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18. Chitinase activity was measured in a radiochemical assay by use of tritiated chitin as the substrate (*8, 10*). For each tissue sample, triplicate assays were performed over a range of different protein concentrations.
19. *Rhizoctonia solani* (AG-4, isolated from peanut), was inoculated into a medium consisting of 800 g of quartz sand, 25 g (dry weight) of Cream of Wheat, 30 g of corn meal, and 75 ml of water, incubated in the light at 25°C for 2 weeks and dried at room temperature for 3 days. Inoculum was stored at 4°C and used for up to a year. Because pathogenicity varied with preparation and age of inoculum, the amount of fungal inoculum required to yield about 50% seedling mortality on control or wild-type plants was empirically determined. Tobacco seedlings (18-day-old) or canola seedlings (7-day-old) were transplanted into inoculated potting medium (metromix 300 or quartz sand); plants sowed in sand were watered with Hoagland's solution [D. R. Hoagland and D. I. Aron, *Calif. Agric. Exp. Circ.* **347** (1950)]. Tobacco plants were grown in environmental chambers at 24°C for 12 hours under mixed cool, white fluorescent and incandescent lights and at 20°C in the dark for 12 hours, with approximately 80% relative humidity. Canola plants were maintained at 20°C under 16-hour days/8-hour nights and 50% relative humidity.
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Binding of ARF and β-COP to Golgi Membranes: Possible Regulation by a Trimeric G Protein

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The binding of cytosolic coat proteins to organelles may regulate membrane structure and traffic. Evidence is presented that a small guanosine triphosphate (GTP)–binding protein, the adenosine diphosphate ribosylation factor (ARF), reversibly associates with the Golgi apparatus in an energy, GTP, and fungal metabolite brefeldin A (BFA)–sensitive manner similar to, but distinguishable from, the 110-kilodalton cytosolic coat protein β-COP. Addition of βγ subunits of G proteins inhibited the association of both ARF and β-COP with Golgi membranes that occurred upon incubation with guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S). Thus, heterotrimeric G proteins may function to regulate the assembly of coat proteins onto the Golgi membrane.

CYTOSOLIC PROTEINS THAT BIND REversibly to membranes of the Golgi complex have been identified (*1, 2*) and shown to exist as a high molecular weight complex in the cytosol referred to as the coatomer (*3*). Cycling between the cytosol and Golgi membrane of at least one of these proteins, the 110-kD coat protein β-COP (*4*), is affected by nonhydrolyzable analogs of GTP such as GTP-γ-S and by BFA (*5–7*). BFA inhibits the association of cytosolic β-COP with Golgi membranes. Aluminum fluoride and GTP-γ-S both promote association of β-COP with Golgi membranes, suggesting that one or more GTP-binding proteins participate in initiating the association of β-COP with the membrane (*6–8*).

Two general classes of regulatory GTP-binding proteins have been defined: the signal transducing trimeric G proteins, believed to exert their effects at the plasma membrane, and the family of small GTP-binding proteins (the RAS superfamily). Several of the small GTP-binding proteins have been implicated in the control of intracellular membrane traffic (*9, 10*). One of these GTP-binding proteins, the adenosine diphosphate ribosylation factor (ARF), is associated with Golgi membrane and is also present in the cytosol (*9*).

We used immunofluorescence microscopy to study the effects of various agents that influence energy status, disrupt the Golgi complex, or alter the activity of G proteins or small GTP-binding proteins on the cellular localization of ARF and β-COP in normal rat kidney (NRK) cells. Immunolabeling of ARF and β-COP in untreated cells revealed a predominant Golgi-like staining pattern, which was juxtanuclear and half-moon shaped (Fig. 1). A low amount of ARF staining was also observed throughout the cytosol. Double labeling with antibodies to mannosidase II confirmed the Golgi distribution of both ARF and β-COP (*11*). Treatment of cells with 50 mM 2-deoxyglucose and 0.05% Na azide (DOGAz) for 10 min at 37°C to

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deplete cellular energy levels, resulted in the reversible redistribution of both ARF and β-COP to a scattered cytoplasmic pattern (Fig. 1, C and D); Golgi membrane markers were unaffected by this treatment (*5, 6*). Addition of BFA, which causes release of β-COP from Golgi membranes (*5*) and redistribution of Golgi membranes into the endoplasmic reticulum (ER) (*12*), reduced the intensity of staining of β-COP and ARF in the Golgi and increased staining of those proteins throughout the cytoplasm (Fig. 1, E and F). These effects occurred within 5 min after BFA treatment, were evident before the Golgi membranes were redistributed into the ER, and were not observed in cells treated with inactive analogs of BFA. If BFA was removed, ARF and β-COP reassociated with Golgi membranes.

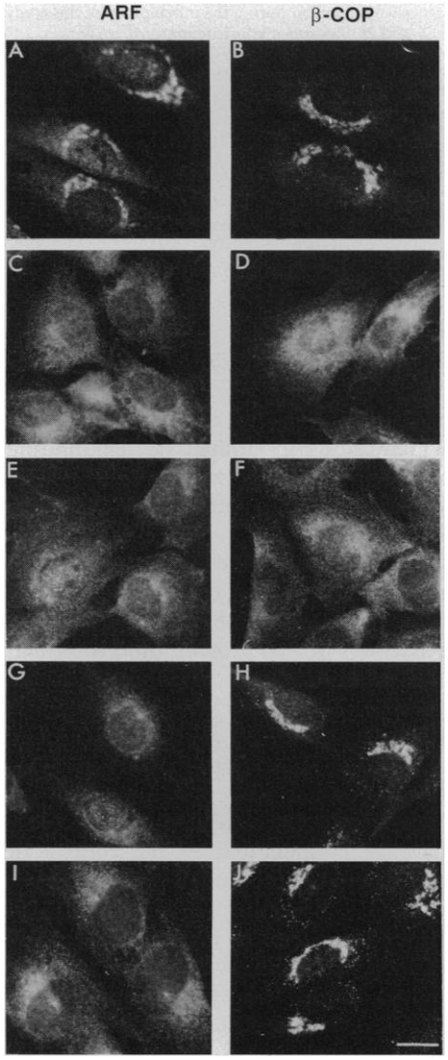

Fig. 1. Differential effects of AlF_n on the association of ARF and β-COP with the Golgi apparatus in cells depleted of ATP or treated with BFA. Distribution of ARF (**A, C, E, G**, and **I**) and β-COP (**B, D, F, H**, and **J**) was assessed by immunofluorescence in untreated NRK cells (A and B) or cells treated with DOGAz for 10 min at 37°C (C and D), BFA (2 μg/ml, 7 μM) for 5 min at 37°C (E and F), AlF_n for 10 min (50 μM $AlCl_3$ and 30 mM NaF) before addition of DOGAz for 10 min (G and H), or AlF_n for 10 min before addition of BFA for 10 min (I and J). NRK cells were incubated in RPMI 1640 with fetal calf serum (10%) with the indicated additions and then fixed and labeled by indirect immunofluorescence with either R5, a rabbit polyclonal antibody to ARF (*9*) or M3A5, a mouse monoclonal antibody to the 110-kD protein (β-COP) as described (*4, 5*). Bar, 10 μm.

Guanine nucleotides and aluminum fluoride (AlF_n) [50 μM $AlCl_3$ and 30 mM NaF; the active species is believed to have 3 to 5 F atoms (*13*)], both activators of regulatory G proteins, have been shown to promote the association of β-COP with the Golgi apparatus and inhibit the redistribution of β-COP into the cytosol observed with energy depletion or BFA (*6, 7*). We tested whether the association of ARF with Golgi membranes would be similarly affected. Although treatment of cells with AlF_n for 10 min before the addition of DOGAz (Fig. 1, G and H) or BFA (Fig. 1, I and J) prevented redistribution of β-COP from Golgi membrane, redistribution of ARF was not affected. Thus, although both ARF and β-COP behave as Golgi-associated proteins whose distribution can be reversibly altered by either energy depletion or treatment of cells with BFA, they can be distinguished by their sensitivity to AlF_n. One possible explanation for this distinction is that the association of β-COP with Golgi membrane is regulated by a trimeric G protein. Although trimeric G proteins (including G_s, G_q, and G_t) are activated by AlF_n (*14*), no small GTP-binding protein, including ARF, Ras, Rab 1, Rab 3, and Rap, is known to be activated by fluoride (*15*).

To further characterize the interaction of ARF and β-COP with Golgi membranes, we measured binding of ARF and β-COP to Golgi membranes in vitro. Isolated Golgi membranes were incubated with cytosol and an adenosine triphosphate (ATP) regenerating system and centrifuged. The sedimented membranes and associated proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting with antibodies to β-COP or ARF. In the absence of membranes, a small amount of β-COP was detected in the sedimented material (*16*). When cytosol was incubated with Golgi membranes in the presence of ATP for 10 min at 37°C, a greater amount of β-COP was associated with the membranes (Fig. 2A). If BFA was present during the incubation, however, β-COP did not associate with the membranes (*7*). An inactive derivative of BFA, B21 (*17*), had no effect on the association of β-COP with membranes, and no binding of β-COP to membranes occurred in

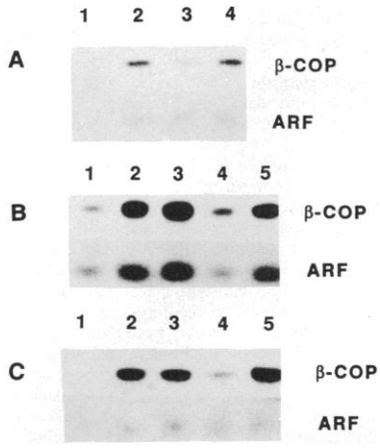

Fig. 2. Effect of BFA, GTP-γ-S, and AlF_n on binding of β-COP and ARF to isolated Golgi membranes. Immunoblots were probed with antibodies to β-COP and ARF. (**A**) Membranes and associated proteins were collected by centrifugation after incubation for 10 min at 37°C of cytosol alone (lane 1), membranes and cytosol (lane 2), or membranes and cytosol in the presence of 200 μM BFA (lane 3) or 200 μM B21 (lane 4). (**B**) Material sedimented after two sequential incubations of 10 min at 37°C. Cytosol was incubated alone and then in the presence of GTP-γ-S (25 μM) (lane 1); a mixture of membranes and cytosol was incubated alone and then with GTP-γ-S (lane 2); membranes and cytosol were incubated with GTP-γ-S and then BFA (200 μM) was added (lane 3); membranes and cytosol were incubated with BFA and then with GTP-γ-S (lane 4); membranes and cytosol were incubated with B21 (200 μM) and then with GTP-γ-S (lane 5). (**C**) Immunoblots of material sedimented after sequential treatments as described in (B) except that AlF_n was added in place of GTP-γ-S. Golgi membranes from Chinese hamster ovary cells (*21*) and bovine brain cytosol (*1*) were incubated under conditions previously defined (*8*) in a final volume of 0.4 ml containing 25 mM Hepes-KOH (pH 7.0), 125 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 M sucrose, 1.0 mM ATP, 5 mM creatine phosphate, creatine kinase (10 units per milliliter), 20 to 24 μg of Golgi membrane protein, and a saturating concentration of cytosol protein (1.2 mg). For incubations with AlF_n, the concentration of KCl was reduced to 25 mM to reveal the optimal effect of fluoride. The membranes were collected by centrifugation at 14,000*g* (Beckman TLA100.2) for 10 min at 4°C, and the sedimented materials resuspended in SDS sample buffer (*22*). Half of the sample was run on SDS-PAGE (8% gel) (*22*), transferred to nitrocellulose, blotted with monoclonal antibody to β-COP (M3A5), and incubated with rabbit antibody to mouse immunoglobulin G (IgG) and then ^{125}I-labeled protein A. The other half of the sample was subjected to SDS-PAGE (13% gel), transferred to nitrocellulose, and incubated sequentially with 1D9, a monoclonal antibody to ARF (*23*), goat antibody to mouse IgG, rabbit antibody to goat IgG, and ^{125}I-labeled protein A. Immunoblots were exposed to Kodak XAR film for 6 to 24 hours at −80°C with an intensifying screen.

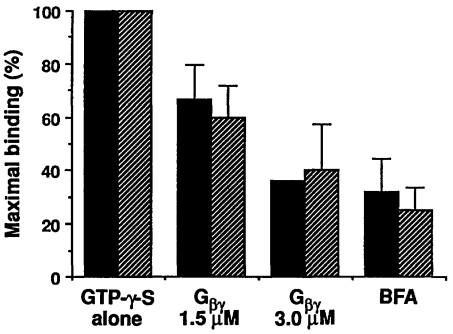

Fig. 3. Inhibition of GTP-γ-S–enhanced binding of β-COP and ARF to Golgi membranes by $G_{βγ}$ subunits. Membranes and cytosol were incubated at 37°C for 5 min with 0, 1.5, or 3.0 μM $G_{βγ}$, or with 200 μM BFA, and then for five more minutes after addition of 25 μM GTP-γ-S. The amount of ARF (solid bars) and β-COP (hatched bars) present on immunoblots of sedimented material was quantitated. The mean and standard error for three separate experiments are shown for all conditions except for ARF bound in the presence of 3.0 μM $G_{βγ}$. Membranes and cytosol were incubated as described in Fig. 2. $G_{βγ}$ was purified from bovine brain as described (*24*), and added from a stock of $G_{βγ}$ (13 mg/ml) containing cholate (1%). After the incubation, the membranes were centrifuged, subjected to SDS-PAGE, and blotted as described in Fig. 2. The bands were cut out of the blots, radioactivity was counted, and the amount of ARF and β-COP bound was expressed as a percentage of maximal amount bound in the presence of GTP-γ-S alone. The resulting amount of cholate had no effect on the amount of GTP-γ-S–enhanced binding of β-COP to membranes but reduced (by 20%) ARF binding to membranes. The data shown for ARF were corrected for this effect of cholate.

the absence of added ATP or if hexokinase and glucose were added to the incubation. A small amount of ARF, relative to the amount of β-COP, associated with membranes after incubation of membranes and cytosol.

Stable binding of both ARF and β-COP to Golgi membranes was increased markedly if GTP-γ-S (25 μM) was included during incubation (Fig. 2B). Four to five times more β-COP was bound to membranes in the presence of GTP-γ-S than in its absence. As observed previously for β-COP (*6, 7*), GTP-γ-S and BFA had opposing effects on the binding of ARF to membranes, and, when both agents were added sequentially, the effect of the agent added first was predominant. Treatment with GTP-γ-S for 10 min before the addition of BFA resulted in enhanced membrane association of β-COP and ARF comparable to that observed with GTP-γ-S alone. If BFA was added 10 min before addition of GTP-γ-S, the amount of β-COP and ARF bound was only 25 to 30% of that bound during incubation with GTP-γ-S alone. The inactive derivative of BFA, B21, did not inhibit binding under these conditions. Under maximal binding conditions, with GTP-γ-S alone, about 10% of the added ARF and β-COP were bound.

Addition of AlF_n to a mixture of cytosol and Golgi membranes caused association of β-COP but not ARF with membranes (Fig. 2C). The amount of β-COP bound to membranes in the presence of AlF_n was about 75% of that observed in the presence of GTP-γ-S. As observed with GTP-γ-S, AlF_n enhanced β-COP's membrane association and protected it from the effects of BFA if added first but could not reverse the effects if added subsequent to BFA. The active species is likely an aluminum fluoride complex since the full effect of fluoride treatment was only observed when $AlCl_3$ was added to the incubation.

The fact that AlF_n and GTP-γ-S induce stable β-COP association suggests that a trimeric G protein might regulate the association of β-COP with Golgi membranes. A specific G protein ($G_{iα3}$) is associated with the Golgi apparatus (*18*), although its function there is unknown. Activation (by AlF_n or GTP-γ-S) of the $G_α$ subunits of trimeric G proteins can be specifically reversed by the addition of excess $G_{βγ}$ heterodimers (*19, 20*). Addition of purified $G_{βγ}$ from bovine brain to membranes and cytosol resulted in the inhibition of the AlF_n-induced association of β-COP with the membranes. Furthermore, incubation of membranes and cytosol with 3.0 μM $G_{βγ}$ for 5 min before the addition of GTP-γ-S antagonized the ability of GTP-γ-S to enhance the binding of both ARF and β-COP to Golgi membranes (Fig. 3), suggesting that a G protein acts upstream to regulate the binding of both proteins to Golgi membranes. The $G_{βγ}$ subunits were nearly as effective as BFA in inhibiting the GTP-γ-S–induced binding of β-COP and ARF to membranes (Fig. 3). Although the concentration of exogenous $G_{βγ}$ subunits required to inhibit binding was high, it is comparable to those used previously (*19*), and the inhibition was abrogated by heat denaturation of the $G_{βγ}$ subunits.

These results suggest the involvement of a trimeric G protein in regulating the binding of both β-COP and ARF to Golgi membranes. Although activation of a putative trimeric G protein by GTP-γ-S or AlF_n is sufficient to promote binding of β-COP to membranes, stable binding of ARF may require the activation of both the trimeric G protein and ARF itself. Perhaps the combination of both types of GTP-binding proteins allows the cell to use the distinct characteristics of each for the regulation of membrane traffic. Identification of the trimeric G protein (or proteins) involved will be necessary to determine whether their function in membrane traffic is analogous to the role of plasma membrane G proteins in signal transduction.

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