Two Distinct Populations of ARF Bound to Golgi Membranes

J. Bernd Helms, David J. Palmer, and James E. Rothman

Program in Cellular Biochemistry and Biophysics, Rockefeller Research Laboratory, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York 10021

Abstract. ADP-ribosylation factor (ARF) is a small molecular weight GTP-binding protein (20 kD) and has been implicated in vesicular protein transport. The guanine nucleotide, bound to ARF protein is believed to modulate the activity of ARF but the mechanism of action remains elusive. We have previously reported that ARF binds to Golgi membranes after Brefeldin A-sensitive nucleotide exchange of ARF-bound GDP for GTPγS. Here we report that treatment with phosphatidylcholine liposomes effectively removed 40–60% of ARF bound to Golgi membranes with nonhydrolyzable GTP, presumably by competing for binding of activated ARF to lipid bilayers. This revealed the pres-ence of two different pools of ARF on Golgi membranes. Whereas total ARF binding did not appear to be saturable, the liposome-resistant pool is saturable suggesting that this pool of ARF is stabilized by interaction with a Golgi membrane-component. We propose that activation of ARF by a guanine nucleotide-exchange protein results in association of myristoylated ARF.GTP with the lipid bilayer of the Golgi apparatus. Once associated with the membrane, activated ARF can diffuse freely to associate stably with a target protein or possibly can be inactivated by a GTPase activating protein (GAP) activity.

TRIMERIC (GTP-binding) G proteins, as well as small molecular weight (monomeric) GTP-binding proteins have been implicated in intracellular protein transport (for reviews see Bourne et al., 1990; Burgoyne, 1992; Pfeffer, 1992; Balch, 1990). Involvement of a trimeric G protein in intra Golgi protein transport was originally proposed by Melançon et al. (1987). Recently, the trimeric G protein $G_{\alpha i3}$ has been localized to the Golgi complex (Ercolani et al., 1990) and has been implicated in transport since its over-expression results in inhibition of secretion of heparan sulphate proteoglycan (Stow et al., 1991). The observations that AlF_4^- activates trimeric G proteins but not small GTP-binding proteins (Kahn, 1991) and that the $\beta\gamma$ subunits of trimeric G proteins inhibit the binding of coat proteins, is further evidence for the involvement of trimeric G proteins in protein transport (Donaldson et al., 1991). In support of this, masto-paran, a peptide that activates trimeric G proteins, was shown to stimulate the association of the coat component β-COP with Golgi membranes (Ktistakis et al., 1992).

The involvement of small molecular weight GTP-binding proteins in transport was first indicated by the identification of the temperature-sensitive yeast sec4 gene product (Salmi-nen and Novick, 1987). Genes of the mammalian homo-logues of the yeast GTP-binding protein YPT1 have been cloned and are grouped in a still growing family of Rab pro-teins (Haubruck et al., 1987; Touchot et al., 1987; Chavrier

et al., 1990). Rab proteins are localized to particular intra-cellular membrane compartments including Golgi mem-branes (Goud et al., 1990; Chavrier et al., 1990) and anti-bodies directed against Rab proteins inhibit specific stages of membrane traffic (Gorvel et al., 1991; Plutner et al., 1991), suggesting that these proteins function in vesicle tar-geting or fusion, perhaps to confer specificity and direction-ality to vesicular transport (Bourne, 1988).

Another family of the small molecular weight GTP-binding proteins is the ADP-ribosylation factor (ARF)[1] family. ARF was originally identified as a cofactor required in ADP-ribosylation of the α subunit of the trimeric G pro-tein G_s by cholera toxin (Schleifer et al., 1982; Kahn and Gilman, 1984) and was subsequently shown to be a GTP-binding protein (Kahn and Gilman, 1986). Two ARF genes have been identified in yeast and yeast mutants lacking the ARF1 gene product exhibit a secretion defective phenotype (Stearns et al., 1990). By immunological techniques it was shown that membrane bound ARF was localized at the Golgi complex in mammalian cells (Stearns et al., 1990). The first indication for the mechanism of action of ARF in vesicular protein transport was provided by Serafini et al. (1991) who showed that ARF is a subunit of the coat of Golgi-derived vesicles. It was subsequently demonstrated that a peptide,

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1. *Abbreviations used in this paper*: ARF, ADP-ribosylation factor; ATPγS, adenosine 5'-*O*-(3-thiotriphosphate); AMPPNP, β-γ-Imidoadenosine 5'-tri-phosphate; GAP, GTPase activating protein; GMPPNP, β-γ-Imidoguano-sine 5'-triphosphate; GTPγS, guanosine 5'-*O*-3-thiotriphosphate; [^{3}H]-myr, [^{3}H]myristoyl; NMT, myristoyl-CoA:protein *N*-myristoyl transfer-ase; PC, phosphatidylcholine.

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derived from the NH$_2$-terminal sequence of ARF, inhibits inter-cisternal Golgi transport (Kahn et al., 1992) as well as protein transport from the ER to the cis-Golgi cisternae (Balch et al., 1992), and endosome–endosome fusion (Lenhard et al., 1992). ARF proteins are also implicated in nuclear vesicle dynamics (Boman et al., 1992). So far, six members of the ARF family have been identified (Tsuchiya et al., 1991) but it is not yet clear whether particular family members are specialized for particular membrane traffic transformations.

The mechanism of action of ARF-protein is subject of current investigation. Recently, it has been shown that ARF is required for the binding of coatomer to Golgi membranes (Donaldson et al., 1992a; Palmer et al., 1993) and that myristoylation of ARF is required for this activity (Palmer et al., 1993). Based on its activity in the cholera toxin catalyzed ADP-ribosylation of G$_s$, ARF likely interacts with the trimeric class of GTP-binding proteins. In agreement with this it has been observed that $\beta\gamma$-subunits of trimeric G proteins inhibited the association of both ARF and β-COP with Golgi membranes (Donaldson et al., 1991). It should be noted however that ARF has a significant homology with the α-subunits of trimeric G proteins, including G$_{s\alpha}$ (Sewell and Kahn, 1988). Thus, it cannot be excluded that the effect of $\beta\gamma$ is directly on ARF rather than via a trimeric G protein.

Purified bovine ARF1, as well as recombinant ARF1, have been characterized in vitro (Kahn and Gilman, 1986; Weiss et al., 1989). It was shown that for spontaneous nucleotide exchange, ARF1 protein requires phospholipids, detergent, and very low concentrations (nM) of Mg^{2+}. It appears that during this exchange reaction, myristoylated ARF.GTP, but not ARF.GDP, is stably bound to the liposomal structures (Kahn, 1991). Although this observation would provide a mechanism for ARF binding to biological membranes, it does not account for the specific localization of ARF at the Golgi complex (Stearns et al., 1990). Also, the intracellular concentration of Mg^{2+} (2–5 mM) and the absence of detergents in biological systems would prevent spontaneous nucleotide exchange. As a result, a Golgi membrane specific nucleotide-exchange enzyme has been postulated to catalyze the exchange of ARF.GDP to ARF.GTP, resulting in binding of ARF to the lipid bilayer of the Golgi complex (Serafini et al., 1991).

Recently, evidence for the presence of a Golgi membrane-bound nucleotide-exchange enzyme has been found (Helms and Rothman, 1992; Donaldson et al., 1992). This enzyme catalyzes the exchange of nucleotides bound to ARF and interestingly, the exchange activity is highly sensitive to Brefeldin A, a fungal metabolite that inhibits a wide variety of membrane transport steps (for review see Pelham, 1991). These results suggest that activation of ARF proteins could be the first committed step in various membrane transformation pathways.

Here we demonstrate that, in the presence of GTP, ARF interacts dynamically with Golgi membranes. A Golgi membrane-bound guanine nucleotide specific–exchange enzyme catalyzes the exchange of GDP for GTP. The GTP is rapidly hydrolyzed, probably after binding. In the presence of the stable GTP analogue guanosine 5'-O-(3-thiotriphosphate) (GTPγS), ARF becomes permanently activated and accumulates on Golgi membranes. The membrane-bound ARF is localized in two distinct pools which can be distinguished by extraction of Golgi membranes with liposomes.

Materials and Methods

Preparation of ARF, Myristoylated ARF, and Golgi Membranes

Recombinant ARF was overexpressed and purified to homogeneity as described by Weiss et al. (1989) except that after overexpression, the bacteria were collected by centrifugation (15 min at 4,000 g at 4°C), washed once with PBS, centrifugated as before, and finally resuspended in 5 ml (per 500 ml of culture) of 20 mM Tris/HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT (DEAE-buffer). The cells were lysed using a French press (8,000 psi cell-pressure, three cycles) in the presence of PMSF (1 mM). The cell-lysate was centrifuged at 100,000 g (1 h at 4°C). The supernatant was diluted 1:1 with DEAE-buffer before purification. Recombinant myristoylated ARF was overexpressed by coexpression of yeast N-myristoyl transferase (Duronio et al., 1990) and purified to homogeneity as described for non-myristoylated ARF (we thank Drs. Gordon and Kahn for providing the pBB131 [N-myristoyl transferase] and pOW12 [ARF] plasmids, respectively). Unlabeled myristic acid (50 μM) was added to the medium during overexpression. Salt-washed rat liver Golgi membranes were prepared as described by Clary and Rothman (1990), except that the extraction solution contained 10 mM Tris/HCl, pH 7.4, and that the extraction was done at 0°C. Protein determination was according to a modification of the Lowry method (Peterson, 1977).

Preparation and Purification of [^{3}H]Myristate-labeled ARF

Myristoylated ARF was radioactively labeled by addition of [^{3}H]myristic acid to the culture medium (0.1 mCi/ml medium) during overexpression of ARF. After induction, the bacteria were collected, washed, and lysed as described for unlabeled myristoylated ARF. The supernatant was diluted 1:1 with DEAE-buffer and passed over a DEAE-Sephacel column (40 ml column/10 ml diluted cell-lysate) at 25 ml/h at 4°C. Fractions containing [^{3}H]myr-ARF, which elutes with the flow-through material, were pooled and concentrated to 20 ml by ultrafiltration (YM10 membrane, Amicon, Beverly, MA). [^{3}H]myr-ARF was dialyzed in 20 mM Tris/HCl, pH 7.4, 50 mM KCl and 0.5 mM DTT, frozen in liquid nitrogen, and stored in aliquots at −80°C.

The specific activity of [^{3}H]myristate-labeled ARF (2,500 cpm/pmol of myristoylated ARF) was calculated by incubating [^{3}H]myr-ARF with Golgi membranes and 20 μM GTPγS in a typical [^{3}H]myr-ARF binding assay (see below). After incubation both the radioactivity, associated with the membranes, as well as the total amount of myristoylated ARF (non-myristoylated ARF fails to bind Golgi membranes (Palmer et al., 1993) associated with Golgi membranes (as determined by quantitative Western blotting using the anti-ARF1 antibody [2048] [Palmer et al., 1993] purified, unlabeled myristoylated ARF was used as a standard) were determined (not shown). From the total amount of ARF in the [^{3}H]myr-ARF preparation (0.3 mg/ml), the total radioactivity (4.78 × 10^6 cpm/ml) and the specific activity (2,500 cpm/pmol), it can be calculated that 13% of the ARF is myristoylated.

[^{3}H]myr-ARF Binding Assay

In a typical ARF binding assay, [^{3}H]myr-ARF (4.5 × 10^3 cpm, or as indicated) was incubated with salt-washed rat liver Golgi membranes (1.7 μg) in 25 mM Hepes/KOH, pH 7.2, 20 mM KCl, 2.5 mM magnesium acetate, ovalbumin (1.6 mg/ml), 0.2 M sucrose and (unlabeled) nucleotides (as indicated in text) in a total volume of 50 μl for 20 min at 37°C. To prevent ARF binding to tube walls, incubations were performed in 1.5-ml silanized tubes. The binding reaction was stopped by transferring the incubations to ice. The reaction mixture was loaded onto a 165-μl cushion of 25% sucrose (wt/vol) in 25 mM Hepes/KOH, pH 7.2, 20 mM KCl, 2.5 mM magnesium acetate) in a 0.5-ml tube and centrifuged for 30 min in a microcentrifuge at 14,000 rpm (4°C) (Eppendorf North America, Inc., Madison, WI). The superna-

tant was removed and the membrane pellet was solubilized in 40 μl of 0.5% Triton X-100 in PBS, and radioactivity was determined by liquid scintillation counting.

Nucleotide-Exchange Assay

Unlabeled myristoylated ARF (amount indicated in figure legend) was incubated with salt-washed rat liver Golgi membranes (1.7 μg) in 25 mM Hepes/KOH, pH 7.2, 20 mM KCl, 2.5 mM magnesium acetate, ovalbumin (1.6 mg/ml), 0.2 M sucrose, and 5 μM GTP[α^{32}P] or GTP[γ^{32}P] (specific activities as indicated in figure legend) in a total volume of 50 μl for 15 min at 37°C. Incubations were performed in 1.5-ml silanized tubes. The nucleotide exchange reaction was stopped by transferring the incubations to ice. Radioactivity, bound to protein, was determined by the nitrocellulose filter trapping assay (Northup et al., 1982).

Extraction of Golgi Membranes with Phosphatidylcholine Liposomes

After incubation of ARF and Golgi membranes as described in the text, the incubations were transferred to ice. Phosphatidylcholine (PC) liposomes (10 μl of a 6-mM stock, unless indicated otherwise) were added to a 50-μl incubation and incubated for 10 min on ice. After incubation, the total reaction mixture (60 μl) was loaded on a sucrose gradient and centrifuged as described in the binding assay. To prepare PC liposomes, 9 mg of L-α-phosphatidylcholine (egg lecithin, 10 mg/ml in ethanol, average molecular mass 750 kD) was dried under a stream of N$_2$. The lipid film was resuspended in 2 ml of 0.2 M sucrose in 10 mM Tris/HCl, pH 7.4 (6 mM PC), and sonicated with a sonifier (20 W output) until optically transparent (Branson Ultrasonics Corp., Danbury, CT).

Liposome Extraction of Golgi Membranes and Separation of Phosphatidylcholine Liposomes on a Sucrose Gradient

In stage I, [^{3}H]myr-ARF (1.8 × 10^6 cpm) and salt-washed rat liver Golgi membranes (68 μg) were incubated in the presence of GTPγS (20 μM) as described under [^{3}H]myr-ARF binding assay in a total volume of 2 ml. After incubation, the membranes were reisolated by sucrose gradient centrifugation as described for the isolation of salt-washed Golgi membranes (see above) and were collected in a total volume of 280 μl. In Stage II, the [^{3}H]myr-ARF loaded membranes (100 μl) were incubated with 100 μl of 6-mM PC liposomes in 25 mM Hepes/KOH, pH 7.2, 20 mM KCl, 2.5 mM magnesium acetate, ovalbumin (1.6 mg/ml), and 0.2 M sucrose in a total volume of 500 μl for 10 min at 0°C. After incubation, the Golgi membranes were pelleted (30 min at 14,000 rpm in an Eppendorf microcentrifuge at 4°C). The supernatant (500 μl) was mixed with 1.1 ml of 2.1 M sucrose. The solution (1.5 M sucrose) was overlayed with 1.5 ml of 1.2 M sucrose and 2.1 ml of 0.2 M sucrose. All sucrose solutions were in 25 mM Hepes/KOH, pH 7.2, 20 mM KCl, 2.5 mM magnesium acetate. The sucrose gradients were centrifuged in a SW55 rotor at 44,000 rpm (180,000 g) for 12 h at 4°C (slow acceleration/deceleration) (Beckman Instrs., Inc., Fullerton, CA). Fractions of 0.5 ml were collected from the bottom of the gradient at 0.5 ml/min and counted for radioactivity by scintillation counting.

Results

Preparation of [^{3}H]Myristate-labeled ARF

Recombinant bovine ARF1 can be myristoylated in bacteria by coexpression of yeast myristoyl-CoA:protein N-myristoyl transferase (NMT) (Weiss et al., 1989; Duronio et al., 1990). When [^{3}H]myristic acid is added to the culture medium during overexpression of myristoylated ARF, the radiolabel readily incorporated into ARF (Fig. 1, lane 3). No labeling of a 20-kD protein was observed when only NMT or ARF were overexpressed in E. coli (Fig. 1, lanes 1 and 2). [^{3}H]Myristic acid is the sole radiolabeled contaminant in the cell-lysate containing [^{3}H]myristoylated-ARF ([^{3}H]-myr-ARF) and it can be efficiently removed from the [^{3}H]-myr-ARF in a single chromatography step using DEAE-
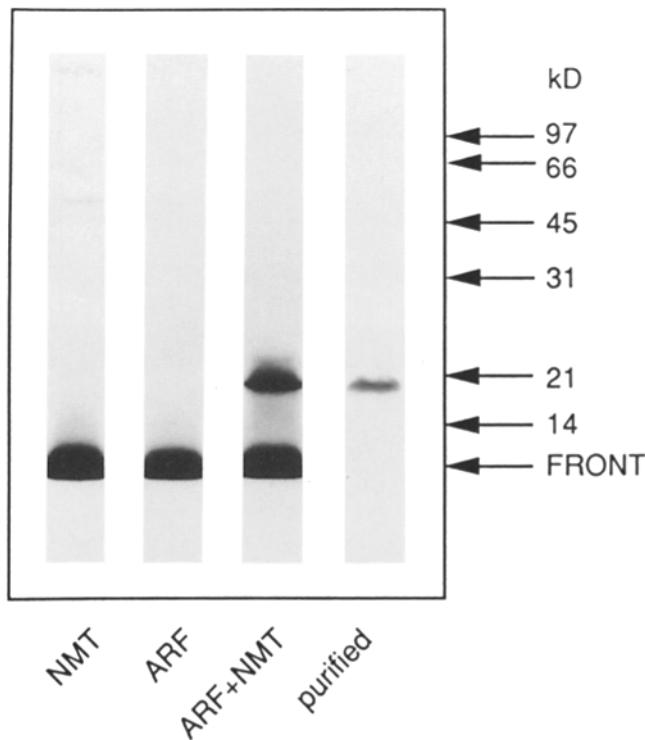

Figure 1. Preparation of [^{3}H]myristate-labeled ARF. (Lanes 1–3) Transfected bacteria were induced with IPTG (1 mM) in the presence of [^{3}H]myristic acid (50 μCi/ml culture) as described in Materials and Methods. Cells were lysed in SDS-gel loading buffer, heated for 5 min at 100°C, and after centrifugation (15 min, 12,000 g rpm), an aliquot of the supernatant (cell-lysate) (40 μg protein) was analyzed by SDS-PAGE (12.5% acrylamide) and fluorography (Enlightning). (NMT) cell-lysate from bacteria, transfected with pBB131; (ARF) cell-lysate from bacteria, transfected with pOW12; (ARF+NMT) cell-lysate from bacteria, transfected with pBB131 and pOW12 (myristylated ARF); and (Purified) [^{3}H]myristoylated ARF (35,000 cpm), purified on DEAE-Sephacel (see Materials and Methods).

Sephacel to produce radiochemically pure [^{3}H]myr-ARF (Fig. 1, lane 4). The presence of ^{3}H-label on ARF was confirmed by immunoprecipitation using a polyclonal anti-ARF1 antibody (2048) described by Palmer et al. (1993) (data not shown).

ARF-binding to Golgi Membranes

Incubation of [^{3}H]myr-ARF with purified salt-washed rat liver Golgi membranes resulted in a low level of membrane-bound ARF (Fig. 2). This level was slightly decreased in the presence of GDP or GTP (Fig. 2). In the presence of GTPγS, a slowly hydrolyzable analogue of GTP, ARF binds and accumulates on membranes to levels ∼threefold higher than with GTP (Fig. 2). GTPγS does not cause precipitation of [^{3}H]myr-ARF as determined by incubation in the absence of Golgi membranes (not shown). With the concentrations of [^{3}H]myr-ARF typically used in these binding assays, accumulation of ARF on membranes in the presence of GTPγS is linear in time for 20–40 min (not shown). The accumulation is specific for guanine nucleotides as binding of ARF was also promoted by the stable GTP analogue β-γ-Imido-guanosine 5'-triphosphate (GMPPNP) but not in the presence of the stable adenine nucleotides adenosine 5'-O-(3-thiotri-
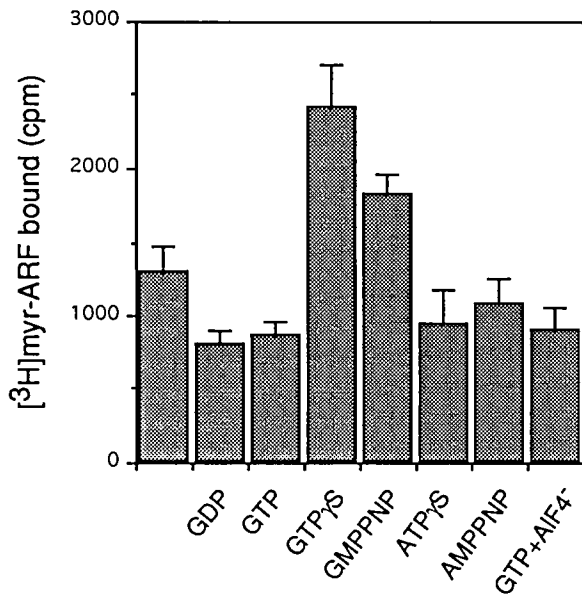
Figure 2. Nucleotide requirement for ARF binding to Golgi membranes. [³H]myr-ARF (82,739 cpm) was incubated with salt-washed rat liver Golgi membranes as described in Materials and Methods in the presence of various nucleotides at 25 μM as indicated in figure. After incubation for 20 min at 37°C the membranes were pelleted through 25% (wt/vol) sucrose cushions and the radioactivity in the membrane-pellet was determined. GMPPNP (β-γ-Imidoguanosine 5'-triphosphate); Aluminum fluoride (50 μM AlCl₃ and 30 mM KF). Data are corrected for pelleting of radioactivity in the absence of membranes (387 cpm) and are shown as the means of duplicate experiments with the error bar representing the higher value.

Figure 3. Liposome-extraction of Golgi membranes. [³H]myr-ARF (38,374 cpm added) was bound to Golgi membranes by pre-incubation with membranes and GTPγS (50 μM) for 20 min at 37°C. The samples were then cooled on ice and increasing concentrations of PC liposomes (prepared as described in Materials and Methods) were added to the incubation. After 10 min on ice, the Golgi membranes were reisolated as described for the binding assay and the radioactivity associated with the membranes was determined. Data are corrected for pelleting of radioactivity in the absence of membranes (245 cpm) and are shown as the means of duplicate experiments.

phosphate) (ATPγS) or β-γ-Imidoadenosine 5'-triphosphate (AMPPNP) (Fig. 2). AlF₄⁻, which activates trimeric G proteins but not small molecular weight GTP-binding proteins like ARF (Kahn, 1991), was also without effect on the level of ARF-binding to membranes (Fig. 2).

Membrane-Bound ARF Is Localized in Two Distinct Pools

Initial experiments showed that the binding of ARF protein to Golgi membranes in the presence of GTPγS was not saturable with respect to increasing ARF concentrations. This raises the possibility that ARF might be inserted directly into the lipid bilayer, perhaps via its myristate group, as has been proposed (Serafini et al., 1991). Indeed we observed that PC liposomes could extract a significant portion of ARF protein, bound to Golgi membranes. With increasing concentrations of PC liposomes, maximally 60% of ARF could be extracted from the membranes (Fig. 3). The liposome-extraction was equally efficient when performed at 0°C or at 37°C and no further membrane-bound ARF could be extracted upon prolonged incubation up to 1 h (data not shown), suggesting a simple (diffusion-mediated) competition event for membrane-bound ARF between Golgi membranes and PC liposomes, and establishing that the extraction was complete under the conditions used.

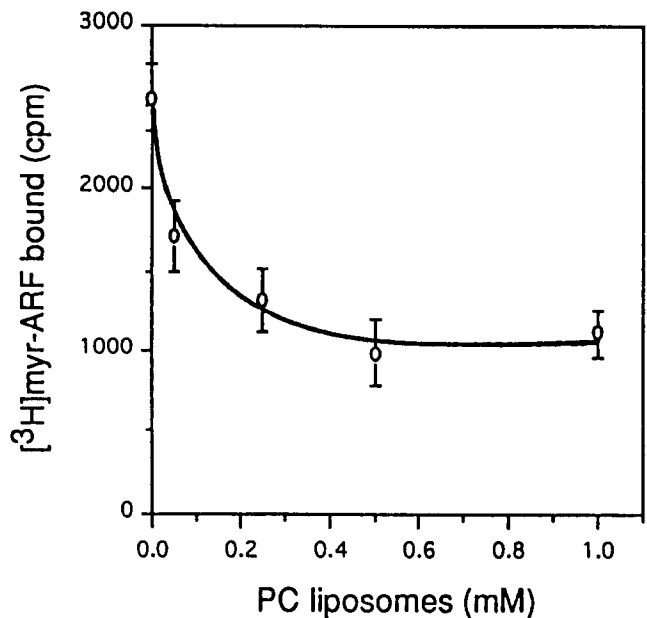The simplest model to explain the liposome extraction of

ARF is that the extractable pool of ARF is freely diffusible in the lipid bilayer of the Golgi membrane. Thus, the lipid bilayer of PC liposomes can compete for Golgi bound ARF. This predicts that after liposome extraction, the [³H]myr-ARF extracted by liposomes will in fact be bound to the PC liposomes. To examine this, PC liposomes were reisolated on a sucrose gradient after liposome-extraction of Golgi membranes (Fig. 4). All the [³H]myr-ARF extracted by PC liposomes migrated to a position in the sucrose gradient identical to the position of PC liposomes (Fig. 4). When soluble (purified) [³H]myr-ARF is incubated with liposomes and GTPγS, ARF did not migrate in the sucrose gradient, indicating that ARF must be activated with GTPγS by a Golgi membrane factor before it is capable of binding PC liposomes (Fig. 4 *A*). Furthermore, after incubation of ARF with GTPγS and Golgi membranes, activated (membrane-bound) ARF was not able to migrate in the gradient (i.e., in the absence of PC liposomes, Fig. 4 *B*). These results strongly suggest the presence of two different pools of ARF bound to Golgi membranes, one pool which can be extracted with PC liposomes ("loosely bound" pool) and one pool which is resistant to liposome extraction ("tightly bound" pool).

Loosely Bound Pool Versus Tightly Bound Pool

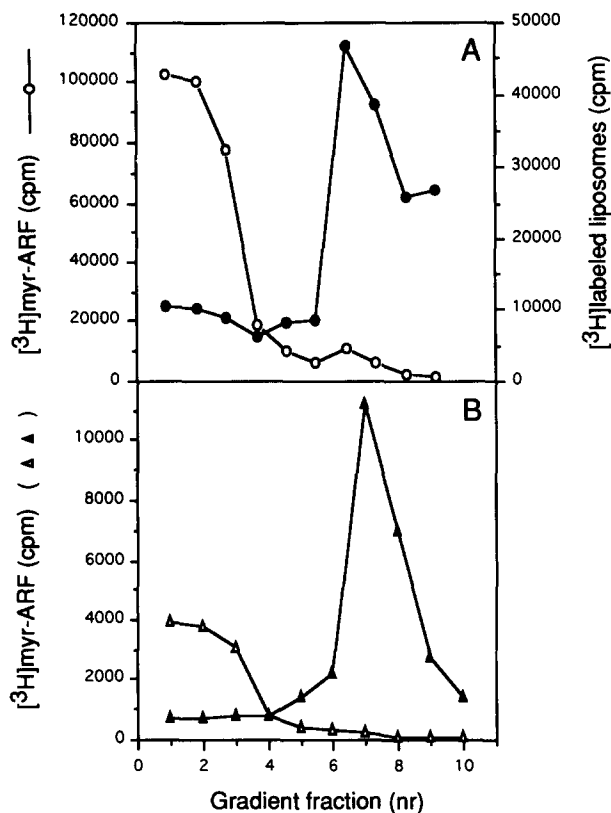To further characterize the different ARF pools on Golgi

Figure 4. Separation of PC liposomes on a sucrose gradient. (A) To determine the migration of PC liposomes (●), the liposomes were radioactively labeled by inclusion of a trace amount of dimyristoyl-[³H]PC (180,000 cpm). The liposomes (500 μl) were mixed with 1.1 ml of 2.1 M sucrose in 10 mM Tris/HCl, pH 7.4 (1.5 M sucrose final), loaded at the bottom of the gradient and centrifuged as described in Materials and Methods (stage II). After centrifugation, 0.5 ml fractions were collected from the bottom of the gradient and the radioactivity was determined by scintillation counting. To determine the effect of PC liposomes on the migration of soluble ARF (○), [³H]myr-ARF (450,000 cpm) was incubated with unlabeled PC liposomes and GTPγS (20 μM) (500 μl) in the absence of Golgi membranes under conditions as described in Materials and Methods (stage II). The mixture was loaded and fractionated on a sucrose gradient as described above. (B) [³H]myr-ARF was bound to salt-washed rat liver Golgi membranes by incubation for 20 min at 37°C in the presence of GTPγS (20 μM) (see Materials and Methods, stage I). After incubation, the Golgi membranes were reisolated on a sucrose cushion as described (Weidman et al., 1989) and incubated in the presence of PC liposomes (▲) or buffer (△) for 10 min at 0°C in a total volume of 500 μl. After incubation, the membranes were pelleted in an Eppendorf centrifuge for 30 min at 14,000 rpm (4°C) and the supernatant was loaded and fractionated on a sucrose gradient as described for A. Radioactivity in each fraction was determined by liquid scintillation counting.

membranes, [³H]myr-ARF was incubated with Golgi membranes, GTPγS, and increasing amounts of unlabeled myristoylated ARF. The amount of membrane-bound ARF was then calculated with or without liposome extraction by determination of membrane-associated ³H-labeled ARF. Fig. 5 shows that the total level of ARF binding (without liposome-extraction) to Golgi membranes was not saturable within the range of ARF-concentrations tested. However,
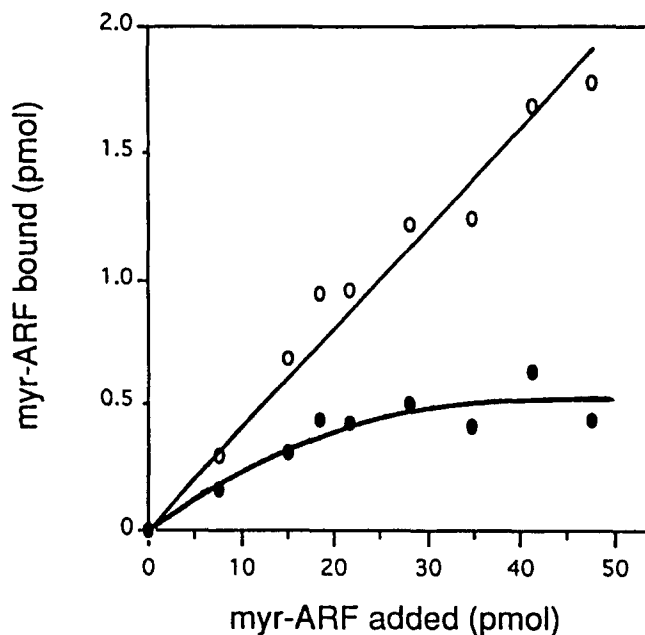

Figure 5. Effect of liposome-extraction on ARF binding to Golgi membranes as a function of ARF concentration. [³H]myr-ARF (34,855 cpm) was mixed with increasing concentrations of unlabeled myristoylated ARF and incubated with Golgi membranes (1.7 μg) and GTPγS (50 μM) as described in Materials and Methods. After incubation, buffer was added to one half of the samples to determine total binding of ARF to membranes (○) whereas PC liposomes were added to the other incubations to quantitate the amount of ARF in the tightly bound pool (●) as described in Materials and Methods. Golgi membranes were reisolated and the radioactivity, associated with the membranes (1.7 μg) was determined by liquid scintillation counting. ARF bound to Golgi membranes (labeled and unlabeled) was calculated by the change in specific activity, due to dilution of [³H]myr-ARF with unlabeled myristoylated ARF, and the actual cpm recovered in the membrane pellet. Data were corrected for precipitation of ARF in the absence of membranes (165 cpm).

when ARF binding is determined after liposome extraction, it appears that the tightly bound pool is saturable (Fig. 5).

The nonsaturable nature of ARF, present in the loosely bound pool, could be accounted for by the association of ARF with only the lipid bilayer. In contrast, the saturability of ARF, present in the tightly bound pool, suggests (stoichiometrical) association with a (limiting) membrane associated factor.

To determine whether ARF can bind to one pool independently of the other, the distribution of membrane-bound ARF in these pools was determined with various experimental conditions. Fig. 6 shows that every experimental condition which results in a decrease in ARF binding, relative to the binding in the presence of GTPγS at 37°C, results in a decrease of both liposome-resistant and total membrane-bound ARF. These data strongly suggest that the two ARF pools on the membrane cannot be modulated independently. The most likely explanation for this phenomena is that membrane-bound ARF, present in the loosely bound pool, can be shifted toward the tightly bound pool. However, entry into the tightly bound pool must be effectively irreversible, for otherwise liposomes would extract both pools.
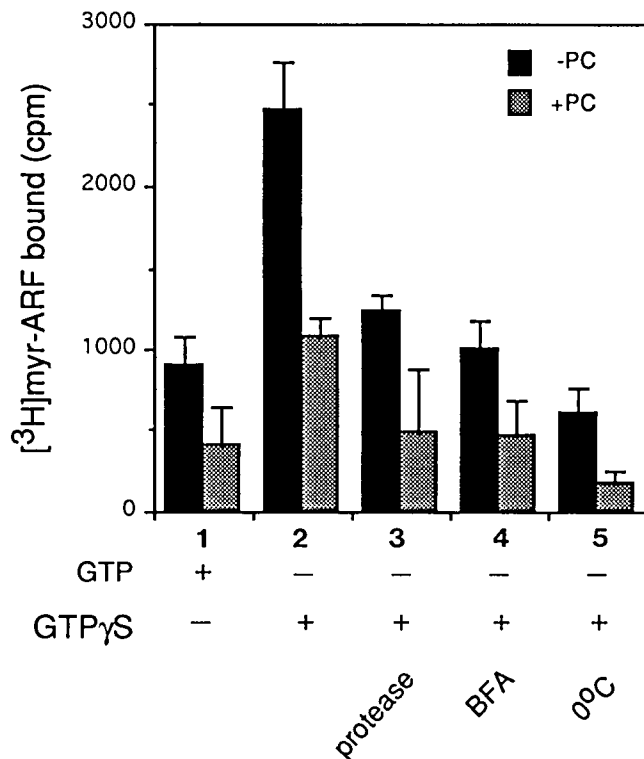
Figure 6. The two different pools of ARF do not arise independently. [³H]myr-ARF (42,973 cpm) was incubated with Golgi membranes in a standard binding assay (Materials and Methods) with modifications as described. After incubation, the samples were cooled on ice and incubated for another 10 min with PC liposomes (▨) or buffer (■) at 0°C. ARF, bound to membranes was quantitated as described in Materials and Methods. (Lane *1*) GTP (25 μM); (lane *2*) GTPγS (25 μM); (lane *3*) membranes were preincubated with Proteinase K (100 μg/ml) for 5 min at 20°C and subsequently with PMSF (2 mM) for 5 min at 20°C before incubation with GTPγS (25 μM); (lane *4*) membranes were preincubated with BFA (200 μM) for 10 min at 37°C before incubation with GTPγS (25 μM); and (lane *5*) GTPγS (25 μM) but at 0°C. Data are corrected for precipitation of [³H]myr-ARF in the absence of membranes (258 cpm) and are shown as the means of duplicate experiments with the error bar representing the higher value.

Rapid Hydrolysis of GTP Bound to ARF after Nucleotide Exchange

We have previously reported that ARF is activated for membrane binding upon Golgi catalyzed GTP-GDP exchange (Helms and Rothman, 1992) but did not characterize the fate of the bound GTP. To examine this here, incubations were performed with nonradioactive myristoylated-ARF and GTP, labeled with ³²P in either the α or the γ positions. Radioactivity, bound to ARF was then determined by nitrocellulose filtration (Northup et al., 1982) and scintillation counting. Fig. 7 confirms that, as expected (Helms and Rothman, 1992), incubation of ARF or Golgi alone with GTP[α³²P] results in little binding of label to protein (Kahn and Gilman, 1986), but a dramatic increase in binding of [α³²P]GTP-derived radiolabeled nucleotide is observed when myristoylated ARF and Golgi membranes are incubated together (Fig. 7) in which condition catalyzed nucleotide exchange can occur. However, no label from GTP[γ³²P] was recovered. Evidently, the γ-phosphate is released from
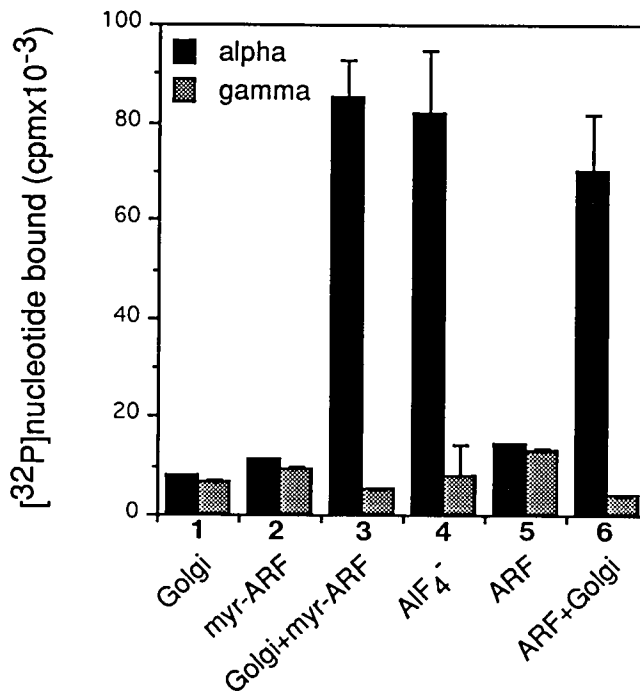

Figure 7. Rapid hydrolysis of GTP bound to ARF after nucleotide exchange. A nucleotide exchange assay was performed as described in Materials and Methods using GTP[α³²P] (■) or GTP[γ³²P] (▨), both 2.4 × 10⁴ cpm/pmol. After incubation for 15 min at 37°C, the reaction was stopped by cooling on ice and the bound radioactivity was determined by the nitrocellulose filter trapping assay (Northup et al., 1982). (Lane *1*) salt-washed rat liver Golgi membranes (1.7 μg); (lane *2*) myristoylated ARF (2 μg); (lane *3*) Golgi membranes and myristoylated ARF; (lane *4*) as lane *3* but in the presence of AlF₄⁻ (50 μM AlCl₃ and 30 mM KF); (lane *5*) non-myristoylated ARF (12 μg); and (lane *6*) Golgi membranes and nonmyristoylated ARF. Data are corrected for binding of labeled nucleotides to nitrocellulose in the absence of Golgi membranes or ARF (2,700 cpm). AlF₄⁻ increased the background signal by 17,200 cpm which is subtracted from the data that include AlF₄⁻. Data are shown as the means of duplicate experiments with the error bar representing the higher value.

GTP by hydrolysis after binding to ARF as mediated by Golgi membranes. Since liver membranes contain a relatively high amount of GTPases (90% hydrolysis of GTP after 15 min, not shown), it remained possible that GTP was hydrolyzed to GDP before membrane catalyzed nucleotide exchange. Therefore, the exchange and hydrolysis experiments were repeated with purified CHO Golgi membranes in the absence or presence of 1 mM pyrophosphate resulting in only 30 and 12% hydrolysis of GTP, respectively, after 15 min of incubation (not shown); with or without pyrophosphate, CHO Golgi membranes catalyzed binding of GTP[α³²P] (similar to rat liver Golgi membranes) but no binding of radioactivity was observed when GTP[γ³²P] was used. This indicates that the failure to incorporate radioactivity from GTP[γ³²P] into ARF was likely due to rapid hydrolysis after binding rather than to binding of GDP after hydrolysis of free GTP before binding to ARF. AlF₄⁻ had no detectable effect on the rate of incorporation of nucleotide as determined from GTP[α³²P] or on the extent of hydrolysis of the bound GTP, as determined from GTP[γ³²P]
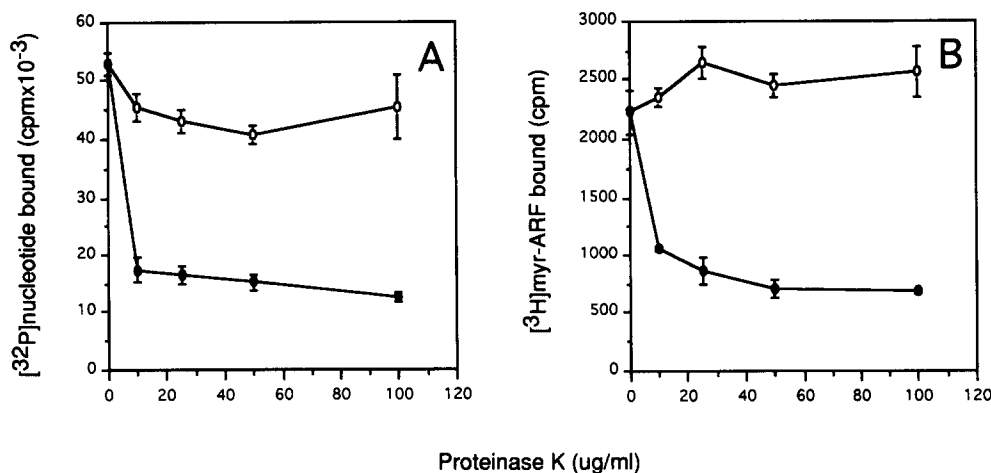
Figure 8. Protease treatment of Golgi membranes inhibits both ARF-binding and nucleotide exchange activity. Salt-washed rat-liver Golgi membranes (1.7 μg/incubation) were incubated for 5 min at 20°C with increasing concentrations of Proteinase K (●) as indicated and subsequently for 5 min at 20°C with PMSF (2 mM). For the mock-digestion (O), membranes were incubated for 10 min at 20°C in the presence of both the indicated concentration of Proteinase K and PMSF (2 mM). After pretreatment, the membranes were used in a standard nucleotide exchange assay using GTP[α^{32}P] (1.3 × 10⁴ cpm/pmol) (*A*) or standard binding assay (*B*). Data for the binding assay are corrected for precipitation of [³H]myr-ARF in the absence of membranes (338 cpm) and are shown as the means of duplicate experiments with the error bars indicating the individual values.

(Fig. 7). Similar results were obtained with CHO Golgi membranes (not shown). Myristoylation of ARF was not required for the activation of ARF by the nucleotide-exchange enzyme (Fig. 7) although it appears that the stimulation is less effective. Whether this is due to the absence of myristate or due to differences in activities between different ARF preparations is not clear. Both the membrane-dependent binding of [α^{32}P]GTP (Fig. 8 *a*) and the binding of myr-ARF to Golgi membranes were eliminated by pretreatment with proteinase K (Fig. 8 *b*) in a similar fashion. These results support the idea that the protein involved in the binding of ARF to Golgi membranes is the guanine nucleotide-exchange enzyme.

Discussion

We have used [³H]myristate-labeled ARF to characterize the binding of ARF to Golgi membranes. Addition of [³H]-myristic acid to the medium during overexpression of ARF resulted in radioactive labeling of a 20-kD protein. The radiolabeled protein was shown to be ARF protein as the radiolabeled 20 kD was not observed without overexpression of ARF. Also, antibodies directed against recombinant ARF1 protein immunoprecipitated the radiolabel. N-myristoyl transferase catalyzes the cotranslational attachment of myristate via an amide bond to the NH₂-terminal Gly²-residue of ARF (Towler et al., 1988). The irreversible nature of N-myristoylation (James and Olsen, 1989; one exception has been described for *D. discoideum* by daSilva and Klein, 1990) allows quantitation of myristoylated-ARF binding to Golgi membranes by determining [³H]myristate radiolabel, associated with membranes.

[³H]myristate-labeled ARF binds and accumulates on Golgi membranes in the presence of the stable GTP-analogue GTPγS (Fig. 2). The accumulation is mediated by a Golgi membrane-bound protein as protease treatment of membranes (Fig. 8) and incubation at 0°C (Fig. 6) prevented ARF binding. Thus enhancement of spontaneous nucleotide exchange by phospholipids, resulting in binding of ARF to membranes (Kahn, 1991) is ruled out under our assay condi-

tions including high Mg²⁺ concentrations (2.5 mM) which inhibits any spontaneous nucleotide exchange (data not shown). The accumulation of ARF in the presence of GTPγS can in principle be explained by either a nucleotide-exchange mechanism (Bourne et al., 1991) or by nucleoside diphosphate kinase (NDK) mediated activation of ARF (Randazzo et al., 1991). NDK uses GTP, ATP, as well as GTPγS and ATPγS as substrates (Wieland and Jacobs, 1989). Our data rule out the involvement of NDK in ARF activation as stable ATP analogues (ATPγS and AMPPNP) cannot induce ARF binding to membranes. Indeed, the only experimental evidence for this mechanism was recently retracted by its authors (Randazzo et al., 1992). In contrast, GMPPNP, which is not an NDK substrate, does enhance ARF binding, suggesting that a guanine nucleotide-specific exchange mechanism is involved. Most likely the exchange mechanism occurs in ARF rather than the possibility that ARF stimulates a nucleotide exchange in some other protein. This possibility seems unlikely as myristoylated ARF.GTP, but not ARF.GDP stably associates with liposomes. Once activated by Golgi membranes, ARF can bind PC liposomes (Fig. 4) providing additional proof for direct activation of ARF. Furthermore, we have recently described a membrane-bound nucleotide-exchange protein (Helms and Rothman, 1992) that catalyzes the exchange of one molecule of GDP, bound to ARF, for one molecule of GTP.

It is generally believed that guanine nucleotide-exchange enzymes stimulate the release of guanine nucleotides, bound to GTP-binding proteins, resulting in a temporarily empty pocket. The subsequent entry of a guanine nucleotide is determined by the availability of guanine nucleotides in the medium (Bourne et al., 1990, 1991). It is interesting to note that in the absence of any nucleotide, the binding of ARF to Golgi membranes is slightly elevated relative to incubations in the presence of GDP/GTP (Fig. 2). This suggests that in the absence of exogenous nucleotides, the entry of a nucleotide in ARF is the rate-limiting step, resulting in an increase of apo-ARF, associated with the exchange enzyme. In summary we conclude that binding of ARF to Golgi membranes is mediated by a guanine nucleotide-exchange protein and

that no other (cytosolic) proteins are required for this process.

Guanine nucleotide-exchange proteins for Ras-proteins have been genetically identified in yeast (Broek et al., 1987; Danak et al., 1991; Hughes et al., 1990). Recently a mammalian Ras nucleotide-exchange protein has been identified (Shou et al., 1992) based on partial sequence homology with yeast exchange enzymes. Ras-exchange activities have been described in various tissue-extracts but only partial purification has been accomplished (Downward et al., 1990). Evidence for a membrane associated, Brefeldin A–sensitive, ARF nucleotide-exchange enzyme has recently been obtained (Helms and Rothman, 1992) using GTP[γ^{35}S] as a substrate. Here we have explored the fate of GTP after nucleotide exchange. As incorporation of radiolabel from GTP[γ^{32}P] was not observed under conditions in which incorporation from GTP[α^{32}P] was observed, it is most likely that almost immediately upon incorporation of GTP into ARF protein, the nucleotide is hydrolyzed. The possibility that GTP was hydrolyzed before entry into ARF protein was made less likely by use of CHO Golgi membranes, raising the possibility of a membrane associated GTPase activating protein (GAP)-activity. The ARF nucleotide-exchange enzyme can use GTP, as well as GDP as a substrate (Helms and Rothman, 1992). This is in agreement with a mechanism in which the nucleotide-exchange enzyme binds to and stabilizes (nucleotide-free) apo-ARF. In vivo, the high intracellular concentration of GTP (GTP >> GDP) will drive the binding of GTP to ARF. In vitro, one can replace GTP for GDP resulting in 'nucleotide exchange' of GDP for GDP. Indeed, Weiss et al. (1989) report that apo-ARF has very similar affinities for GDP and GTP. Under the most stringent conditions in our experiments (catalysis of nucleotide exchange by CHO membranes in the presence of pyrophosphate) ~90% of the free nucleotide is GTP and 10% is GDP (after 15 min of incubation). Thus, although we cannot formally exclude the possibility of very specific GDP binding, there is no basis for this. Rather, it seems likely that in our assay conditions ARF binds at least nine molecules of GDP per molecule of GDP so that greater amounts of GDP must be produced by hydrolysis of bound GTP. These data could provide an explanation for the observation that GTPγS, but not GTP, induces accumulation of [^{3}H]myr-ARF on Golgi membranes (Fig. 2). In the presence of GTP, [^{3}H]myr-ARF interacts dynamically with Golgi membranes resulting in the binding of GTP to ARF. Rapid hydrolysis of the ARF bound GTP, however, produces ARF.GDP that dissociates from the membranes, preventing the ARF from accumulating.

Myristoylation of ARF is not required for interaction with the nucleotide-exchange enzyme (Fig. 7). In agreement with this, we have observed that incubation of Golgi membranes with myristoylated ARF and GTP[γ^{35}S] (5 μM) results in a distribution of protein-associated radioactivity over Golgi membranes (18% of total) and in the supernatant (82% of total) (data not shown). This distribution closely resembles the proportion of ARF that is myristoylated in the ARF preparation (13%) indicating that myristoylated ARF binds membranes. In contrast, nonmyristoylated ARF is activated by the exchange enzyme but fails to associate with the lipid bilayer.

In a typical binding reaction (1,500–2,500 cpm accumula-

tion, 150 pmol of ARF added, 20 min incubation) it can be calculated (specific activity 2,500 cpm/pmol myristoylated ARF) that 0.030–0.050 pmol of myristoylated ARF accumulates per minute of incubation. In a standard nucleotide-exchange reaction (75,000–150,000 cpm binding of nucleotide to ARF, 150 pmol of ARF added, 15 min incubation, 2.4 × 10^4 cpm/pmol) it can be calculated that 0.2–0.4 pmol of ARF exchanges nucleotides per min of incubation. Assuming that myristoylated and nonmyristoylated ARF have similar affinity constants for the nucleotide-exchange enzyme and that the myristoylated ARF preparation is 13% myristoylated (similar to that of [^{3}H]myr-ARF), 0.026–0.052 pmol of myr-ARF binds a nucleotide. These data are consistent with the model that for every nucleotide that enters a myristoylated ARF protein, one myristoylated ARF protein binds the membrane.

PC liposome extraction of Golgi membranes results in reduction of 40–60% (variation between experiments) of membrane-bound [^{3}H]myr-ARF. Rather than some non-specific (detergent) effect of liposomes on the integrity of the Golgi membranes, the extraction is specific by several criteria. Addition of increasing concentrations of PC liposomes results in saturation of extraction (maximally 60%, Fig. 3) whereas a detergent-effect would result in 100% "release" of ARF from membranes. Rather than the fact that PC liposomes disrupt the integrity of Golgi membranes resulting in solubilization of [^{3}H]myr-ARF, it has been shown that [^{3}H]-myr-ARF actually binds the PC liposomes (Fig. 4). Rat-liver Golgi membranes contain equal amounts of lipids and proteins (based on weight, Zambrano et al., 1975). Thus it can be calculated from Fig. 3 that 50% of the liposome-sensitive pool (i.e., a 30% reduction of ARF binding) is extracted when total amount of Golgi lipids equals the amount of lipids in PC liposomes. Liposome-extraction is complete at a 10–20-fold excess of PC liposome lipids over Golgi lipids. These data strongly suggest that an equilibrium is established between binding of activated ARF.GTPγS to the two types of membranes.

In the presence of GTPγS, total ARF binding to Golgi membranes is not saturable within the range of ARF concentrations tested (Fig. 5). This suggests that ARF is not stoichiometrically bound to a membrane protein but rather catalytically inserted into the lipid bilayer as proposed by Serafini et al. (1991), providing a virtually unlimited capacity for ARF binding. Although the rate of catalytic action of the nucleotide-exchange enzyme should be saturable at some ARF concentration, this could occur at much higher concentrations than saturation of stoichiometric binding to a receptor. Similar observations have been made with rab4, showing that an 80-fold overexpression of rab4 over the endogenous protein still resulted in an exclusive membrane localization (van der Sluijs et al., 1992).

PC liposome extraction of Golgi membranes reveals the presence of a saturable ARF-binding site (tightly bound pool) by removing the nonsaturable loosely bound pool. In the present study, the loosely bound pool accumulates on the membrane as hydrolysis of ARF bound GTPγS is blocked. In vivo, the size of the loosely bound pool might be determined by the rate of input of GTP into ARF by the nucleotide-exchange enzyme versus the hydrolysis of GTP, bound to ARF, by a membrane-bound GAP-activity, and most likely by interaction of ARF.GTP with other compo-

PC VESICLES

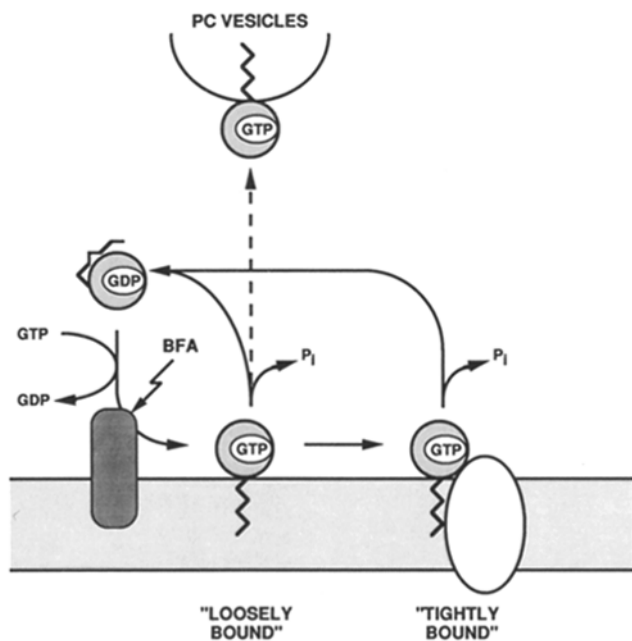"LOOSELY BOUND"   "TIGHTLY BOUND"

Figure 9. Model for the mechanism of ARF binding to Golgi membranes. Open oval represents the putative membrane receptor. The heavily shaded object inserted in the membrane represents the BFA-sensitive nucleotide-exchange enzyme. ARF is represented by shaded circles with either GDP or GTP bound to it.

nents (like coatomer). Since we have shown that hydrolysis of ARF bound GTP is a very efficient step, the loosely bound pool might even be small, relative to the tightly bound pool. From Fig. 5 it can be calculated that saturable ARF binding to the tightly bound pool occurs at ≈0.5 pmol myr-ARF/1.7 μg membrane protein. Assuming a 1:1 interaction of ARF with the receptor and a molecular mass of 30–60 kD, the abundance of such a component would be 1–2% of salt-washed rat liver Golgi membrane protein by mass.

Fig. 9 describes a model for the mechanism of ARF binding to Golgi membranes. The model is based on Serafini et al. (1991) and has been expanded in this study. ARF binding is mediated by a Brefeldin A-sensitive nucleotide-exchange enzyme (Helms and Rothman, 1992). Activation of ARF results in catalytic insertion in the lipid bilayer of Golgi membranes. Part of this loosely bound pool associates with a membrane receptor since we have shown here that both ARF pools do not arise independently (Fig. 6). Myristoylation of ARF is not required for interaction with the nucleotide-exchange protein (Fig. 7). Similarly, peptides derived from the NH$_2$-terminal sequence of ARF proteins do not need to be myristoylated in order to inhibit inter-cisternal Golgi protein transport (Kahn et al., 1992) or protein transport from the ER to the *cis*-Golgi cisternae (Balch et al., 1992), or transport between endosomes (Lenhard et al., 1992). However, myristoylation is required for binding of coatomer to Golgi membranes (Palmer et al., 1993). These data imply that although myristoylation of ARF is not required for interaction with the nucleotide-exchange enzyme, stable association of ARF with Golgi membranes by its myristoyl moiety is a necessary intermediate before interaction with the receptor (otherwise, activated, but nonmyristoylated ARF could interact with the membrane receptor as

well, resulting in stimulation of coatomer binding to membranes).

Coatomer induces alteration of the distribution of ARF, bound to Golgi membranes (Orci et al., 1993) resulting in movement of ARF from a (large) cisternal pool into a coat protein–coated region. Similar to this study, this implies the presence of two pools of ARF on the membrane. It will be important to study the interaction of ARF with coatomer and the membrane-receptor to elucidate its role in the formation of this complex.

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