Heterotrimeric G Proteins Interact with the Small GTPase ARF
POSSIBILITIES FOR THE REGULATION OF VESICULAR TRAFFIC*

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Trimeric G proteins have emerged as important regulators of membrane trafficking. To explore a role for Gβγ in endosome fusion, we have taken advantage of β-adrenergic receptor kinase (βARK), an enzyme translocated to membranes by interaction with Gβγ. The COOH terminus of βARK (βARKct) has a Gβγ-binding domain which blocks some Gβγ-mediated processes. We found that βARKct and peptide G, a peptide derived from βARKct, inhibit in vitro endosome fusion. Interestingly, peptide G and ARF share sequence similarity. Peptide G and βARKct reversed ARF-mediated inhibition of endosome fusion and blocked ARF binding to membranes. Using an ARF fusion protein, we show that both Gβγ and GαS interact with the small GTPase ARF, an interaction that is regulated by nucleotide binding. We conclude that G proteins may participate in the regulation of vesicular trafficking by directly interacting with ARF, a cytosolic factor required for transport.

Vesicular membrane trafficking among intracellular compartments is now recognized to involve multiple small GTP-binding proteins including members of the Ras-like superfamily such as Rab, ARF, and Sar1 (reviewed by Goud and McCaffrey, 1993; Pryer et al., 1992; Nuoffer and Balch, 1994). The ARF family, which includes several distinct ARF proteins, seems to control the assembly of coat components on transport vesicles. ARF (ADP-ribosylation factor) was originally discovered as a cofactor required for the ADP-ribosylation by cholera toxin of the heterotrimeric G protein Gs (Kahn and Gilman, 1984). The initial evidence for a role for ARF in vesicular transport came from genetic studies in yeast where deletion of the ARF1 gene resulted in a secretory defect (Stearns et al., 1990a, 1990b). Using several in vitro assays that reconstitute transport between different compartments, it has been shown that ARF is an essential component required for transport (Balch et al., 1992; Lenhard et al., 1992; Donaldson and Klausner, 1994). ARF is also required for the assembly of the coat complex on non-clathrin-coated vesicles (COP-coated vesicles) mediating transport between Golgi compartments (reviewed by Rothman and Orci, 1992; Kreis and Pepperkok, 1994; Donaldson and Klausner, 1994) and in the association of AP-1 adaptors to Golgi membranes, raising the possibility that ARF may also be required for the assembly of clathrin coats at the trans-Golgi network (Stamnes and Rothman, 1993; Traub et al., 1993).

A growing body of evidence indicates that heterotrimeric GTP-binding proteins (G proteins) play a crucial role in vesicular trafficking (reviewed by Bomsel and Mostov, 1993; Barr et al., 1992; Burgoyne, 1992; Nuoffer and Balch, 1994). Previous work from our laboratory indicates that fusion among endosomes and between phagosomes and endosomes is controlled by G proteins (Colombo et al., 1992, 1994a; Beron et al., 1995). Moreover, multiple G proteins seem to participate in different steps of transport (Stow et al., 1991; Leyte et al., 1992; Carter et al., 1993). We have reported that one of the G proteins involved in endosomal fusion is Gαs (Colombo et al., 1994b). The role of Gαs has also been implicated in trafficking in polarized cells (Pimplikar and Simons, 1993; Bomsel and Mostov, 1993; Barros and Sztul, 1993; Hansen and Casanova, 1994) and in the secretory pathway (Leyte et al., 1992). However, the actual mechanism by which these proteins regulate traffic remains poorly understood.

Classically, trimeric G proteins transduce extracellular signals to appropriate effector molecules inside the cell. G proteins are comprised of three subunits, Gα, Gβ, and Gγ. Binding of GTP causes the activation of the G protein and the subsequent dissociation of Gα from Gβγ (Gilman, 1987). It is now widely accepted that signals by both Gα and Gβγ are physiologically relevant. Several recent reports clearly demonstrate the prominent involvement of Gβγ in several transmembrane signaling systems. An increasing number of G protein-coupled effectors which appear to be modulated by Gβγ subunits have been identified (reviewed by Clapham and Neer, 1993; Sternweis, 1994). On the other hand, Gβγ specifically mediates the translocation of cytosolic β-adrenergic receptor kinase (βARK),1 one of the G protein-coupled receptor kinases, to the plasma membrane. This translocation allows the phosphorylation of activated receptors as part of the desensitization process (Inglese et al., 1993). A fragment of βARK corresponding to the last 222 C-terminal amino acids was found to contain the "Gβγ-binding domain" (Pitcher et al., 1992). A fusion protein corresponding to this Gβγ-binding domain blocks binding of βARK to Gβγ (Koch et al., 1993) and prevents receptor phosphorylation. It has recently been shown that this reagent interferes with multiple Gβγ-mediated processes such as Gβγ-dependent activation of

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1The abbreviations used are: βARK, β-adrenergic receptor kinase; GST, glutathione S-transferase; GTPγS, guanosine 5′-O-(thiotriphosphate); DNP, dinitorphenol; BSA, bovine serum albumin; PBS, phosphate-buffered saline; AIF, aluminum fluoride; GDPβS, guanosine 5′-O-(2-thiodiphosphate); RK, rhodopsin kinase.
adenyl cyclase type II, βARK2 regulated octafloy signal transduction, and atrial K+ channel activation (Reuveny et al., 1994; Boekhoff et al., 1994; Inglese et al., 1994).

In an attempt to study the possible role of Gβγ in the mechanism or regulation of endosome fusion we used βARK C-terminal fusion protein and peptides derived from the Gβγ-binding domain in a cell-free assay that reconstitutes fusion between endosomes. His6-βARK fusion protein completely blocked endosome fusion while His6-rhodopsin kinase had no effect. A single 28-amino acid peptide (Peptide G) derived from the targeting domain of βARK was also found to inhibit fusion. Alignment of the cytosolic small GTP-binding protein ARF and peptide G reveals that they share sequence similarity. Our results suggest that a direct collaboration among heterotrimeric G proteins and ARF may regulate vesicular transport.

RESULTS Suggest that a direct collaboration among heterotrimeric G proteins and ARF, a cytosolic factor required for endosome fusion. In order to address this provocative hypothesis, we constructed GST-ARF fusion proteins and studied their direct interaction with purified G proteins. Our results indicate that both Gβγ and Gα interact with the small GTPase ARF. Activation of Gα subunits by either GTPγS or aluminofluoride complexes completely blocked ARF-Gα interaction, indicating that the heterotrimer is the most likely candidate for ARF-G protein interaction. Our results suggest that a direct collaboration among heterotrimeric G proteins and ARF may regulate vesicular transport.

EXPERIMENTAL PROCEDURES Cells and Materials—J 774, E-clone (mannose receptor positive), a macrophage cell line, was grown to confluence in minimum essential medium containing Earle’s salts and supplemented with 10% fetal calf serum. HDP-1, a mouse IgG1 monodonal antibody specific for dinophenol was isolated and mannosylated as described previously (Diaz et al., 1988; Colombo et al., 1992b). Gβγ-interferon was isolated from rat preputial glands and derivatized with dinitrophenol (DNP) using dinitrophenyl (DNP)-BSA. Cytosol samples (200 μg) were gel filtered through a 1-ml Sephadex G-25 spin column just before use in the fusion assay. Protein concentration after filtration was 3–5 mg/ml. The His6-fusion proteins, His6-RK carboxyl terminus and His6-βARK carboxyl terminus containing the terminal 91 amino acids of RK and the terminal 222 amino acids of βARK1, were prepared and purified as described (Inglese et al., 1994). Peptides G5, G6, and G7, corresponding to specific βARK1 and βARK2 sequences were synthesized and purified as described previously (Koch et al., 1993). Recombinant myristoylated ARF1 and ARF4 were prepared and purified essentially as described (Randazzo et al., 1992). G6 subunits were purified by gel filtration in the brain as described previously (Casey et al., 1989). Recombinant G5 subunits were a generous gift from Dr. M. Linder (Washington University, St. Louis, MO) and Dr. J. Garrison (University of Virginia, Charlottesville, VA). All other chemicals were obtained from Sigma.

Preparation of Endocytic Vesicles—Early endosomes were loaded with mannosylated anti-DNP IgG or with DNP-β-glucuronidase by a 5 min uptake at 37°C as described previously (Diaz et al., 1988; Colombo et al., 1992b). After ligand uptake, the macrophages (1 × 106 cells) were washed sequentially with 150 mM NaCl, 5 mM EDTA, 10 mM phosphate buffer, pH 7.0, and with 250 mM sucrose, 0.5 mM EDTA, 20 mM HEPES-KOH, pH 7.0 (homogenization buffer), and homogenized in the latter buffer (2 ml) using a cell homogenizer (Colombo et al., 1992b). Homogenates were centrifuged at 800 × g for 5 min to eliminate nuclei and intact cells, and then pelleted for 1 min at 37,000 × g in a Beckman L 100 microcentrifuge. The supernatants were centrifuged for additional 5 min at 50,000 × g. The pellets of this second centrifugation were enriched with 5-min endosomes. Endosomal fractions containing each preparation were homogenized in 10% GST and incubated with gel filtered cytosol. The samples were incubated at 37°C for 45 min and the reaction was stopped by cooling on ice. To measure the immune complexes formed, the vesicles were solubilized by adding 50 μl of solubilization buffer (1% Triton X-100, 0.2% mbenzethionium chloride, 1 mM EDTA, 0.1% BSA, 0.15 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 750 μg/ml DNP-BSA. For immunoprecipitation the samples were transferred to multwell plates coated with rabbit anti-mouse IgG. After 30–45 min of incubation at room temperature, the wells were washed three times with 300 μl of solubilization buffer, and enzyme activity was measured using 4-methylumbelliferyl β-D-glucuronide as substrate in a Microplate fluorometer 7600, Cambridge Technology, Inc. (Colombo et al., 1992b). Fusion was expressed in arbitrary fluorescence units.

βARK Binding Assay—An enriched endosomal fraction was prepared by sequential centrifugation as described previously (Colombo et al., 1992b). The endosomal fraction (10–20 μg of total protein) was resuspended in the fusion buffer described above, containing gel filtered cytosol (1–2 mg protein/ml). Incubations were carried out in 1.5 ml tubes (Beckman, polyallomer). Incubation volumes were 50 μl. After 5 min of prernuculation with the reagents to be tested, 20 μM GTPγS was added, and the samples were incubated for additional 20 min at 37°C. After incubation, the samples were washed with 1 ml of homogenization buffer containing 20 μM GTPγS and 1 mM MgCl2. The membranes were recovered by centrifugation for 5 min at 50,000 × g. Proteins were subjected to SDS-polyacylamide gel electrophoreses under reducing conditions and transferred onto nitrocellulose in 25 mM Tris, pH 8, 192 mM glycine, and 5% methanol at 150 mA for 1 h. ARF was detected using a rabbit polyclonal affinity purified antibody against ARF. Kindly provided by J. Rothman (diluted 1:500) and horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:1,500). The visualization was performed using the ECL detection system (Amersham Corp.) according to the manufacturer’s instructions.

Construction and isolation of GST fusion proteins—cDNA corresponding to human ARF4 (a gift from Richard Kahn, NIH) and ARF4 with the first 17 amino acids deleted were amplified by the polymerase chain reaction using 5′ primers containing BamHI sites. The GST gene fusion vector pGEX-3T (Pharmacia Biotech Inc.) was used to construct cDNAs in which the amplified cDNAs were ligated with the 3′-end of the coding region of GST. The clones used in these experiments were very similar in sequence (Koch et al., 1993). Fusion proteins constructs were introduced into the Escherichia coli strain J101 and induced with isopropyl-1-thio-galactopyranoside to produce GST fusion proteins.

Recombinant C-terminal half of ARF1 (ARF1ct) protein was expressed as follows: the C-terminal half of the ARF1 cDNA was amplified by polymerase chain reaction using 5′ primers containing BamHI sites. The GST gene fusion vector pGEX-3T (Pharmacia Biotech Inc.) was used to construct cDNAs in which the amplified cDNAs were ligated with the 3′-end of the coding region of GST. The clones used in these experiments were very similar in sequence (Koch et al., 1993). Fusion proteins constructs were introduced into the Escherichia coli strain J101 and induced with isopropyl-1-thio-galactopyranoside to produce GST fusion proteins.

The fusion proteins were purified by glutathione-Sepharose either by standard techniques or using the Sarksmyl method (Frangioni and Nedd, 1993). The samples were dialyzed against PBS and, if necessary, concentrated in a Centricon-10 (Amicon). GST-ARK COOH-terminal and GST-ARK COOH-terminal fusion proteins were constructed and purified as described previously (Koch et al., 1993). GST-Rab5 fusion protein, constructed and purified as described (Barbieri et al., 1994), was kindly provided by Mary K. Cullen (Washington University, St. Louis, MO).

Detection of Binding of Gβγ to Fusion Proteins—Binding of Gβγ subunits to the purified GST fusion proteins was done essentially as described previously (Pitcher et al., 1992; Touhara et al., 1994). Briefly, purified bovine brain Gβγ subunits (200–300 nm) were incubated with the GST fusion proteins (600–700 nm) for 30 min on ice in PBS containing 0.1% BSA. When indicated, purified recombinant Gα subunits were also added to the binding assay. Glutathione-Sepharose (20 μl of a 50% slurry in PBS) was added, and then the mixture was incubated on ice for 60 min. The Sepharose beads containing bound GST or fusion fusion proteins were subsequently washed four times with PBS/BSA (400 μl), subjected to SDS-polyacylamide gel electrophoreses and transferred to nitrocellulose membranes as described above. Antibodies against Gβγ subunits kindly provided by Gary Johnson (National Institute of Dental and Oral Health, Bethesda, MD) were used at a 1:10,000 dilution of the primary antibody against the COOH-terminal end of Gα subunits kindly provided by Dr. Gary Johnson. Blots were developed with goat anti-rabbit IgG coupled to horseradish peroxidase.
and detected with the ECL detection system (Amersham) according to the manufacturer’s instructions.

RESULTS

The Gγβ-binding Domain of βARK Inhibits Endosome Fusion—Previous work (Colombo et al., 1992a, 1994a, 1994b) from our laboratory indicates that heterotrimeric G protein(s) regulate fusion among endosomes. Gα has long been associated with signal transduction pathways. More recently, Gβγ has emerged as a major participant in signal transduction via its interaction with several effectors within the cell (reviewed by Clapham and Neer, 1993; Sternweis, 1994). βARK is a cytosolic enzyme that is targeted by Gβγ to the membrane (Inglese et al., 1993). A fragment of βARK corresponding to 222 amino acids of the COOH-terminal domain contains the targeting domain for binding to Gβγ (Koch et al., 1993) and other Gβγ-mediated processes.

To assess the possible involvement of Gβγ in the mechanism of endosome fusion, the COOH-terminal βARK fusion protein was tested in the in vitro endosome fusion assay. Fig. 1A shows that a 6His-COOH-terminal βARK1 fusion protein (βARK1ct) completely blocks fusion between endosomes (closed circles). The inhibitory potency of βARK1ct in the in vitro fusion assay (EC₅₀ 10–15 μM) was similar to the inhibitory activity against Gβγ activation of βARK (Koch et al., 1993). Interestingly, the 6His-βARK2ct corresponding to the same region of βARK2, another member of the G protein-coupled kinase family, was a better inhibitor of endosome fusion (triangles). In contrast, no effect was observed with the COOH-terminal domain of rhodopsin kinase (open circles). This result is consistent with earlier observations showing that the COOH-terminal domain of RK (RKct) does not bind to Gβγ. RKct lacks the Gβγ-binding domain and consequently does not interact with Gβγ subunits (Pitcher et al., 1992). The differential effect observed with βARKct and RKct fusion proteins appears to rule out any nonspecific effect of these polypeptides.

In order to identify the critical regions involved in βARK binding to Gβγ, Koch and collaborators (1993) synthesized several peptides corresponding to the targeting domain. A single 28-amino acid peptide (Peptide G1) derived from the targeting domain of βARK1 was found to inhibit Gβγ activation of βARK with an IC₅₀ of 76 μM. In contrast, peptide G2, containing only the first 15 amino acid residues of peptide G1, was inactive. Fig. 1B shows that peptide G1 was also inhibitory of endosome fusion with a similar EC₅₀ (open circles). No inhibitory effect was observed with peptide G1 (open circles). As observed with βARK2, peptide G2 corresponding to the same region of βARK2 was a more potent inhibitor of endosome fusion (triangles).

Since the COOH-terminal domain of βARK selectively binds to Gβγ, the inhibitory effect observed with the fusion protein and peptide G suggests that a Gβγ-mediated process is involved in in vitro endosome fusion. The results further suggest that βARKct and peptide G are likely blocking the interaction of Gβγ subunits with a factor(s) required for in vitro endosome fusion.

The Small GTP-binding Protein ARF and the Gγβ-binding Domain of ARF Share Sequence Similarity—ARF is a Ras-like small GTP-binding protein that was originally identified as the protein cofactor required for efficient ADP-ribosylation of Gα₁ by cholera toxin (Kahn and Gilman, 1984). It is now clear that ARF has an important role in vesicle transport (reviewed by Nuoffer and Balch, 1994; Rothman and Orci, 1992). Work in our laboratory indicates that ARF is required for in vitro endosome fusion and that in the presence of GTPγS, ARF inhibits fusion (Lenhard et al., 1992). Recently, we have shown that ARF plays a regulatory role in receptor-mediated endocytosis (D’Souza-Schorey et al., 1995). Given that ARF is a cytosolic protein involved in fusion between endosomes we compared the sequence of peptides G with members of the ARF family. When peptides G were aligned with ARF a surprising similarity was found among the sequences (Fig. 2). A segment of five amino acids (ELRDA) from peptide G₁ was identical to a fragment corresponding to amino acids 115–119 of ARF1 (see box in Fig. 2). Equivalent sequence similarity was observed with other members of the ARF family.

βARKct and Peptide G Interferes with ARF Binding to Membranes—Previous work has shown that addition of GTPγS inhibits several assays that reconstitute vesicular transport including transport through the Golgi and fusion between endosomes, in a cytosol-dependent fashion (Rothman and Orci, 1992; Mayorga et al., 1989). The sensitivity to GTPγS of several cell-free assays is conferred in part by ARF, a cytosolic protein (Taylor et al., 1992). Since peptide G and ARF have sequences in common, we speculated that peptide G would compete with
ABR function. If that were the case, addition of peptide G might be expected to compete both the GTP-γS- and ARF-dependent inhibition of fusion. As predicted the inhibitory effect of GTP-γS was reversed by addition of increasing concentrations of peptide G1 (Fig. 3A). Similarly, the inhibitory effect of ARF was reversed by addition of peptide G1 (Fig. 3B). Moreover, βARKct also reversed the inhibitory effect of both GTP-γS and ARF (data not shown). The observation that both peptide G and the fusion protein containing the COOH-terminal domain of the βARK produce a similar effect rules out the possibility that the effects observed in our assay are due to detergent-like effects sometimes attributed to certain peptides.

As another approach to directly show that peptide G was competing with ARF for interaction with membranes, we studied the binding of ARF to endosomal membranes by Western blot assay. Fig. 3C shows that incubation of enriched endosomal membranes with cytosol in the presence of 2 μM GTP-γS resulted in binding of ARF (lane a). Preincubation of the membranes for 5 min at 37°C before the addition of GTP-γS with peptide G1 or G2 (lanes b and c) inhibited the binding of ARF to endosomal membranes. As expected no inhibition of ARF binding was observed with the control peptide G1, (lane d).

Taken together our results indicate that peptide G interferes with ARF function by blocking the interaction of the protein with the membrane. Binding of Gβγ Subunits to ARF Fusion Proteins—Based on the sequence similarity between peptide G and ARF and, since peptide G blocks binding of βARK to Gβγ subunits (Koch et al., 1993), the results suggest that Gβγ is one of the membrane components that may interact with ARF. In order to address this question, we performed an in vitro binding assay using a GST-ARF fusion protein to study the direct interaction between the proteins. We constructed GST-ARF4 and an amino-terminal deletion mutant GST-ARF4 (Δ1-17) with the first 17 amino acids deleted. In order to better define the domain that is involved in the interaction of ARF with Gβγ, a third fusion protein GST-ARF1ct, which contained the carboxy-terminal half of ARF1, was constructed based on the sequence alignments between peptide G and ARF. Fig. 4 shows a diagrammatic representation of the GST-ARF fusion proteins used in the in vitro binding assay. As shown in Fig. 5A both ARF mutants, ARF4 (Δ1-17) and ARF1ct (lanes 4 and 5, respectively), bound Gβγ although to a lesser extent than GST-βARK1ct (lane 1, positive control). Also, GST-ARF4 bound Gβγ to an extent similar to the mutated forms of ARF (data not shown). The corresponding region of ARF (GST-ARF1ct), which does not bind Gβγ, and GST alone were used as negative controls (lanes 2 and 3, respectively). In order to assess the specificity of the interaction between Gβγ and ARF, Rab5, another small GTP-binding protein involved in fusion among endosomes, was tested in the binding assay. GST-Rab5 was negative for binding to Gβγ subunits (lane 6). Although these results indicate that the binding of Gβγ to immobilized ARF is specific, only a small amount of the available Gβγ subunits bound to GST-ARF. However, the binding of Gβγ to GST-ARF4 (Δ1-17) was markedly increased by addition of recombinant Gα subunits such as Gαs and Gα13 (Fig. 5A, lanes 7 and 8), suggesting that ARF interacts more efficiently with the heterotrimer than with Gβγ alone. This was an unexpected observation given previous results showing that Gα completely inhibited the binding of Gβγ to βARK (Touhara et al., 1994). Nevertheless, there is a precedent for a possible Gα-ARF association given the fact that ARF is the co-factor necessary for the ADP-ribosylation of Gα by cholera toxin (Kahn and Gilman, 1984, 1986). Therefore, Gα may associate directly with ARF increasing the binding of Gβγ.

Gβγ binding to ARF-GST was specifically competed by purified recombinant ARF1 and ARF4 (Fig. 5B), but not by BSA indicating the specificity of the ARF-Gβγ association. Gαs in the GDP-bound Form Interacts with ARF—As mentioned above addition of Gα subunits enhanced the binding of Gβγ to ARF. In order to study the possibility that Gα was also part of the complex, we added recombinant Gαs to the assay in the presence of Gβγ subunits. As shown in Fig. 6A, Gαs was detected using a specific antibody generated against the COOH-terminal domain of Gαs. We next asked if Gαs was able to associate with ARF in the absence of Gβγ and if this interaction were specific. Fig. 6B shows that Gαs binds to GST-ARF in the absence of Gβγ, and that a marked increase in binding is observed when both subunits were added to the assay. Essentially, no binding was observed when GST alone was used, indicating the specificity of the protein association. It is known that GTPases function as molecular switches changing their conformation when they are activated. Aluminum fluoride (AlF) is a classical activator of heterotrimeric G proteins but does not activate members of the small GTPase family such as ARF (Kahn et al., 1992). Therefore, in order to independently activate the heterotrimeric G protein, the effect of AlF was tested in the binding assay. As shown in Fig. 6C, activation of Gαs by AlF completely abolished ARF-Gαs association both in the presence or the absence of Gβγ. As expected, AlF did not affect ARF-Gβγ association.

Given that both ARF and heterotrimeric G proteins are regulated by nucleotide binding we next studied the effect of either GTP-γS or GDP-βS. Similar to the effect observed with AlF,
Peptide G₁ reverses GTP₆S- and ARF-mediated inhibition of fusion by inhibiting ARF binding to the membranes. A, endosome fusion was tested in the presence of 0.8 mg/ml cytosol supplemented with 20 μM GTP₆S to inhibit fusion. The inhibitory effect of GTP₆S was reversed by addition of increasing concentrations of peptide G₁. Endosome fusion was measured as described under “Experimental Procedures.” Fusion is expressed in relative units. B, endosomal vesicles were resuspended in cytosol (0.2 mg/ml) containing 20 μM GTP₆S. Fusion was assessed in the presence (closed circles) or absence (open circles) of 15 μg/ml purified recombinant myristolated ARF1. The inhibitory effect of ARF was reversed by addition of increasing concentrations of peptide G₁. The results are representative data of a experiment performed three times. C, enriched endosomal fraction (10–20 μg of total protein) was resuspended in fusion buffer, containing 1 mg/ml cytosolic proteins. Samples were incubated for 5 min at 37 °C in the presence of: lane a, no additions; lane b, 50 μM peptide G₂; lane c, 25 μM peptide G₂; lane d, 50 μM G₁ (control peptide). After preincubation, 20 μM GTP₆S was added, and the samples were incubated for additional 20 min at 37 °C. After incubation, the samples were washed with 1 ml of homogenization buffer containing 20 μM GTP₆S and 1 mM MgCl₂, and the membranes were recovered by centrifugation for 5 min at 50,000 × g. The membrane proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with anti-ARF antibodies. Data represent one of three similar experiments.

GTP₆S almost completely blocked G₁₆S-ARF association (data not shown); essentially no effect was observed with GDP₆S. Our results clearly indicate that G₁₆S, in the GDP-bound form associates with ARF either in the presence or the absence of G₁₆S subunits. Activation of G₁₆S by either GTP₆S or AIF completely blocked ARF-G₁₆S interaction, indicating that the heterotrimer is the most likely candidate for ARF-G protein interaction. GTP₆S inhibited ARF-G₁₆S association (Fig. 6D) suggesting that ARF interacts with G₁₆S in the GDP-bound state.

DISCUSSION
The G₁₆S subunits of heterotrimeric G proteins modulate the activity of several signal-transducing effector molecules such as phospholipase C, phospholipase A2, certain isoforms of adenylyl cyclase and cardiac muscarinic potassium channels (reviewed by Clapham and Neer, 1993). G₁₆S also mediates the membrane translocation of the β-adrenergic receptor kinases (βARK1 and βARK2) where they phosphorylate activated receptors (Inglese et al., 1993). The COOH-terminal domain of βARK (βARKct) contains the targeting domain for binding to G₁₆S (Pitcher et al., 1992), and a fusion protein corresponding to this targeting domain blocks the binding of βARK to G₁₆S (Koch et al., 1993).
FIG. 6. A, binding of Gαs to GST-ARF. Purified bovine brain Gβγ (300 nM) with or without purified recombinant Gαs (700 nM) was incubated with GST-ARF4(1–17) as described in Fig. 5. B, Gαs interacts with ARF in the presence or absence of Gβγ. GST-ARF4(1–17) or GST alone was incubated with purified bovine brain Gβγ (300 nM), purified recombinant Gαs (500 nM) or both as described. C, activation of Gαs by AIF blocks Gαs-ARF association. GST-ARF4(1–17) was incubated with 300 nM of purified bovine Gβγ subunits and/or with 700 nM recombinant Gαs, for 30 min at 30°C in PBS containing 0.01% Lubrol, 10 mM MgCl₂, in the presence or the absence of AIF (100 μM AlN₃(SO₄)₂ + 10 mM KF). D, activation of ARF by GTPγS inhibits Gβγ-ARF association. GST-ARF4(1–17) was preincubated for 90 min at 37°C in 50 mM HEPES-K, pH 7.5, containing 0.01% Lubrol, 1 mM DTT, and 10 mM MgCl₂, in the presence of 50 μM GTPγS, 50 μM GDPβS or no additions. Nucleotide exchange on ARF was stopped by cooling at 4°C. Subsequently, 300 nM of purified bovine Gβγ subunits were added, and the samples were incubated for additional 30 min at 4°C. The binding of the proteins to glutathione-Sepharose and the detection were performed as described under “Experimental Procedures.” Western blot analysis showing Gβγ and/or Gαs binding to GST fusion proteins. Data represent one of three similar experiments.

et al., 1993). Moreover, βARKct appears to act as a general Gβγ antagonist, inhibiting Gβγ-mediated signals other than βARK translocation such as Gβγ-dependent activation of adenyl cyclase type II, βARK2-regulated olfactory signal transduction, and atrial K+ channel activation (Reuveny et al., 1994; Boekhoff et al., 1994; Ingelse et al., 1994; Koch et al., 1994).

In this report we present evidence that the COOH-terminal portion of βARK (βARKct) and peptides corresponding to the Gβγ-targeting domain of βARK inhibit in vitro endosome fusion. The results suggest that a Gβγ-mediated signal is involved in either the mechanism or the regulation of endosome fusion. Indeed, our results suggest that βARKct and peptides from the Gβγ-binding domain (peptides G) block the interaction of Gβγ with a factor(s) required for endosome fusion. We believe that one of these factors is ARF for the following reasons: (i) peptide G and ARF share sequence homology, (ii) peptide G reverses GTPγS- and ARF-mediated inhibition of endosome fusion, (iii) peptide G inhibits ARF binding to membranes. Supporting evidence for a direct interaction between ARF and Gβγ was provided by an in vitro binding assay using ARF-GST fusion proteins. Our study establishes that Gβγ binds to ARF and that this interaction is specifically competed by purified recombinant ARF and enhanced by Gαs.

While the binding of Gβγ to immobilized ARF is specific, only small amounts of the available Gβγ subunits bound to GST-ARF. However, the binding was increased by the addition of Gαs. A trivial explanation is that most of the Gβγ has been simply denatured during its preparation. Another possibility is that ARF binds only to a specific subset of the Gβγ combinations comprising the heterogeneous preparation isolated from bovine brain. An interesting possibility is that Gβγ may require interaction with another protein to be in the right conformation for binding. The Gβγ-binding domain of βARK shares homology with the novel pleckstrin homology domain (PH domain). This domain is found in a variety of signaling molecules such as RasGAP, Ras-GRF, SOS, and others (Shaw, 1993; Musacchio et al., 1993). Recently, it has been shown that proteins with PH domains bind to Gβγ in vitro (Touhara et al., 1994). Protein-protein interactions between proteins containing a PH domain and Gβγ may play a significant role in cellular signaling. Although the presence of a PH domain has not been described for ARF, it is tempting to speculate that putative ARF accessory proteins such as ARF-GAP or ARF-GRF may indeed contain such a domain and that they may regulate ARF activity in conjunction with Gβγ. Current models for the interaction between ARF and target membranes propose that activation of ARF by a protease- and brefeldin A-sensitive membrane-bound nucleotide-exchange factor (Helms and Rothman, 1992; Donaldson et al., 1992b; Randazzo et al., 1993) results in association of ARF-GTP with the lipid bilayer. Our results indicating that ARF in the GDP form interacts with Gβγ suggest that these proteins may form a multimeric complex that allows the interaction of ARF with its nucleotide exchange factor resulting in ARF activation.

The results presented in this report are the first direct evidence indicating that both Gαs and Gβγ associates directly with ARF. There is a precedent for this connection in that ARF is the co-factor necessary for the ADP-ribosylation of Gα by cholera toxin and a possible interaction with Gαs has been previously suggested (Kahn and Gilman, 1984, 1986).
ingly, during the purification of ARF from bovine brain, ARF eluted in two peaks, one coincidental with Gαs. Addition of AIF was necessary to obtain a single peak of ARF activity (Kahn and Gilman, 1984, 1986). The more likely target for AIF is the GDP-form of Gαs. In agreement with the results of Kahn and Gilman, our data indicate that Gαs in the GDP-bound conformation associates with ARF since activation of Gαs by either GTPγS or AIF completely blocked ARF-Gαs interaction. Recently, Finazzi and collaborators (1994) have shown that AIF plus GTP stabilizes the active state of ARF by preventing the rapid hydrolysis of the GTP loaded onto ARF. These authors have postulated that an AIF-sensitive target may lead to a persistent activation of ARF by inhibiting an ARF GAP or by making the ARF-GTP either insensitive or inaccessible to ARF GAP. While the exact role and mechanism of action of Gαs remains to be defined, our results of complete inhibition of Gαs-ARF association by AIF suggest the intriguing possibility that Gαs in the GDP-bound form may regulate ARF GTPase activity.

An interesting outcome of our experiments relates to the role of the amino-terminal domain of ARF in mediating ARF function. It has been reported that the amino terminus of ARF is critical for function since deletion of this domain results in a global reduction of ARF activities (Kahn et al., 1992). A synthetic peptide derived from the amino terminus of ARF inhibits ARF activity including chola toxin activation, as well as intra-Golgi transport (Kahn et al., 1992) and fusion between endosomes (Lenhard et al., 1992). Moreover, the amino-terminal 13 residues of ARF1 are required for cofactor activity in the ADP-ribosylation by chola toxin when Gαs is the substrate (Randazzo et al., 1994). However, Vaughan and collaborators (Hong et al., 1994) have shown that the amino terminus of ARF is not necessary for in vitro activation of chola toxin using as a substrate agmatine. Although the basis for this disparity is not clear, this latest result suggests that other domains, besides the amino terminus, are likely involved in the interaction of ARF with the toxin. Our results indicate that the ARF domain involved in ARF-Gβγ interaction does not require the amino-terminal 17 amino acids since Gαsβγ binds to GST-ARF4 and to the truncated ARF mutants (ARF4Δ1–17 and ARF1Δct) to a comparable extent. However, we cannot rule out the possibility that the presence of GST at the amino terminus may interfere with the proper folding and binding capacity of this domain.

It has been demonstrated that ARF plays an essential role in regulating cotumer (Donaldson et al., 1992a; Palmer et al., 1993) and AP-1 recruitment onto Golgi membranes (Traub et al., 1993; Stammes et al., 1993). Moreover, a number of studies have provided evidence for the involvement of heterotrimeric G proteins in coat assembly (Donaldson et al., 1991; Kitstakis et al., 1992). Association of ARF and Gβγ-COP with Golgi membranes is sensitive to a number of reagents that modulate heterotrimeric G protein function (Donaldson et al., 1991; Kitstakis et al., 1992). In addition to GTPγS, AIF, known to specifically activate trimeric G proteins (Kahn, 1991), enhances the binding of Gβγ-COP to Golgi membranes (Serafini et al., 1991). These findings and the observation that Gβγ inhibits both ARF and Gβγ binding (Donaldson et al., 1991) suggest that G proteins regulate coat protein binding. We have also recently shown that both heterotrimeric G proteins and ARF regulate priming of endosomal membranes for fusion (Lenhard et al., 1994). Addition of Gβγ resulted in inhibition of GTPγS-mediated priming of endosomes. In contrast, addition of ARF to the assay enhanced priming in the presence of cytosol. These observations suggest that ARF enhances binding of cytosolic factors required for fusion onto the endosomal membrane. All though the linkage between ARF binding and coat assembly with heterotrimeric G proteins has been proposed based on the data summarized above, to date no direct evidence for the interaction between ARF and heterotrimeric G proteins has been presented. Our data would support a model in which heterotrimeric G proteins regulate binding of essential proteins at least in part, by directly interacting with ARF.

Finally, several recent observations implicate a signal transduction mechanism in the regulation of vesicular traffic. The findings from Bomsel and Mostov (1993) indicating that binding of dIgA to the plgR stimulates the formation of transcytotic vesicles suggest that ligand binding generates a signal that is transduced to the intracellular sorting machinery. Interestingly, in Chinese hamster ovary cells transfected with muscarinic receptors, endosomal trafficking was inhibited by carbacol (Haraguchi and Rodbell, 1991). More specifically, antigen-induced activation of the IgE receptor and activation of protein kinase C regulate the GTP-dependent binding of ARF and β-COP to Golgi membranes (De Matteis et al., 1993). Furthermore, the recent identification of phospholipase D as an effector of ARF (Brown et al., 1993; Kahn et al., 1993) raises the possibility that a novel signal transduction pathway may regulate intracellular membrane traffic. Our results of a direct interaction between ARF and trimeric G proteins suggest that ARF may be a nexus linking heterotrimeric G proteins and downstream effectors (i.e. PLD). Given the enormous potential for specificity with 24 possible combinations of Gβγ and several ARFs, our present observations, together with those of others, provide a novel prospect by which trimeric G proteins and ARF provide fine control of vesicular traffic and its response to extracellular signals.

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Note Added in Proof—While this paper was under review, we became aware of a paper reporting similar results (Franco, M., Paris, S. and Chabre, M. (1995) FEBS Lett. 362, 286–290).

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