplikar and Simons, 1993). The precise roles of heterotrimeric G proteins in regulating vesicle trafficking are not yet understood. One point at which they do appear to have a role is in regulating the dynamic binding of cytosolic coat proteins to Golgi membranes. It is possible that $G\alpha_{i\text{-}3}$, together with other G proteins, is involved in regulating vesicle budding and/ or fusion in the Golgi by controlling the binding of these cytosolic proteins.

Intra-Golgi vesicular transport is a GTP-dependent process (Melancon et al., 1987). The non-clathrin-coated carrier vesicles are formed following the binding of a series of coatomer proteins, such as β-COP and ARF, from the cytosol onto the Golgi membranes (Rothman and Orci, 1992; Duden et al., 1991; Serafini et al., 1991; Orci et al., 1991; Donaldson et al., 1991a). The binding of coatomer proteins to Golgi membranes is inhibited by the fungal metabolite brefeldin A (BFA) (Klausner et al., 1992). Several studies have shown that activation of G proteins with GTPγS (guanosine 5′-*O*-(3-thiotriphosphate)), or aluminium fluoride (AlFn), will inhibit the effects of BFA and stabilize the membrane binding of vesicle-associated proteins such as β-COP, ARF and γ-adaptin (Donaldson et al., 1991a, 1992b; Ktistakis et al., 1992; Robinson and Kries, 1992).

We have described another cytosolic protein, p200, which is localized in the cytoplasm and on Golgi membranes (Narula et al., 1992) in a variety of cells. The p200 protein can also translocate between the cytosol and Golgi membranes, since it is rapidly and reversibly dissociated from the Golgi membranes in the presence of BFA. Although the function of p200 is not yet known, it is possible that it may also be involved in vesicle trafficking. It was therefore important to establish whether the binding of p200 to Golgi membranes is regulated by G proteins. The present studies were undertaken to investigate the binding of cytosolic p200 to Golgi membranes and the potential role of heterotrimeric G proteins in regulating this binding. The translocation of p200 from the cytosol to Golgi membranes was studied in intact cells and in an in vitro binding assay. Our results show that the binding of p200 is indeed regulated by pertussis toxin-sensitive heterotrimeric G proteins and the data specifically implicate $G\alpha_{i\text{-}3}$ as the member of this family responsible for this binding.

MATERIALS AND METHODS

Cell culture

Normal rat kidney (NRK) cells and LLC-PK_1 cells, a polarized epithelial cell line derived from pig kidney, were grown as previously described in Dulbecco's modified Eagle's medium with 10% FCS, in 5% CO_2/95% air (Ercolani et al., 1990; Narula et al., 1992).

Antibodies and reagents

Polyclonal antibodies to specific decapeptides from $G\alpha_i$ subunits were kindly provided by Dr A. Spiegel (NIH) or purchased from Dupont (Wilmington, DE) The EC antibody specifically recognizes the carboxy terminus of the $\alpha_{i\text{-}3}$ subunit (Spiegel et al., 1990) and it was used for immunoblotting and immunolocalization of $G\alpha_{i\text{-}3}$ as previously described (Stow et al., 1991). The M3A5 monoclonal antibody to β-COP (Duden et al., 1991) was kindly provided by Dr Thomas Kreis (EMBL). The mannosidase II antibody was provided by Dr Brian Burke (Harvard). The AD7 monoclonal antibody, which recognizes the p200 protein, has been characterized in our laboratory and was described previously (Narula et al., 1992). Goat anti-rabbit IgG and goat anti-mouse IgG conjugated to FITC, HRP or alkaline phosphatase were obtained from Vector Laboratories (Burlingame, CA).

GTPγS (guanosine 5′-*O*-(3-thiotriphosphate) and other nucleotides were obtained from Calbiochem (San Diego, CA) and pertussis toxin was from List Biologicals (Campbell CA). Mastoparan, an amphiphilic tetradecapeptide from wasp venom, was obtained from Sigma (St Louis, MO).

Isolation of rat liver Golgi membranes

Highly purified rat liver Golgi membranes were prepared as previously described (Stow et al., 1991); briefly, total microsomes were separated on a discontinuous sucrose gradient (Ehrenreich et al., 1973) and the Golgi membrane fraction was collected from the 0.90/0.40 M interface. This fraction was assessed morphologically and enzymatically and was found to contain >90% Golgi membranes, many of which were recovered as stacked Golgi cisternae. The cytosol fraction was collected as the supernatant from rat liver microsomal membranes pelleted at 100,000 ***g*** for 1 hour, this supernatant was then spun a second time at 100,000 ***g*** to remove all membranes. In some experiments, Golgi membranes were separated on sucrose density gradients into crude fractions corresponding to light and heavy Golgi membranes (Hendricks et al., 1992).

In vitro assay for protein binding on Golgi membranes

Samples of Golgi membranes (20 μg membrane protein) and saturating amounts of rat liver cytosol (1200 μg protein) were incubated together in buffer alone or in the presence of 100 μM GTPγS or AlFn ($AlF_{3\text{-}5}$, added as 30 mM NaF + 50 μM $AlCl_3$) or 100 μM mastoparan in buffer containing 30 mM Hepes, 20 mM KCl, 5 mM Mg acetate, 20 mM Tris-HCl, pH 7.4, for 30 minutes at 37°C. We determined that the addition of exogenous ATP or an ATP regenerating system was not necessary, and had no specific effect on protein binding in this assay. In some experiments, Golgi membranes (20 μg) were pretreated with 20 μl (2 μg) activated pertussis toxin or with buffer alone for 180 minutes at 4°C in 12.5 mM Tris-HCl (pH 7.5), 10 mM thymidine, 1 mM EDTA, 1 mM ATP and 0.1 mM GTP. Following incubations, the Golgi membranes were recovered by centrifugation at 100,000 ***g*** for 30 minutes; membrane pellets and samples of supernatants (cytosol fractions) were solubilized in SDS-PAGE sample buffer and loaded onto 5-15% gradient SDS-PAGE gels. Proteins were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford MA) for western blotting. Transfers were stained with Coomassie Blue to ensure that transferred lanes were matched for protein content and then destained. Immunoblotting was then performed with the specific primary antibodies to $G\alpha_{i\text{-}3}$, β-COP and p200, followed by detection with either alkaline phosphatase-conjugated antibodies or with a HRP-chemiluminescence (Amersham, Arlington Heights, IL) detection system. All antibody incubations were carried out in blotting buffer containing 5% non-fat milk, 1% Triton X-100, 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5. Fluorographs of western blots were quantified using densitometry.

Immunofluorescence

Cultures of NRK or LLC-PK_1 cells on coverslips were stained by immunofluorescence using methods described previously (Narula

et al., 1992). Cells fixed in 4% paraformaldehyde were permeabilized with 0.1% Triton X-100 and then incubated sequentially in diluted specific antibodies followed by goat anti-mouse IgG-FITC with appropriate washes. Coverslips were mounted in 50% glycerol containing 1% *N*-propyl gallate to prevent fading and viewed by epifluorescence microscopy.

RESULTS

Redistribution of p200 in cells treated with aluminium fluoride

The distribution of p200 and β-COP was examined in NRK cells treated with AlFn to activate heterotrimeric G proteins. AlFn is known to activate heterotrimeric G proteins (Gilman, 1987) and it has been reported that AlFn does not activate small molecular mass G proteins, including ARF and Rab proteins (Kahn, 1991). Intact cells were incubated in the presence of AlFn (added as 50 μM $AlCl_3$ and 30 mM NaF) for various periods of time prior to fixation and immunofluorescence staining with specific antibodies. Control cells and AlFn-treated cells were stained with antibodies to localize mannosidase II, as a marker for Golgi cisternal membranes, and $G\alpha_{i\text{-}3}$, to determine the effect of activation on its localization. The distribution of mannosidase was similar in control and AlFn-treated cells, where

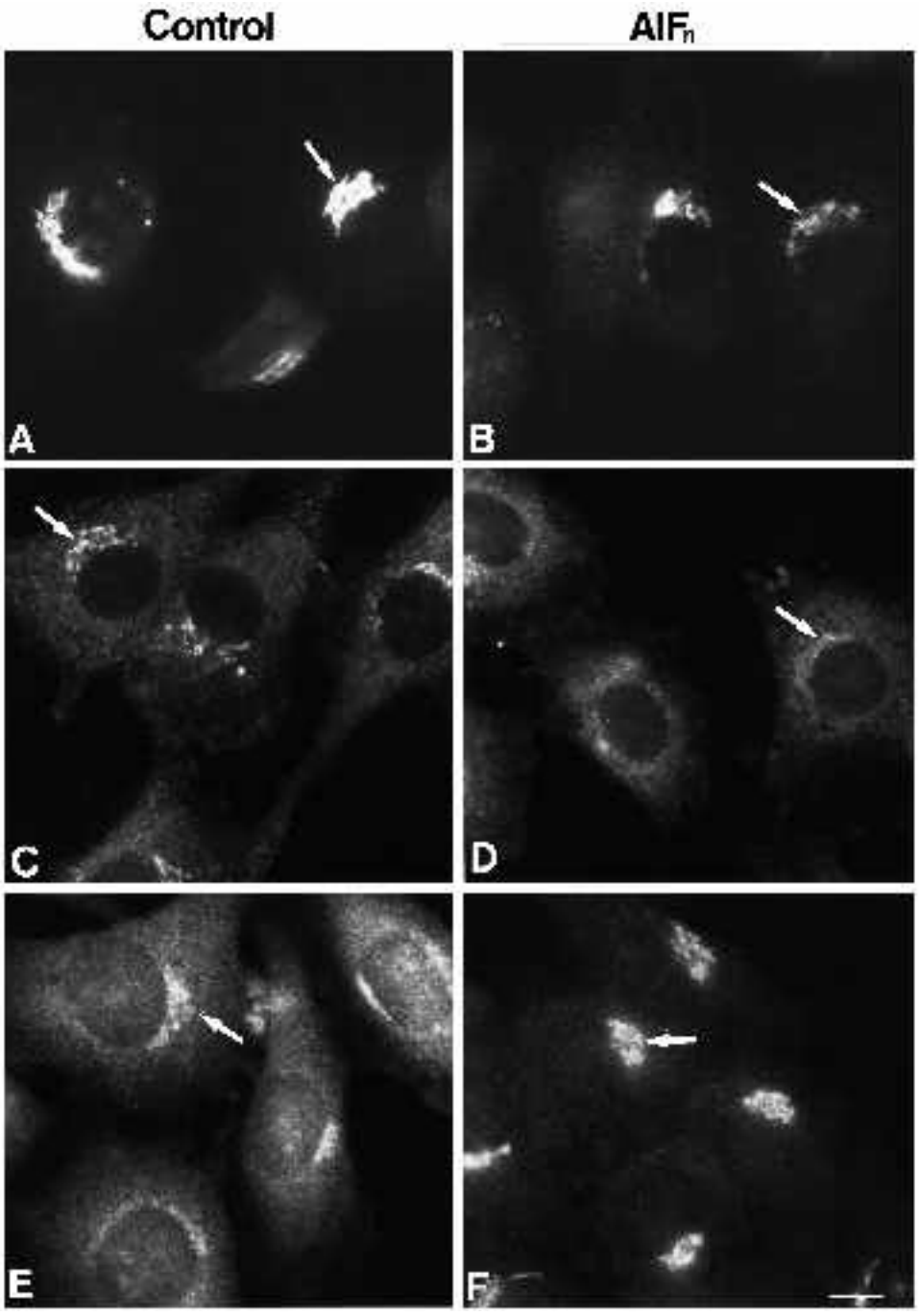

Fig. 1. Localization of Golgi-associated proteins in AlFn-treated NRK cells. NRK cells, either untreated (control) or incubated for 30 minutes with 30 mM NaF + 50 μM $AlCl_3$ (AlFn), were fixed with 4% paraformaldehyde and stained by immunofluorescence with antibodies to mannosidase II (A and B), $G\alpha_{i\text{-}3}$ (C and D) or p200 (E and F). There is staining of mannosidase in the perinuclear Golgi complex in control and AlFn-treated cells (arrows); the staining is less reticular and more diffuse following AlFn treatment (B). Reticular $G\alpha_{i\text{-}3}$ staining of perinuclear Golgi (arrow) in control cells (C), in AlFn-treated cells $G\alpha_{i\text{-}3}$ is still localized in the perinuclear Golgi complex (arrow) but the staining is more diffuse (D). Control cells show p200 staining over the crescent-shaped, perinuclear Golgi complex (arrow) and diffuse staining of the cytoplasm (E). Following AlFn treatment, p200 staining over the Golgi (arrow) is more intense and is contracted into a tighter, ball-like, perinuclear structure; the diffuse cytoplasmic staining is markedly depleted (F). Bar, 10 μm.

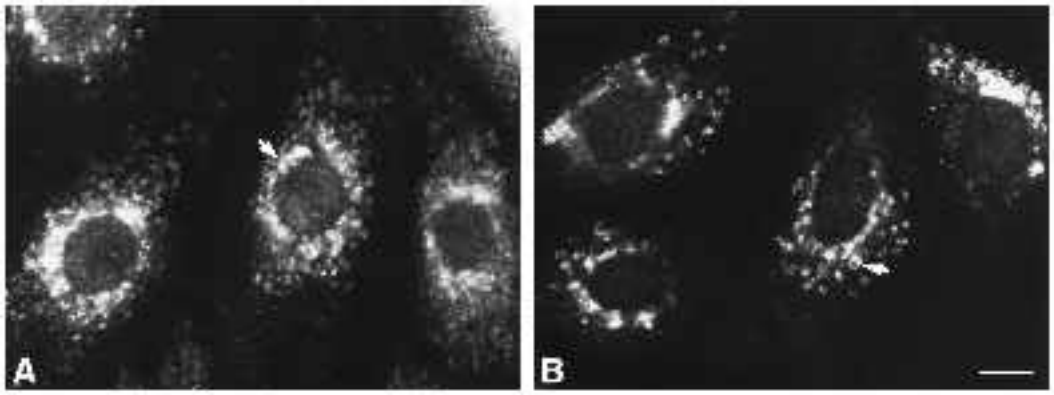

Fig. 2. Immunofluorescence localization of p200 in AlFn-treated LLC-PK_1 cells. The Golgi complex in untreated LLC-PK_1 epithelial cells is typically a very dispersed structure, in contrast to the Golgi complex in the NRK cells, which is usually confined to a single perinuclear patch (see Fig. 1.). (A) p200 staining of Golgi membranes (arrow), which emanate out from the perinuclear area, and diffuse p200 staining of the cytoplasm of untreated cells. (B) In AlFn-treated cells note that p200 staining of Golgi membranes is more intense and is contracted into a series of discrete ring-like structures (arrow) and cytoplasmic staining has disappeared. Bar, 10 μm.

it was localized in the perinuclear Golgi complex. The normal reticular appearance of this Golgi staining (Fig. 1A) was less distinct in AlFn-treated cellls (Fig. 1B). In control NRK cells, $G\alpha_{i\text{-}3}$ was localized in the perinuclear Golgi complex where the staining appeared as a reticular pattern, consistent with its localization on cisternal membranes in the Golgi complex (Fig. 1C). In AlFn-treated cells, $G\alpha_{i\text{-}3}$ remained associated with the Golgi complex, although the staining of $G\alpha_{i\text{-}3}$ was somewhat less reticular than in untreated cells (Fig. 1D). In control NRK cells, prominent p200 staining was found throughout the cytoplasm and around the perinuclear Golgi complex (Fig. 1E). Following AlFn treatment, the p200 staining in the cytoplasm was dramatically diminished and there was concomitantly more intense staining over the Golgi complex, suggesting that p200 relocated from the cytoplasm to the Golgi area (Fig. 1F). The shape of the Golgi-associated p200 staining in the AlFn-treated cells was changed markedly. In control cells, a wide crescent-shaped Golgi area was stained with p200 antibody, while in contrast, the Golgi staining was contracted into a tight, intense ball-like structure in AlFn-treated cells (Fig. 1F). The effect of AlFn on p200 distribution was reversible. In cells treated for 1 hour with AlFn, then incubated for 2 hours in normal medium, there was staining of p200 over a crescent-shaped Golgi complex and diffuse staining in the cytoplasm, similar to the pattern seen in untreated cells (data not shown).

Alterations in the localization and appearance of p200 staining following AlFn treatment were also demonstrated in another cell line, LLC-PK_1 epithelial cells. Whereas the Golgi complex in NRK cells is typically perinuclear and crescent shaped (see Fig. 1A), Golgi membranes in LLC-PK_1 cells are more loosely organized and dispersed over a wider area of the cell. The staining of p200 in control LLC-PK_1 cells shows the presence of scattered Golgi membranes and diffuse staining of the cytoplasm (Fig. 2A). Following incubation of LLC-PK_1 cells in AlFn, the amount of p200 staining in the cytoplasm was diminished and there was more intense staining of discrete, ring like elements of the Golgi complex (Fig. 2B) showing that, in these cells also, p200 relocated.

The immunofluorescent staining suggests that AlFn-induced activation of G proteins results in a redistribution of p200, altering the proportions of this protein residing either in the cytoplasm or associated with the Golgi complex.

Activation of heterotrimeric G proteins induces binding of p200 to membranes

An in vitro assay was used to determine whether AlFn activation of heterotrimeric G proteins could induce cytosolic p200 protein to bind to Golgi membranes, producing the redistribution of p200 seen in intact cells. First, LLC-PK_1 cells were homogenized and fractionated into a microsomal membrane fraction (100,000 ***g*** pellet) and thesemembranes were incubated together with rat liver cytosol in the presence of buffer alone or in the presence of AlFn. The membranes were recovered by centrifugation, then membrane and cytosol fractions were analyzed by SDS-PAGE and immunoblotting to detect p200 (Fig. 3). There was little p200 recovered on freshly prepared microsomal membranes, compared to the abundance of p200 in rat liver cytosol. Incubation of membranes and cytosol in buffer alone resulted in only a small amount of cytosolic p200 binding to membranes, however in the presence of AlFn a large amount of cytosolic p200 bound to the LLC-PK_1 microsomal membranes and was recovered in the mem-

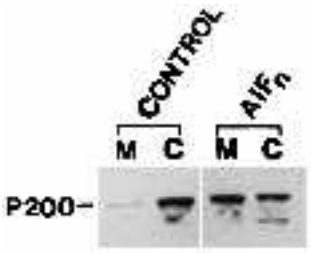

Fig. 3. Binding of cytosolic p200 to LLC-PK_1 microsomal membranes. LLC-PK_1 microsomal membranes (100 μg protein) were incubated with rat liver cytosol in the presence of buffer alone (CONTROL) or with 30 mM NaF + 50μM $AlCl_3$ (AlFn). Membranes (M) and samples of the cytosol/buffer (C) were subjected to SDS-PAGE and immunoblotting to detect p200. Note that, while little p200 protein was initially bound to the microsomal membranes in cytosol and buffer alone, with AlFn, a significant amount of p200 from rat liver cytosol is bound to LLC-PK_1 membranes and there is depletion of p200 from this sample of cytosol.

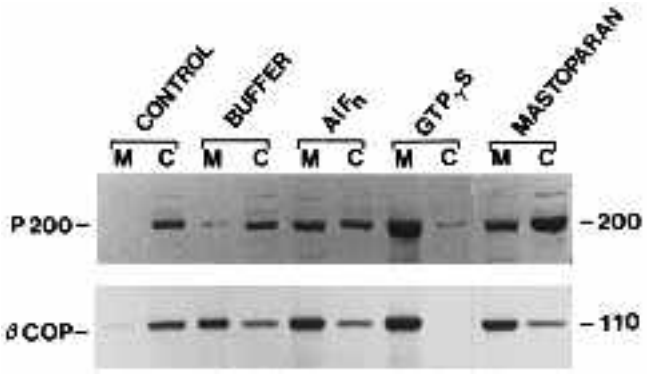

Fig. 4. Effect of G protein activation on binding of p200 and β-COP to Golgi membranes. Immunoblotting of p200 (200 kDa) and β-COP (110 kDa) proteins on fractions of rat liver Golgi membranes and cytosol. Control lanes contain freshly prepared rat liver Golgi membranes (20 μg protein) and cytosol (100 μg protein). Samples of membranes and cytosol were incubated together, as described in the text, for 30 minutes at 37°C in buffer alone (BUFFER) or with AlFn (30 mM NaF + 50 μM $AlCl_3$), or 100 μM GTPγS or 100 μM mastoparan. Membranes and cytosol were separated by centrifugation and the total membrane pellets (M) and samples of the supernatant (cytosol/buffer) (C) were subjected to SDS-PAGE and immunoblotting with specific antibodies to p200 or β-COP and alkaline phosphatase-conjugated antibodies. The membrane pellets (M) can be compared to show the relative amounts of p200 and β-COP bound to the Golgi under different conditions. The samples of cytosol loaded were not matched and thus the relative amounts of p200 and β-COP in cytosol fractions (C) cannot be quantitatively compared. There is little detectable p200 or β-COP on freshly prepared membranes (CONTROL). Some β-COP, but little p200, is on membranes incubated with cytosol and in buffer alone (BUFFER). Activation of G proteins by GTPγS caused maximal amounts of p200 and β-COP to bind to Golgi membranes; AlFn and mastoparan also caused significant amounts of both proteins to bind to the membranes.

brane pellet (Fig. 3). Thus AlFn activation of heterotrimeric G proteins induced the binding of cytosolic p200 from rat liver to membranes derived from LLC-PK_1 cells, indicating that soluble p200 is capable of binding to membranes and that its membrane binding site(s) is conserved between these species and tissues.

Next, the in vitro assay was used to test binding of cytosolic p200 to highly purified Golgi membranes from rat liver. Since activation of G proteins has also been shown to induce binding of β-COP to Golgi membranes, the binding of p200 and β-COP was compared in these assays. A representative experiment (Fig. 4) shows immunoblotting of p200 and β-COP on rat liver Golgi membranes and in cytosol fractions. Quantification of the p200 and β-COP binding in a total of five binding assays is summarized in Table 1. Both p200 and β-COP were most abundant in the cytosol (Fig. 4) and freshly isolated rat liver Golgi membrane fractions contained very little or no p200 or β-COP. Membranes and cytosol were then incubated together at 37°C for 30 minutes in the presence of buffer alone, some β-COP (20%) and very little (<10%) p200 bound to the Golgi membranes. Incubation of membranes and cytosol in the presence of GTPγS, which is a non-hydrolyzable

Table 1. Relative amounts* of p200 and β-COP bound to Golgi membranes by activation of G proteins

	Control	Buffer	GTPγS	AlFn	Mastoparan
p200	1%	10±5%	100%	70±6%	72±4%
β-COP	3%	20±8%	100%	68±5%	55±5%

Amounts of p200 and β-COP bound to Golgi membranes were quantified by densitometric analysis of immunoblots as described in the text.

*Amount of protein bound in the presence of GTPγS was designated as 100%; other amount calculated relative to this value ± error.

nucleotide for both heterotrimeric and monomeric G proteins, induced maximal binding (designated as 100%) of both p200 and β-COP to the Golgi membranes. More selective activation of heterotrimeric G proteins with AlFn also induced a significant amount of cytosolic p200 (70%) and β-COP to bind to Golgi membranes. Mastoparan stimulates guanine nucleotide exchange and consequently activates heterotrimeric G proteins, particularly G_o and G_i (Higashijima et al., 1988). Addition of mastoparan to the incubations also induced a 72% increase of p200 binding to membranes, compared to a 55% increase in β-COP binding. Thus, the binding of p200 to purified Golgi membranes was induced by a non-hydrolyzable analog of GTP, and by selective activation of heterotrimeric G proteins with AlFn or mastoparan. Notably, in comparison to β-COP, the binding of p200 was more strongly induced by activation of heterotrimeric G proteins, probably Gi proteins, through the action of the peptide mastoparan.

In order to test whether the active heterotrimeric G proteins were located on the Golgi membranes or in the cytosol, Golgi membranes were pretreated with AlFn or mastoparan prior to combining the membranes and cytosol. Golgi membranes were incubated in buffer with AlFn or mastoparan for 10 minutes, the membranes were then washed and used for incubations with cytosol. Pretreatment of membranes with AlFn or mastoparan induced a significant amount of p200 binding, 68% and 69%, respectively, resulting in the same amount of binding as that induced by adding these activators and cytosol together. This indicates that the heterotrimeric G proteins that are being activated to induce binding of p200 to Golgi membranes are located on the Golgi membranes themselves and not in the cytosol.

In order to show that pelleting of p200 with Golgi membranes represented specific binding to membranes, rather than non-specific aggregation of proteins, membranes were recovered by centrifugation and then by flotation through a sucrose gradient. The gradient gave a crude separation of membranes into heavy and light Golgi fractions. Fractions collected from the gradients were analysed by SDS-PAGE and immunoblotting to detect p200. In all cases, there was no p200 remaining in the loading zone at the bottom of the gradient, thus there was no evidence for precipitation of large protein aggregates involving p200 (Fig. 5). All of the p200 was floated through the gradient, and recovered in the fractions along with other Golgi membrane markers, suggesting that p200 was specifically bound to membranes. Membranes recovered from incubations of Golgi membranes and buffer alone contained p200 in both heavy and light fractions. Membranes recovered after incubations with

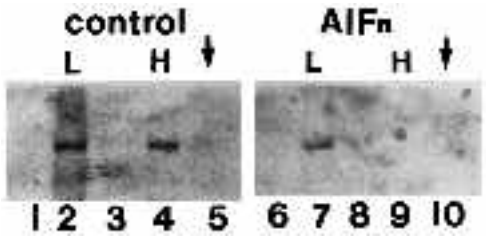

Fig. 5. Recovery of membrane-bound p200 in Golgi membrane fractions. Golgi membranes were incubated in the presence of cytosol and buffer alone (control) (200 μg protein) or in the presence of cytosol and AlFn (AlFn) (20 μg protein). Golgi membranes recovered by centrifugation were refloated through a sucrose gradient. Lanes 5 and 10 (arrows) indicate the loading zone at the bottom of the gradients. Lanes 1-4 and 6-9 show fractions, collected at various densities within the gradients, which were analyzed by SDS-PAGE and immunoblotting with p200 antibody. The stained band corresponding to p200 is present in lanes 2 and 4 corresponding to light (L) and heavy (H) Golgi fractions from the control incubation. In membranes incubated with AlFn, p200 was recovered from the light Golgi fraction, lane 7, and was now absent from the heavy Golgi fraction, lane 9. There was no p200 detected in the loading zones (arrows) at the bottom of both gradients.

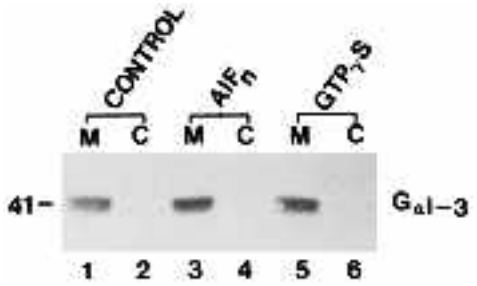

Fig. 6. Detection of activated $G\alpha_{i-3}$ on Golgi membranes. Rat liver Golgi membranes (M) and cytosol (C) were incubated together in buffer alone (CONTROL; lanes 1 and 2) or with AlFn (lanes 3 and 4) or with GTPγS (lanes 5 and 6) and then analyzed by SDS-PAGE and immunoblotting to detect the 41 kDa, $G\alpha_{i-3}$ with the specific peptide antibody (EC) followed by an alkaline phosphatase-conjugated second antibody. $G\alpha_{i-3}$ was recovered in the Golgi membrane fractions (M) under all conditions of G protein activation; $G\alpha_{i-3}$ subunit was not detected in the cytosol.

AlFn contained p200 mainly in the light Golgi fraction showing that this p200 was associated with membranes but also showing that there was a change in the buoyant density and configuration of these membranes following binding of cytosolic proteins (Fig. 5).

Activated $G\alpha_{i-3}$ is bound to rat liver Golgi membranes

We have previously shown that the pertussis toxin-sensitive $G\alpha_{i-3}$ subunit is present on rat liver Golgi membranes, where it is the only pertussis toxin-sensitive subunit (Stow et al., 1991). Immunoblotting of the 41 kDa, $G\alpha_{i-3}$ protein in rat liver Golgi membrane and cytosol fractions was performed to determine the effect of G protein activation on the distribution of this subunit (Fig. 6). Membrane and cytosol fractions from the same experiment as shown in Fig. 4 were used for immunoblotting with the $G\alpha_{i-3}$ antibody. This confirmed the presence of $G\alpha_{i-3}$ on the isolated Golgi membranes and showed the complete absence of the α_{i-3} subunit in the cytosol. Incubation of the rat liver Golgi membranes with AlFn and GTPγS, to activate G proteins, did not dislodge $G\alpha_{i-3}$, which remained in the membrane fraction (Fig. 6). There was no displacement of $G\alpha_{i-3}$ from the membranes into the cytosol after any of the treatments performed to activate or inactivate G proteins.

Pertussis toxin inhibits the binding of p200 to Golgi membranes

Pertussis toxin ADP-ribosylates $G\alpha_{i-3}$ on rat liver Golgi membranes, as we have previously shown (Stow et al., 1991), which should result in the inactivation and uncoupling of the Gα subunit from its potential receptor. If such a receptor signal transduction pathway is coupled to $G\alpha_{i-3}$ on the Golgi, and is responsible for regulating cytosolic protein binding, pertussis toxin would be expected to modify p200 membrane binding. In order to test directly the effect of pertussis toxin on p200 membrane binding, rat liver Golgi membranes were pretreated with pertussis toxin and then used in the same in vitro assay described above. Quantification of the Golgi membrane binding is shown in Fig. 7. Since there was little binding of p200 to Golgi membranes in buffer alone, any effect of pertussis toxin on this binding was not measurable. There was no effect of pertussis toxin in the presence of GTPγS, which causes maximal binding of p200 to membranes. However pertussis toxin did block a significant amount of p200 binding induced by activation of heterotrimeric G proteins with AlFn and, particularly, with mastoparan. Pertussis toxin reduced the AlFn-induced binding by 40% and the mastoparan-induced binding by 75%. In contrast, the effect of pertussis toxin on β-COP binding was less dramatic. There was no significant effect of pertussis toxin in the presence of GTPγS and, in this case, no significant inhibition of β-COP binding induced by AlFn or mastoparan.

The effect of pertussis toxin on the distribution of p200 was also shown by immunofluorescence staining in intact cells (Fig. 8). NRK cells were pretreated overnight with 2 μg/ml pertussis toxin, which results in complete ADP-ribosylation of all pertussis toxin-sensitive $G\alpha_i$ subunits in the cell, including $G\alpha_{i-3}$ on the Golgi (Stow et al., 1991). While pertussis toxin treatment alone had no detectable effect on the p200 staining pattern at the light microscopy level, it did result in a marked inhibition of the AlFn-induced alterations in p200 localization and Golgi complex structure. The AlFn-induced depletion of cytoplasmic p200 staining and the contraction of the p200-stained Golgi membranes (Fig. 8B) did not occur in cells pretreated with pertussis toxin (Fig. 8C), instead these cells retained the appearance of untreated cells, with diffuse staining of p200 throughout the cytoplasm and staining of a crescent-shaped Golgi complex. Thus pertussis toxin protected p200 in cells from the effects of AlFn; there was no relocation of p200 to the Golgi, consistent with the effect of pertussis toxin on isolated membranes in the in vitro assay.

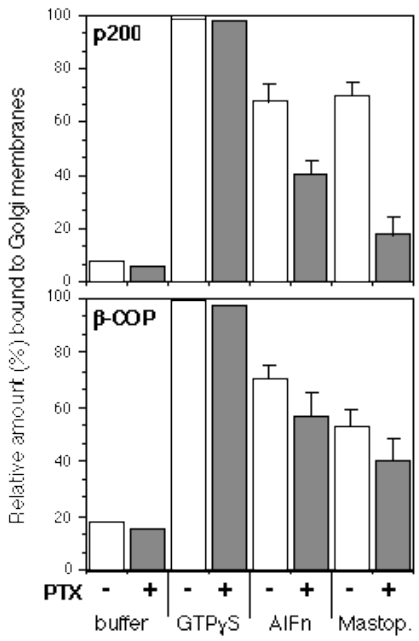

Fig. 7. Effect of pertussis toxin on membrane binding of p200 and β-COP. Rat liver Golgi membranes and cytosol were incubated in the in vitro assay (see Fig. 4; Table 1). The amounts of p200 (top panel) and β-COP (lower panel) bound to Golgi membranes were determined by SDS-PAGE, immunoblotting and densitometry as described in the text. $G\alpha_{i\text{-}3}$ on rat liver Golgi membranes was ADP-ribosylated by preincubating membranes with pertussis toxin (PTX) prior to incubation with cytosol and G protein activating agents. The amounts bound (shown as the mean and standard error) in the presence of buffer, AlFn or mastoparan are expressed relative to the maximal amount bound (100%) in the presence of GTPγS. PTX treatment (shaded columns) did not significantly alter the amount of p200 or β-COP binding in the presence of buffer alone where there was little binding; the maximal amounts of p200 and β-COP bound by GTPγS were also not affected by PTX. The amount of p200, but not of β-COP, bound to membranes in the presence of AlFn or mastoparan was significantly reduced when membranes were treated with PTX, compared to untreated membranes.

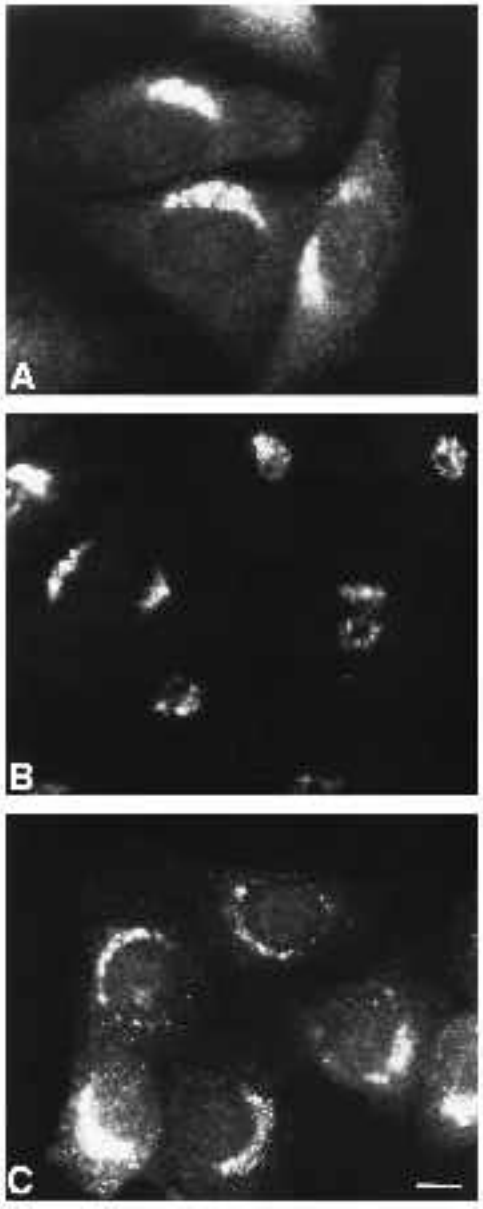

Fig. 8. Immunofluorescence localization of p200 in pertussis toxin-treated NRK cells. NRK cells were fixed and stained with the p200 antibody following no treatment (A), treatment with AlFn for 30 minutes (B) or pretreatment with pertussis toxin overnight followed by treatment with AlFn for 30 minutes (C). p200 staining of perinuclear Golgi and cytoplasm in control cells (A). In AlFn-treated cells, p200 has disappeared from the cytoplasm and is in a tight ball-like configuration of Golgi-associated staining (B). Note that in cells pretreated with pertussis toxin, prior to AlFn, p200 staining is similar to that in control cells with diffuse cytoplasmic staining of p200 and p200 Golgi staining in a crescent-shaped perinuclear area (C).

DISCUSSION

The data presented here show that the binding of p200 to Golgi membranes is regulated by the activation of heterotrimeric G proteins since GTPγS, AlFn and mastoparan were able to induce translocation of p200 from the cytosol onto Golgi membranes. The fact that p200 is bound to Golgi membranes through a G protein-regulated mechanism, similar to that reported for other vesicle-associated proteins, β-COP and γ-adaptin (Donaldson et al., 1991a; Robinson and Kreis, 1992) provides new, compelling evidence that p200 may also be involved in the formation of vesicles for Golgi trafficking.

In intact cells, AlFn-induced G protein activation resulted in depletion of cytoplasmic staining and intense p200 staining of a contracted Golgi complex. The depletion of cytoplasmic staining reflects the movement of p200 from cytosol to the Golgi, which was also the result seen in the presence of AlFn in the in vitro binding assay. The marked change in the shape of the Golgi staining following AlFn signifies a rearrangement or reconfiguration of specific membrane domains upon binding of p200. It was also noted that isolated Golgi-derived membranes containing p200 were altered in their buoyant density following AlFn treatment, with the appearance of more p200 in the light Golgi

fraction and less in the heavy Golgi fraction recovered from the in vitro assay. It has been reported previously that the immunofluorescence Golgi staining of β-COP undergoes a similar condensation or contraction in GTPγS-treated cells (Donaldson et al., 1991b). Since β-COP is known to be associated with the Golgi-derived vesicles that accumulate in the presence of GTPγS (Orci et al., 1991), this contracted staining of β-COP on the Golgi is consistent with the conversion of some Golgi membranes to clusters of vesicles following activation of G proteins. This precedent, together with the immunoperoxidase staining of p200 on the budding ends of Golgi cisternae (Narula et al., 1992), suggests that p200 is also associated with accumulations of Golgi-derived vesicles in AlFn-treated cells. A definitive localization of p200 at the ultrastructural level, however, is required to further elucidate its association with Golgi membranes and to establish whether p200 binds to either β-COP-coated Golgi vesicles or other Golgi-derived vesicles.

It is of interest to note that the staining patterns of mannosidase and $G\alpha_{i\text{-}3}$ did not change in a similar fashion to that of p200 in AlFn-treated cells. The staining of these other proteins, both of which are found in or on Golgi cisternae (Stow et al., 1991) remained spread over the crescent-shaped Golgi complex, although their staining was more diffuse and less reticular than in untreated cells. This further supports the localization of p200 and $G\alpha_{i\text{-}3}$ on distinct membrane domains within the Golgi (Narula et al., 1992; Stow et al., 1991). Secondly, it suggests that $G\alpha_{i\text{-}3}$ may be regulating the binding of p200 from a remote site, for instance by altering the status of the whole Golgi membrane, rather than by directly altering a specific binding site.

The data obtained in the in vitro binding assay show that selective activation of heterotrimeric G proteins that are located on Golgi membranes, with AlFn and mastoparan, induces binding of cytosolic p200 to Golgi membranes. We also found that pertussis toxin had a significant effect on the binding of p200 to Golgi membranes. The traditional view of pertussis toxin action is that ADP-ribosylation of $G\alpha_i$ results in the uncoupling of α_i from its receptor (Sunyer et al., 1989), thus inactivating the receptor-linked signal transduction pathway. It has been shown in a number of G_i-mediated pathways that pertussis toxin maximally inhibits receptor (or mastoparan)-induced $G\alpha_i$ activation (Weingarten et al., 1990), variably inhibits AlFn-activation (Kremer et al., 1988), but cannot overcome GTPγS activation (Brown and Birnbaumer, 1990; Cantiello et al., 1989). This paradigm for pertussis toxin action fits the data shown in Fig. 7 for p200 binding, strongly implicating $G\alpha_i$ as the major G protein involved in p200 binding. This result again is supported by the immunofluorescence staining in intact, AlFn-treated cells, where pertussis toxin treatment protected cells from the translocation of p200 from the cytosol to the Golgi complex.

We attributed the effects of pertussis toxin on the binding of p200 to the $G\alpha_{i\text{-}3}$ subunit, since this is the only pertussis toxin-sensitive Gα subunit that we were able to detect on rat liver Golgi membranes. The $G\alpha_{i\text{-}3}$ subunit was detected on isolated rat liver Golgi membranes by ADP-ribosylation and by immunoblotting with specific antibodies (Stow et al., 1991), while other closely-related pertussis toxin-sensitive Gα subunits were not detected on rat liver Golgi membranes using peptide antibodies (Stow and de Almeida, 1993). $G\alpha_{i\text{-}3}$ is tightly bound to Golgi membranes and is not found in the cytosol. Although $G\alpha_{i\text{-}3}$ is found on Golgi membranes in many cell types, in some cells additional $G\alpha_i$ subunits are also associated with the Golgi complex. In PC-12 cells several pertussis toxin-sensitive subunits have been found on TGN membranes including $G\alpha_{i\text{-}3}$, $\alpha_{i\text{-}2}$ and α_o (Leyte et al., 1992).

The nature of the G protein activation, the localization of the G protein and the effects of pertussis toxin, are consistent with $G\alpha_{i\text{-}3}$ being one of the G proteins regulating p200 binding. The results of these experiments do not show whether $G\alpha_{i\text{-}3}$ alone is responsible for this binding or whether other G proteins, either heterotrimeric or monomeric, are also involved in p200 binding. One interpretation of our data is that, in the presence of activated or GTP-bound $G\alpha_{i\text{-}3}$, p200 is able to bind to Golgi membranes. Since there was little or no binding of p200 when cytosol and membranes were incubated together without exogenous activators of G proteins, we can assume that freshly isolated Golgi membranes contain $G\alpha_{i\text{-}3}$, (and possibly other G proteins) predominantly in a GDP-bound form. In the presence of non-hydrolyzable GTPγS, or AlFn or mastoparan, which induce or mimic $G\alpha_{i\text{-}3}$ in its GTP-bound form, the necessary signal is generated to alter Golgi membranes, making them receptive for binding of soluble p200 and perhaps for generation of vesicles.

The variety of G protein subunits involved in cytosolic protein binding, and ultimately in vesicle trafficking, already appears to be complex. The effects of AlFn and mastoparan on p200 binding could also include activation of the $G\alpha_s$ subunit, or even other non-toxin-sensitive subunits. $G\alpha_s$ is present on isolated rat liver Golgi membranes (Stow and de Almeida, 1993), although no function has yet been demonstrated for it in our hands. Both inactivation of $G\alpha_i$ by ADP-ribosylation with pertussis toxin and activation of $G\alpha_s$ by cholera toxin ADP-ribosylation stimulated cell-free vesicle and granule formation from TGN membranes (Leyte et al., 1992), suggesting that $G\alpha_s$ and $G\alpha_i$ subunits may have opposing regulatory roles in vesicle formation. A recent study (Pimplikar and Simons, 1993) shows that in polarized cells, pertussis toxin stimulates basolateral targetting of viral proteins, similar to its effect on HSPG secretion (Stow et al., 1991), and in contrast, cholera toxin specifically affected apical transport of viral proteins. The effects of activation or inactivation of heterotrimeric G proteins on trafficking and secretion, polarized targetting and TGN-vesicle formation are consistent with each other (Stow et al., 1991; Pimplikar and Simons, 1993; Barr et al., 1991). However, it is still difficult to reconcile the inhibitory effect of G protein activation on vesicle trafficking in intact cells, with the apparent stimulatory effect on cytosolic protein binding to isolated membranes. Since the binding assay measures the involvement of G proteins in only a small step of vesicle trafficking pathways, there may be many other steps of G protein regulation not yet considered. The definition of G protein regulation of these processes will require a more detailed knowledge of which steps in protein binding, vesicle budding and targetting are coupled to G protein-mediated signal transduction events and identification

of potential receptor and effector proteins. GTPγS was more effective in inducing p200 and β-COP binding than the selective activators AlFn or mastoparan, hence, activation of both monomeric and heterotrimeric G proteins may be required to regulate this binding. Indeed, the monomeric G protein ARF, in its GTP-bound form, is required for β-COP binding (Donaldson et al., 1992a), and major roles for both ARF and ARF exchange factors have been proposed for the regulation of coatomer binding and vesicle formation (Donaldson et al., 1992b; Helms and Rothman, 1992). It will be interesting to determine whether ARF is also required for p200 binding.

In this study we directly compared p200 and β-COP binding to Golgi membranes in the binding assay and their binding in response to heterotrimeric G protein activation was similar. A number of previous studies provided the initial evidence that β-COP binding to Golgi membranes is regulated, at least in part, by activation of heterotrimeric G proteins (Donaldson et al., 1991a; Ktistakis et al., 1992; Robinson and Kries, 1992). The main difference between the binding of p200 and β-COP found in the present study was in their sensitivity to pertussis toxin. In our assay, pertussis toxin inhibited AlFn- and mastoparan-induced binding of p200 but there was no significant effect of pertussis toxin on the binding of β-COP to Golgi membranes. This difference could potentially be explained by the involvement of different or opposing G protein subunits in the regulation of these two proteins. In contrast to our results, a previous study implicated $G\alpha_i$ proteins in the regulation of β-COP binding by showing that pertussis toxin counteracted the mastoparan-inhibition of BFA on β-COP-Golgi association (Ktistakis et al., 1992). It is difficult to directly compare the results of these studies since both were carried out under very different conditions but this again highlights the possible complications of comparing Golgi systems from different cell types where potentially different sets of G proteins are present.

The results of the present study show that another protein, p200, can now be added to the list of cytoplasmic proteins known to be regulated by Golgi-associated G proteins. Our data strongly implicate the pertussis toxin-sensitive $G\alpha_{i\text{-}3}$ protein as a major regulator of p200 binding. Since we have previously shown that $G\alpha_{i\text{-}3}$ influences the secretory pathway, it is probable that p200 is also a component of this intracellular pathway.

We wish to acknowledge our collaboration in this work with Dr Karl Matlin and Dr Brian Burke and we thank them for helpful discussions. We would like to thank Dr T. Kreis and Dr A. Spiegel for the generous gift of antibodies. This work was supported by NIH grant PODK38452.

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(Received 27 June 1993 - Accepted 7 September 1993)