Dominant Inhibitory Mutants of ARF1 Block Endoplasmic Reticulum to Golgi Transport and Trigger Disassembly of the Golgi Apparatus

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Using three different trans dominant mutants of bovine ARF1 affecting GDP exchange or GTP hydrolysis we demonstrate the central role of ARF1 in controlling vesicular traffic from the endoplasmic reticulum (ER) to the Golgi apparatus and between successive Golgi compartments. Overexpression of ARF1(Q71L), a mutant likely to be restricted to the GTP-bound form, resulted in the accumulation of vesicular stromatin virus glycoprotein in pre-Golgi intermediates, inhibited transport between successive Golgi compartments, and led to a striking association of β-COP with pre-Golgi intermediates and the Golgi stack. In contrast, ARF1(T31N), a mutant which is likely to have a preferential affinity for GDP compared to the wild-type protein, inhibited export from the ER and triggered a brefeldin A-like phenotype, resulting in the redistribution of β-COP from Golgi membranes to the cytosol and the collapse of the Golgi into the ER. This mutant, which may efficiently sequester an ARF-specific guanine nucleotide-exchange protein (ARF-GEF), suggests that ARF and ARF-GEF are essential for export from the ER. These results are discussed in the context of the GDP and GTP-bound forms of ARF in controlling both membrane structure and vesicular traffic through the early secretory pathway.

Multiple GTP-binding proteins belonging to the rab (reviewed in Zerial et al., 1993), ARF (Stearns et al., 1990a; Waters et al., 1991a; Kahn et al., 1992; Balch et al., 1992), and SAR1 (reviewed in Pryer et al., 1992) protein families control vesicular traffic through the exocytic pathway of eukaryotic cells. These small GTP-binding proteins are likely to function as molecular switches, controlling the assembly and disassembly of protein complexes involved in vesicle budding, targeting and/or fusion. The composition of these complexes or their specific roles in vesicular traffic remain to be elucidated.

The ARF gene family now includes at least 6 members of a closely related group with homologies ranging from 80 to 95% (Kahn et al., 1991; Tsuchiya et al., 1991). ARF was originally discovered as the cofactor required for the ADP-ribosylation of the heterotrimeric G-protein G1 (Kahn and Gilman, 1986; Kahn et al., 1988). More recent evidence suggests that ARF proteins play a critical role in vesicular traffic in yeast (Stearns et al., 1990b) and in mammalian cells (Balch et al., 1992; Kahn et al., 1992; Taylor et al., 1992). ARF is a component of nonclathrin-coated vesicles which accumulate in the presence of GTPγS (Waters et al., 1991b; Taylor et al., 1992) and appears to be essential in the recruitment of coatomer, a coat protein complex containing α-, β-, γ- and δ-COP proteins, to Golgi membranes (Donaldson et al., 1992a; Palmer et al., 1993; Orci et al., 1993). ARF not only participates in the recruitment of nonclathrin coat complexes, but has recently been demonstrated to facilitate the binding of β-adaptin, an adaptor protein associated with clathrin-coated vesicles (Robinson and Kreis, 1992; Stamnes and Rothman, 1993).

In addition to its role in vesicular traffic, ARF plays a key role in the maintenance of Golgi structure. Brefeldin A (BFA) triggers the reversible collapse of the cis/medial Golgi compartments to the ER (Donaldson et al., 1980; Lippincott-Schwartz et al., 1990; Graham et al., 1993; reviewed in Lippincott-Schwartz, 1993). While the target for BFA remains to be directly demonstrated, several lines of evidence strongly suggest that it inhibits the activity of an ARF-specific guanine nucleotide-exchange protein (ARF-GEF) (Donaldson et al., 1992b; Helms and Rothman, 1992), indicating that the ability to recruit and maintain a population of ARF on Golgi membranes through the activity of GEF is fundamental to Golgi structure/function (Helms et al., 1993).

While in vitro assays which reconstitute ER to Golgi (Balch et al., 1992) and intra-Golgi transport (Kahn et al., 1992; Helms et al., 1993; Palmer et al., 1993) strongly implicate a role for ARF in vesicular traffic, less attention has been given to its role in vivo. ARF proteins, like all members of the ras superfamily, contain highly conserved guanine nucleotide-binding domains involved in GDP exchange and hydrolysis of GTP (Bourne et al., 1990, 1991). Amino acid substitutions in each of these domains lead to inactive GDP or activated GTP-bound forms. These mutants frequently have a trans dominant negative phenotype and have proven to be exceptionally powerful probes for elucidation of GTP-binding protein structure and function. The most stringently characterized protein in this regard has been ras (reviewed in Barbacid, 1987; Lowy and Willumsen, 1993), although considerable progress has been made in understanding the function of the small GTP-binding proteins SEC4 and YPT1 in yeast (Schmitt et al., 1986, 1988; Walworth et al., 1989), and rab1 (Tisdale et al., 1992), rab4 (van der Sluijs et al., 1992), and rab5 (Bucci et al., 1992) in mammalian cells through analysis of mutant function in vivo and in vitro (reviewed in Zerial 1993 and Pryer et al., 1992).

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† The abbreviations used are: GDPγS, guanosine-5'-O-(3-thiotriphosphate); VSV-G, vesicular stromatin virus glycoprotein; ER, endoplasmic reticulum; Man₄α, the ER form of VSV-G containing oligosaccharides with 9 mannose residues; endo H, endoglycosidase H; GEF, guanine nucleotide-exchange protein; PCR, polymerase chain reaction; BFA, brefeldin A; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
In the present paper, we address the specific role of ARF1 in vesicular traffic between the ER and the Golgi, and control of Golgi structure in vivo. We utilize a transient expression system in conjunction with a series of trans dominant mutant forms of ARF1 to analyze their effects on protein transport. This approach has a distinct advantage over previous pharmacological approaches (which have utilized more general reagents such as GTPγS, AIFα, or BFA) to explore ER/Golgi structure/function. It allows us to identify the specific functional role(s) of the different GDP and GTP-bound states of ARF1 in the control of membrane function.

We provide evidence that a substitution which is widely used to convert ARF1 to a form with a preferential GDP (ARF1(T31N)), inhibits export from the ER, triggers the release of β-COP to the cytoplasm, and promotes the disassembly and collapse of the Golgi compartment into the ER. Using both morphological and biochemical criteria, we find that this mutation in all respects mirrors the effects of BFA. Given the likelihood that ARF1(T31N) sequesters the function of an ARF-specific GEF, these results provide the direct evidence that ARF-GEF is essential to ER to Golgi traffic, as well as Golgi membrane function and structure. In contrast, a substitution which is likely to constitutively lock ARF in the GDP-bound form (ARF1(Q71L)) does not inhibit export from the ER. Rather, this mutant leads to the accumulation of vesicular stomatitis virus glycoprotein (VSV-G) in pre-Golgi transport vesicles and carriers and promotes binding of β-COP to pre-Golgi intermediates and Golgi membranes. These results provide evidence for the essential role of ARF and β-COP in mediating the organization and function of the Golgi stack in vesicular traffic, and suggest that both ARF and β-COP are essential for export from the ER in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials—**Tran35S-label (specific activity > 1,000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA) and α-[32P]GTP (specific activity 3,000 Ci/mmol) from DuPont-New England Nuclear Research. Endoglycosidase H (endo H) was obtained by Boehringer Mannheim, fluorescein isothiocyanate-conjugated Lens culinaris lectin from EX Laboratories (San Mateo, CA), Texas Red goat α-mouse antibody from P. Hauri, Biocenter (Basel, Switzerland), monoclonal antibody against p53 from H. P. Hauri, Biosensor (Basel, Switzerland), monoclonal antibodies against the cytoplasmic tail of VSV-G (our laboratory). All other reagents were obtained from Sigma unless otherwise indicated.

The recombinant expression plasmid pW012 (Weiss et al., 1991) carrying the bovine ARF1 coding sequence under control of the T7 promoter was kindly provided by R. Kahn, National Cancer Institute (Bethesda, MD). The pAR-G (pETα) plasmids encoding VSV-G and ts045-VSV-G were obtained from J. Rose, Yale University (New Haven, CT) and C. Machamer, Johns Hopkins University (Baltimore, MD). The polyclonal rabbit α-VSV-G antibody was raised against a 15-amino acid COOH-terminal peptide of the cytoplasmic tail of VSV-G (our laboratory). All other antibodies used in this study were generous gifts from the following laboratories: the polyclonal antibody against β-COP from T. Kreis, University of Geneva (Geneva, Switzerland), the monoclonal antibody against p53 from H. P. Hauri, Biosensor (Basel, Switzerland), monoclonal antibodies recognizing the cytoplasmic tail (P5D4) or a luminal domain of VSV-G protein (8G5) from K. Howell, University of Denver (Denver, CO) and from B. Wattenberg, Upjohn (Kalamazoo, MI), respectively.

**Construction of ARF Mutants—**Point mutations were introduced into bovine ARF1 by PCR site-directed mutagenesis using Phu polymerase (Stratagene) under standard conditions and the pW012 plasmid as a template. The T31N, Q71L, N126I, and D129N substitutions were introduced using a two-step recombinant PCR technique. In the primary PCR reactions overlapping 3' and 5'-DNA fragments were generated. The 5'-oligonucleotide primer, 5'-GGA GAT CAT ATA ATG GGC AAC ATG TCT GC-3' and the anti-sense mutagenic oligonucleotides, 5'-GAT GGT GCT CTT CTC CCG AGC ATG TAG G3-3' (T31N, introduced point mutation is underlined), 5'-GCG CAT GAT CCT GGC CCC GGC CCG CAC GAG CCG CAG CTT GCC CAC CTA GAC GGC AAC ATG TCT GC-3' (N126I), and 5'-CATTAGG CTT CTC TCT GTA TGC AAA C-3' (D129N), served as primers for amplification of the 5'-fragments.

The 3'-fragments were generated using oligonucleotides complementary to the above mutagenic primers in combination with the 3'-end anti-sense oligonucleotide primer 5'-CCC CTC GGA TTC CTT GTT CCG G-3', which was in addition used to introduce a BamHI restriction site (underlined in the sequence) at the 3'-end of the ARF mutant genes. In the second fusogenic PCR reaction the appropriate pair of overlapping fragments were then combined with the 5'-end oligonucleotide primer and the 3'-anti-sense oligonucleotide primer to generate full-length mutant sequences. The G2A mutation was generated by PCR amplification using the mutagenic 5'-end oligonucleotide primer 5'-CCC CCT TTC AGA CAT ATG GCG AAT ATC TTC G-3' and the 3'-anti sense oligonucleotide primer described above. All final PCR products were subsequently treated with T4 DNA polymerase and after solution from an agarose gel subcloned into the EcoRI restriction site of pBluescript SK(+). Mutations were confirmed by sequencing of the entire coding sequence by the chain termination method (Sanger et al., 1977). All ARF mutant sequences were then introduced into the pET3a vector (Stratagene) as NdeI/BamHI fragments for expression from the T7 promoter as described previously (Studer et al., 1996; Koshravi-Far et al., 1991; Tisdale et al., 1992). The G2A/T31N and G2A/Q71L double mutations were generated using the pET3a constructs of ARF1(T31N) and ARF1(Q71L) as a template, respectively. The G2A mutations were introduced by PCR amplification using the mutagenic 5'-end oligonucleotide primer and the 3'-anti-sense oligonucleotide primer. Both single and double mutants were subsequently subcloned as described above.

Mutations were confirmed by sequencing of the entire coding sequence by the chain termination method (Sanger et al., 1977).

**Transfection Procedure—**HeLa cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies Inc.) supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and streptomycin in a 5% humidified CO2 incubator. Infection with recombinant vaccinia virus and transfection of HeLa cells were performed as described by Tisdale et al. (1992). Briefly, HeLa cells (3 × 10⁷) were plated in 60-mm cell culture dishes the day prior to transfection. Before infection the cells were washed twice with DMEM and then infected with vaccinia T7 RNA polymerase recombinant virus (vT7-3; Fuerst et al., 1986) at a multiplicity of 10 plaque-forming units/cell in 1 ml of DMEM for 30 min with intermittent rocking in a 37°C CO2 incubator. The infection media was removed and the cells co-transfected with 7 pg of recombinant PET3 plasmid DNA encoding ARF wild-type or a particular mutant construct, and 7 μg of an expression vector carrying VSV-G (pAR-G). Both plasmid DNAs had been premixed for 15 min at room temperature in 2 ml of DMEM and 35 μl of transfection reagent (TransfectACE) prepared as described (Rose et al., 1991). Two hours post-transfection the inoculum was removed and saved. The cells were detached from the culture dish with PBS containing 5 mm EDTA (PBS/EDTA), washed once with DMEM, and resuspended in the transfection media from the parental dish and 4 ml of DMEM, 4% fetal calf serum. One-third of the cells (5 × 10⁶) was then replated in a 35-mm tissue culture dish and incubated overnight for pulse-labeling experiments. Two-thirds of the cells (1 × 10⁶) were replated in a 60-mm dish and used for Western blot analysis to determine the expression level for each individual experiment. Cells were incubated for an additional 3 h to allow expression of VSV-G protein and ARF proteins.

To study the time course of inhibition two 10-cm dishes of HeLa cells (3 × 10⁹/10-cm dish) were infected with recombinant vaccinia virus at a multiplicity of 10 plaque-forming units/cell in 2 ml of DMEM and either transfected with 15 μg of pAR-G alone or co-transfected with pAR-G and the particular ARF expression plasmid, 15 μg each in 4 ml of DMEM as described above. Two h post-transfection the inoculum was removed and the cells attached to the culture dishes with PBS/EDTA. The cells were combined, washed with DMEM, and resuspended in the transfection media from the parental dishes and 4 ml of DMEM, 4% fetal calf serum. For each indicated time point 5 × 10⁶ cells were replated in 35-mm dishes for pulse-labeling experiments and 1 × 10⁶ cells to 60-mm dishes to determine the expression level by Western blot analysis. Cells were further incubated for the total times as indicated under "Results."

**Transport Analysis—**Transport analysis was performed as described earlier (Tisdale et al., 1992). Briefly, after post-transfection (see above) the cells were washed twice with PBS and lysed in lysis buffer (80 mm Tris-HCl, pH 8.0, 200 mm NaCl, 1 mm PMSF, 10 μg/ml aprotinin, 10 mm phenylmethylsulfonyl fluoride). VSV-G protein was subsequently immunoprecipitated using a monoclonal antibody to VSV-G (clone 8G5) and
The three GTP-binding domains highly conserved between members of the ras superfamily of small GTP binding proteins are indicated as G1, G2, and G3. The acceptor site of post-translational myristoylation is marked by myr. Amino acid residues within these domains subjected to mutagenesis are indicated by large bold letters highlighted by shaded boxes. The corresponding amino acid substitutions are shown in boxed letters for each single mutant protein.

**TABLE I**

**ARF1 mutant proteins generated for this study**

| bovine ARF1 | myr | G1  | G2  | G3  | Analogous ras mutation |
|-------------|-----|-----|-----|-----|------------------------|
|             |     |     |     |     |                         |
| net G        |     | 2   |     |     |                         |
| G27         |     |     | 31  |     |                         |
| G71         |     |     |     | 126 |                         |
| G129H       |     |     |     | 129 |                         |

**RESULTS**

**Generation and Characterization of Bovine ARF1 Mutants**—To address the potential role of ARF proteins in the regulation of vesicular transport between the endoplasmic reticulum and the Golgi apparatus, we performed site-directed mutagenesis within domains likely to be essential for ARF function. A series of mutants were generated in three guanine nucleotide binding motifs which are highly conserved between members of the ras superfamily (Table I) (Bourne et al., 1990, 1991). The generation of analogous mutations in other members of the GTP-binding protein family has previously documented the potency of this approach to study the diverse cellular functions of these proteins (Der et al., 1988; Schmitt et al., 1986, 1988; Walworth et al., 1989; Buccii et al., 1992; van der Sliujs et al., 1992; Tisdale et al., 1992). In particular, intensive mutational and structural analyses of H-ras has assigned residues in each of these domains (Table I: G1, G2, and G3) with defined functions in the ras superfamily (Table I: GI, G2, and G3) with defined functions in the ras superfamily.
The Q61L substitution in the G3 domain impairs H-ras GTP hydrolysis leading to a constitutively GTP-bound form (Der et al., 1986), whereas mutations in the G3 domain (N116I or D119N) alter the nucleotide exchange rate (Der et al., 1988) (Table I).

In addition, in contrast to most other GTP-binding proteins which are prenylated or palmitoylated at the carboxyl terminus (reviewed in Der and Cox (1991)), ARF proteins are myristoylated at their amino termini on a glycerine residue at position 2 (Table I). Myristoylation has been shown to be important for the reversible interaction of ARF with membranes (Kahn et al., 1992) and to be critical for its biological function in vitro (Balch et al., 1992; Palmer et al., 1993). The substitution of this residue by alanine should prevent membrane attachment and should yield a cytosolic, nonfunctional protein.

The analogous mutations in bovine ARF1 which were generated for the present study are summarized in Table I. To test the biochemical properties of the three GTP binding mutants of ARF1 we performed a qualitative GTP overlay technique using ARF bound to nitrocellulose filters (Wagner et al., 1992). As expected, bacterially expressed ARF1(T31N) and ARF1(N126I) mutant proteins showed only very weak binding of [α-32P]GTP when immobilized on nitrocellulose membranes (data not shown). No binding was detected in the presence of 1000-fold molar excess of GDP (Fig. 1). An identical result was observed for ARF1(D129N) (data not shown). In contrast, both wild-type and mutant ARF1(Q71L) recombinant proteins showed strong [α-32P]GTP binding in the absence (data not shown) or the presence of a large excess of unlabeled GDP (1000-fold) (Fig. 1), suggesting a high affinity for GTP over GDP. These results indicate that the predicted phenotypes based on guanine nucleotide binding of other small GTP-binding proteins is applicable for ARF1, one of the most distant relatives of the ras superfamily (Valencia et al., 1991).

Transient Expression of ARF1 Wild-type and Mutant Proteins in HeLa Cells—In order to examine the effects of the generated mutant forms of bovine ARF1 on vesicular transport from the ER to the Golgi apparatus in mammalian cells, we used a recombinant T7 vaccinia virus system (Fuerst et al., 1986) to transiently express the wild-type and mutant forms in HeLa cells. This approach has previously been applied to document the inhibitory effects of trans dominant negative mutant forms of rab1 and rab2 on the transport of VSVG from the ER to the Golgi stack (Tisdale et al., 1992). VSV-G has been used extensively as a marker protein to study the biochemical and molecular basis for transport through the exocytic pathway in vivo (Beckers et al., 1987; Plutner et al., 1992; Davidson and Balch, 1993). VSV-G is a type I transmembrane protein containing two N-linked carbohydrate chains. Vectorial transport of VSVG from the ER through sequential cis, medial, and trans Golgi compartments can be measured by the processing of the two oligosaccharide chains from the high mannose (Man9) endo H-sensitive form found in the ER and pre-Golgi intermediates (S in Fig. 2A) to endo H-resistant forms found in the Golgi stack. These processing intermediates can be readily distinguished by SDS-PAGE (Schwaninger et al., 1991; Plutner et al., 1992; Davidson and Balch, 1993). The first endo H-resistant (R) form corresponds to the transport of VSVG to the early cis Golgi compartment where one or both of the oligosaccharides chains becomes endo H resistant by the action of resident αl,2-mannosidases and glycosyltransferases (R in Fig. 2, A and B) (Schwaninger et al., 1991; Tisdale et al., 1992; Davidson and Balch, 1993). Subsequent transport of VSVG to the trans Golgi network results in the appearance of the fully processed form containing two complex, endo H-resistant oligosaccharides and glycosyltransferases (R in Fig. 2, A and B) (Schwaninger et al., 1991; Tisdale et al., 1992; Davidson and Balch, 1993). The appearance of sequential processing intermediates allows us to directly examine the effects of mutant constructs on both ER to Golgi and intra-Golgi transport within the same experiment (Tisdale et al., 1992; Davidson and Balch, 1993).

HeLa cells infected with the recombinant vaccinia virus (VT7-5) were co-transfected with an expression vector carrying either wild-type or mutant ARF1 genes and a vector encoding VSVG. Cells were incubated for 3–6 h to allow sufficient time for protein expression (Tisdale et al., 1992). Transfected cells were subsequently pulse-labeled with [35S]methionine for 10 min, followed by a chase in the presence of unlabeled methionine for 1.5 h. Using this protocol, between 30 and 60% of the total cells are transfected. The efficiency of co-transfection of VSVG with plasmids encoding ARF constructs is close to 100% (Tisdale et al., 1992). The expression of wild-type and mutant ARF1 proteins was monitored by Western blot analysis (Fig. 2C) using an affinity purified polyclonal antibody generated against bacterially expressed recombinant ARF1 wild-type protein. Overexpression in this system can be observed as early as 3–4 h post-transfection (Fig. 3; Tisdale et al., 1992). In general, expression levels of the wild-type and mutant proteins by 5–6 h of post-transfection varied between 2 and 15-fold over endogenous levels depending on the vector and the transfection efficiency within each particular experiment.

As shown in Fig. 2, A and B, expression of the ARF1 wild-type protein in vivo partially (12%) inhibited transport of VSVG to the Rf form when expressed at a level 2–5-fold that of the endogenous pool. A correlation between the extent of inhibition of transport and the levels of expression of wild-type ARF1 is shown in Fig. 3A. Expression could be detected between 3 and 4 h post-transfection with ARF1(wild-type) protein continuing to accumulate over the 6-h time course. Transport to the Rf form was inhibited by only 16% even when the expression level was 12-fold the endogenous pool (Fig. 3A). Although no variation in the steady state level of the cis/medial Golgi Rf intermediate form could be detected throughout the time course, reflecting its rapid transit through these compartments, at 6 h post-transfection ~36% of VSVG remained in the endo H-sensitive form (S in Fig. 3A) compared to the control in which generally 20–24% remained in the endo H-sensitive form after a 90-min chase. Curiously, we have reproducibly detected a slight stimulation of transport at early time points of transfection where it is difficult to detect overexpression (Fig. 3A, 3-h

2 C. Nuoffer, H. Davidson, and W. E. Balch, submitted for publication.

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Fig. 1. [α-32P]GTP binding of ARF1 wild-type and mutant proteins. Bacterial cells were transformed with ARF1 wild-type or the indicated ARF1 mutant expression constructs and induced with IPTG for expression of recombinant protein as described under "Experimental Procedures." Total cellular protein before (−IPTG) or after (+IPTG) induction was separated by SDSPAGE, transferred to nitrocellulose filters, and after renaturation allowed to bind [α-32P]GTP in the presence of unlabeled GDP (1000-fold excess). Bound [α-32P]GTP was visualized by autoradiography.
myristoylated at the amino terminus) had no effect on transport (Fig. 2, A and B). In this case, VSV-G protein was efficiently processed to the endo H-resistant R₁ and R₂ forms in a manner comparable to the control lacking any recombinant ARF1 plasmid (Fig. 2, A and B).

Mutant Forms of Bovine ARF1 Defective in GTP-binding Potently Inhibit ER to Golgi and Intra-Golgi Transport—In contrast to the rather weak inhibition observed by overexpression of the wild-type ARF1 protein, mutant forms defective in GTP binding or hydrolysis resulted in striking inhibition of VSV-G transport. In this experiment the levels of expression of the different mutants varied from 2 to 5-fold (Fig. 2B). Overexpression of the GDP-bound ARF1(T31N) mutant, the GTP-bound form ARF1(Q71N), and a mutant form with a high guanine nucleotide-exchange rate (ARF1(N126I)) efficiently (>99%) inhibited maturation of VSV-G to the terminally processed R₂ form (Fig. 2B). In all cases, a significant fraction of the total VSV-G remained in the endo H-sensitive, pre-Golgi S form (Fig. 2B). However, even at high levels of expression, partial processing of VSV-G to the R₁ form (20–30%; Figs. 2 and 3, C and D) was observed for both the ARF1(T31N) and ARF1(N126I) mutants. In contrast, the ARF1(Q71L) mutant protein efficiently prevented processing of VSV-G to the R₁ form (5–10%, Figs. 2, A and B, and 3B). The lack of substantial accumulation of VSV-G in the R₁ form in the presence of each of these mutants indicates that both ER to Golgi and intra-Golgi transport are substantially impaired, with transport through sequential Golgi compartments being particularly sensitive.

Analysis of the time course of inhibition indicates that low levels of each of the mutant proteins potently inhibit ER to Golgi and intra-Golgi transport (Fig. 3, B–D). Unlike the wild-type ARF1 protein for which only partial inhibition of transport to the R₁ form (16%) was observed even when the protein was present at a concentration 15-fold over the endogenous pool after 6 h of transfection (Fig. 3A), complete (>99%) inhibition of transport to the R₂ form was observed after a 2–5-fold overexpression by all three mutants after only 3 h of expression (Fig. 3, B–D). Inhibition of transport of VSV-G from the ER to the cis Golgi R₁ form by the ARF1(T31N) and ARF1(Q71L) mutants showed maximal effects by 3–4 h post-transfection when the expression levels were 5-fold or less than the endogenous pool (Fig. 3, B and C). Although the onset of the ARF1(N126I) mutant phenotype was also quite rapid (61% inhibition of transport by 3 h), the level of inhibition increased after 4–6 h of expression (to 75% inhibition) (Fig. 3D).

Only partial inhibition of VSV-G processing to the R₁ form was observed when VSV-G was co-transfected with the ARF1(D129N) mutant (Fig. 2B), consistent with the less potent phenotype of the equivalent mutation in H-ras (Der et al., 1988). In this case, ~18% of the total VSV-G could be detected in the mature endo H-resistant R₂ form compared to complete inhibition in the presence of the other mutants (Fig. 2, A and B).

To determine whether myristoylation was critical for the function of the various ARF1 mutants, double mutants were constructed. For this purpose, the myristoyl acceptor site was mutated to Ala in the T31N and Q71L mutants to form ARF1(G2A/T31N) and ARF1(G2A/Q71L). In contrast to the striking effects of the myristoylated forms (Figs. 2 and 3), no inhibition of transport could be detected at levels of expression 2–5-fold the endogenous pool (data not shown). These results demonstrate that the effects observed on VSV-G transport are a specific consequence of co-expression of VSV-G with the different trans dominant ARF mutants, and that myristoylation is critical for membrane association.

The data described above indicate that the GDP, GTP and
"activated" mutant forms of ARF1 either directly or indirectly perturb both ER to Golgi and intra-Golgi vesicular traffic. Given that the onset of inhibition of intra-Golgi transport (processing of VSV-G to the R1 form) compared to ER to Golgi transport (processing to the R2 form) was more rapid, complete, and required a lower level of expression, these results suggest that each of these mutants may have a compound effect on both Golgi function in vesicular traffic and possibly Golgi structure.

**Morphological Analysis of VSV-G Transport in the Presence of ARF1 Mutants**—To determine the effects of the different mutants on ER and Golgi structure and function, we examined the morphological effects of the ARF1 mutants on transport in *vivo*. As demonstrated above and as established previously (Tisdale et al., 1992),2 co-expression of VSV-G with different plasmids occurs with >90% efficiency, allowing us to directly analyze the effects of the different ARF1 mutant constructs on the transport of VSV-G and Golgi structure in individual cells using indirect immunofluorescence (Tisdale et al., 1992).2 For this purpose, HeLa cells were transfected with an expression vector carrying a temperature-sensitive form of VSV-G (ts045) which fails to exit the ER when cells are incubated at the restrictive temperature (39.5 °C). Under these conditions, ts045-VSV-G shows a reticular distribution characteristic of the ER (Fig. 4a). Shift of the cells to the permissive temperature (32 °C) 4 h after transfection at the restrictive temperature results in the synchronized release of ts045-VSV-G from the ER and transport to the compact, juxta-nuclear Golgi stack during an ensuing 90-min incubation (Fig. 4c, arrows). In this case, ts045-VSV-G overlaps with the distribution of *L. culinaris* lectin (Fig. 4, compare c (VSV-G) to d (L. culinaris lectin), a protein which binds with high-affinity to N-linked oligosaccharides containing terminal N-acetylglucosamine, serving as a strong marker for cis/medial Golgi compartments (Yamamoto et al., 1982). The lectin will also recognize, albeit with reduced affinity, N-linked oligosaccharides with terminal Man residues associated with pre-Golgi compartments (Osawa and Tsuji, 1987).

When cells were co-transfected with the GTP-bound mutant form ARF1(Q71L) for 4 h and then shifted to the permissive temperature for 90 min, ts045-VSV-G did not enter the Golgi stack (Fig. 4, compare e (VSV-G) to f (L. culinaris lectin)). Rather, in >90% of co-transfected cells ts045-VSV-G protein accumulated in punctate structures throughout the cytoplasm

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**Fig. 3. Time course of transport inhibition by overexpression of ARF1 wild-type and mutant proteins.** A, recombinant vaccinia virus-infected HeLa cells were either transfected with a plasmid encoding VSV-G protein (pAR-G) or co-transfected with both pAR-G and the ARF1(wt) expression construct (pOW12) as described under "Experimental Procedures." Cells were co-transfected for the indicated times prior to transfer to ice and processing for transport and Western blot analysis as described in the legend to Fig. 2 and under "Experimental Procedures." Control cells were incubated for 6 h. B-D, recombinant vaccinia virus infected cells were either transfected with a plasmid encoding VSV-G (pAR-G) alone (ctl) or co-transfected with pAR-G and the indicated ARF mutant expression construct. Control cells were incubated for 6 h and co-transfected cells for the indicated times prior to processing for subsequent analysis of VSV-G transport and level of ARF protein expression as described under "Experimental Procedures." B, time course of transport inhibition in the presence of ARF1(Q71L). C, time course of transport inhibition in the presence of ARF1(T31N). D, time course of transport inhibition in the presence of ARF1(N126I). Percent inhibition of transport (%R, + Rr) and expression levels were determined as described in the legend to Fig. 2.
FIG. 4. VSV-G protein is transported to pre-Golgi intermediates in cells overexpressing ARF1(Q71L). HeLa cells were infected with recombinant vaccinia virus and either transfected with a plasmid encoding the temperature-sensitive mutant form of VSV-G protein (pAR tsO45-G) alone (a-d) or co-transfected with both pAR tsO45-G and the ARF1(Q71L) expression construct (e-h) as described under “Experimental Procedures.” Cells were incubated for 4 h at the restrictive temperature (39.5 °C) and then shifted to the permissive temperature for 1.5 h to allow release of tsO45-VSV-G from the ER and transport to the Golgi apparatus. Cells were fixed, saponin permeabilized, and the distribution of tsO45-VSV-G (a, c, e, and g), Golgi membranes (L. culinaris lectin) (b, d, and f), and p53 (h) were viewed by double immunofluorescence microscopy as described under “Experimental Procedures.” a and b, distribution of tsO45-VSV-G (a) and Golgi membranes (b) after 5.5 h continuous incubation at the restrictive temperature (39.5 °C). c and d, distribution of tsO45-VSV-G (c) and Golgi membranes (d) after 1.5 h at the permissive temperature (32 °C). e, f, g, and h, overlapping distribution of tsO45-VSV-G (e and g), Golgi membranes (f), and p53 (h) in cells overexpressing the ARF1(Q71L) mutant. Arrowheads indicate the juxta-nuclear Golgi region. Small arrows show punctate structures in which tsO45-VSV-G and p53 colocalize (e, g, and h) or the distribution of the Golgi stack (b-d). Large bold arrows denote co-transfected cells.

(Fig. 4e, arrows), and in punctate structures within and around the compact, juxta-nuclear Golgi stack (Fig. 4e, arrowhead). To address the possibility that this distribution of tsO45-VSV-G found in punctate structures in the presence of ARF1(Q71L) overlapped with p53 (Schweizer et al., 1988, 1990). p53 preferentially resides in pre-Golgi transport intermediates composed of clusters of vesicles and tubular elements which are distributed throughout the cytoplasm and enriched in the juxta-nuclear Golgi region (Schweizer et al., 1990). As shown in Fig. 4 (compare g (VSV-G) to h (p53)), tsO45-VSV-G and p53 show a striking overlap both in terms of peripheral punctate staining as well as in punctate staining in the juxta-nuclear Golgi region. These results suggest that tsO45-VSV-G is efficiently transported to pre-Golgi intermediates, but fails to enter the cis Golgi compartment. They are also consistent with the ability of the ARF1(Q71L) mutant protein to inhibit the processing of VSV-G to the Golgi associated R1 (and R2) forms at early time points of transient expression (Figs. 2, A and B, 3B). Since the juxta-nuclear Golgi stack typically remained intact (Fig. 4f), overexpression of the GTP-bound form of ARF appears to have no effect on Golgi structure, at least as detectable using indirect immunofluorescence.

A different morphological phenotype was obtained by overexpression of the ARF1(T31N) and ARF1(N126I) mutants. In the case of the ARF1(T31N), tsO45-VSV-G failed to mature to the position normally observed for the juxta-nuclear Golgi stack, being retained in a more uniform distribution characteristic of the ER and perinuclear ER (Fig. 5, a (VSV-G) and b (L. culinaris lectin)). In this figure the large bold arrows indicate cells co-transfected with VSV-G and the mutant plasmid; the small arrows denote the position of the Golgi stack revealed by lectin staining. In some cells we also observed partial maturation of VSV-G to punctate intermediates which overlapped with p53 (Fig. 5, c (VSV-G) and d (p53), arrows). However, the extent of maturation of VSV-G to punctate, pre-Golgi intermediates was, qualitatively, considerably less than that observed in the
Overexpression of either ARF1(T31N) or ARF1(N126I) mutant protein results in retention of VSV-G protein in the ER and disassembly of the Golgi complex. Recombinant vaccinia virus infected HeLa cells were co-transfected with both a plasmid encoding tsO45-VSV-G (pAR-tsO45-G) and either the ARF1(T31N) (a-d) or ARF1(N126I) (e-f) mutant expression constructs. Cells were incubated and processed for double immunofluorescence microscopy as described in the legend to Fig. 4 and under "Experimental Procedures." a, b, e, and f, distribution of tsO45-VSV-G (a and e) and Golgi membranes (L. culinaris lectin) (b and f) in the presence of ARF1(T31N) mutant protein (a and b) and ARF1(N126I) mutant protein (e and f). Large bold arrows mark transfected cells, small arrows indicate the juxta-nuclear Golgi membranes. c and d, distribution of tsO45 (c) and p53 (d) in cells overexpressing ARF1(T31N) mutant protein. Small arrows indicate punctate structures in which tsO45-VSV-G partially colocalizes with p53 (a-d) or the distribution of the Golgi stack (e, f).

In the presence of ARF1(T31N), ~60% of the cells on the coverslip showed a distribution reflecting complete retention in the ER, ~30-40% showed partial (weak) maturation to dispersed pre-Golgi intermediates and only 10–15% of transfected cells expressing tsO45-VSV-G showed transport to the juxta-nuclear Golgi position. These results are likely to reflect the differential levels of expression of the ARF1 mutant forms in individual cells as a consequence of variation in the efficiencies of transfection, but emphasize the effects of this mutant on inhibition of export from the ER.

A similar distribution of tsO45-VSV-G to that of cells overexpressing ARF1(T31N) was observed in the presence of the ARF1(Q71L) mutant (Fig. 4, g and h). In the presence of ARF1(T31N), ~60% of the cells on the coverslip showed a distribution reflecting complete retention in the ER, ~30-40% showed partial (weak) maturation to dispersed pre-Golgi intermediates and only 10–15% of transfected cells expressing tsO45-VSV-G showed transport to the juxta-nuclear Golgi position. These results are likely to reflect the differential levels of expression of the ARF1 mutant forms in individual cells as a consequence of variation in the efficiencies of transfection, but emphasize the effects of this mutant on inhibition of export from the ER.

The morphological effects of the T31N and N126I mutants were very similar to those obtained when cells are treated with the drug BFA, which causes the inhibition of ER to Golgi transport, collapse of the Golgi into the ER, and release of the coatomer subunit β-COP from Golgi membranes (Donaldson et al., 1990, 1991; Lippincott-Schwartz, 1993). To address the possibility that these mutants were indeed mimicking the effects of BFA, we first examined the effects of BFA on the redistribution of lectin-positive Golgi membranes and β-COP in HeLa cells. In these experiments we utilized tsO45 VSV-G to identify cells co-expressing the respective mutant protein (Fig. 6, a, c, e, g, and i, large bold arrows). For clarity, cells were kept at the restrictive temperature throughout the duration of the experiment to retain VSV-G in the ER. Similar to results observed in other cell lines, BFA caused the compact, juxta-nuclear Golgi stack to disperse (data not shown) and the redistribution of β-COP to the cytosol (Fig. 6, compare b (+BFA, small arrows) to d (+BFA)) in both transfected and nontransfected cells.

An identical result to BFA was observed in cells co-transfected with either the ARF1(T31N) or ARF1(N126I) mutants.
ARF Regulation of Vesicular Traffic and Golgi Structure

In this case, β-COP was completely dispersed to a diffuse cytoplasmic staining pattern (Fig. 6, h (T31N) and j (N1261), large bold arrows). However, we have observed that in cells expressing the ARF1(N126I) mutant, that β-COP was frequently found in punctate aggregates scattered throughout the cytoplasm (Fig. 6j, see cell with large bold arrow). These structures did not overlap with compartments of the secretory pathway (data not shown), reminiscent of the aggregation of β-COP when cells are depleted of ATP (Hendrick et al., 1993). In contrast, when the distribution of β-COP in cells expressing the ARF1(Q71L) mutant was examined, β-COP was strongly associated with the compact, juxta-nuclear Golgi stack in >90% of the transfected cells examined (Fig. 6f, compare β-COP distribution (small arrows) in transfected cells (large bold arrow) to nontransfected cells). In these cells, β-COP could also be detected in punctate structures which overlap with p53 containing pre-Golgi intermediates (Fig. 6f, small arrowheads) (data not shown). Cells expressing the Q71L mutant which were shifted to the permissive temperature showed strong overlap of β-COP with pre-Golgi intermediates containing VSV-G (data not shown).

BFA Triggers Partial Processing of VSV-G to the Rl Form in HeLa Cells—It is now well recognized that BFA triggers the delivery of Golgi-associated α1,2-mannosidases and glycosyltransferases normally residing in the cis/medial Golgi compartments to the ER (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Lippincott-Schwartz, 1993). To examine whether the residual processing of VSV-G to the Rl form in cells expressing the ARF1(T31N) or ARF1(N126I) mutants (Fig. 2B) was possi-

Fig. 6. Mutations in GTP-binding domains of ARF1 affect the distribution of the coatomer component β-COP. Recombinant vaccinia virus-infected HeLa cells were transfected either with a plasmid encoding tsO45-VSV-G (pAR-tsO45-G) alone (a-d) or co-transfected with pAR-tsO45-G and ARF1(Q71L) (e and f), ARF1(T31N) (g and h), and ARF1(N126I) (i and j). Cells were incubated and processed for double immunofluorescence to determine the distribution of VSV-G and β-COP as described in the legend to Fig. 4, and under “Experimental Procedures.” a-d, after 4 h post-transfection at the restrictive temperature (39.5 °C), cells were rapidly transferred to ice. a-d, distribution of VSV-G (a, cell with large bold arrow) and β-COP (b) after incubation for an additional 1.5 h at 39.5 °C in absence of BFA; distribution of VSV-G (c, cell with large bold arrow) and β-COP (d) after incubation for 1.5 h (39.5 °C) in the presence of BFA (10 μg/ml final concentration). e and f, distribution of VSV-G (e, cell with large bold arrow) and β-COP (f, small arrows) in a cell expressing the ARF1(Q71L) mutant protein. Punctate structures resembling the pre-Golgi intermediates are indicated (arrowheads). g and h, distribution of VSV-G (g, cell with large bold arrow) and β-COP (h) in cells expressing the ARF1(T31N) mutant protein. i and j, distribution of VSV-G (i, cells with large bold arrow) and β-COP (j) in cells expressing the ARF1(N126I) mutant protein. In h and j, small arrows indicate β-COP distribution in non-transfected cells.

In this case, β-COP was completely dispersed to a diffuse cytoplasmic staining pattern (Fig. 6, h (T31N) and j (N126I), large bold arrows). However, we have observed that in cells expressing the ARF1(N126I) mutant, that β-COP was frequently found in punctate aggregates scattered throughout the cytoplasm (Fig. 6j, see cell with large bold arrow). These structures did not overlap with compartments of the secretory pathway (data not shown), reminiscent of the aggregation of β-COP when cells are depleted of ATP (Hendrick et al., 1993). In contrast, when the distribution of β-COP in cells expressing the ARF1(Q71L) mutant was examined, β-COP was strongly associated with the compact, juxta-nuclear Golgi stack in >90% of the transfected cells examined (Fig. 6f, compare β-COP distribution (small arrows) in transfected cells (large bold arrow) to nontransfected cells). In these cells, β-COP could also be detected in punctate structures which overlap with p53 containing pre-Golgi intermediates (Fig. 6f, small arrowheads) (data not shown). Cells expressing the Q71L mutant which were shifted to the permissive temperature showed strong overlap of β-COP with pre-Golgi intermediates containing VSV-G (data not shown).

BFA Triggers Partial Processing of VSV-G to the Rl Form in HeLa Cells—It is now well recognized that BFA triggers the delivery of Golgi-associated α1,2-mannosidases and glycosyltransferases normally residing in the cis/medial Golgi compartments to the ER (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Lippincott-Schwartz, 1993). To examine whether the residual processing of VSV-G to the Rl form in cells expressing the ARF1(T31N) or ARF1(N126I) mutants (Fig. 2B) was possi-
Promotes Vesicle Formation-The tide-binding region which is essential for the function of all within all members of the ras superfamily would alter the highly conserved guanine nucleotide-binding domains found would be anticipated to block the normal guanine nucleotide cycle necessary for ARF function. Fig. 2 and under "Experimental Procedures." Naturally, during synthesis ARF is likely to be loaded with GTP due to the fact that the steady-state guanine nucleotide pool in cells is largely GTP. In this instance, ARF(Q71L) may bypass the requirement for GEF, being directly recruited to the membrane surface either via specific receptors which recognize the "active" GTP-bound form, or simply through an initial more nonspecific stable association with the lipid bilayer as a consequence of its active conformation (Helms et al., 1993). In either case, an excess of ARF(Q71L) on the membrane would provide a stable, saturating pool of the GTP-bound form of ARF to recruit coat components and initiate vesicle formation, suggesting that GTP hydrolysis by ARF per se is not required for budding. A prediction of this interpretation is that the distrib...
bution of β-COP in cells transfected with the ARF1(Q71L) mutant will be insensitive to BFA, a result which we have observed. However, since these carrier vesicles are defective in targeting or fusion to a downstream acceptor compartment, these results suggest that GTP hydrolysis promoted by a membrane-associated ARF-specific GAP is essential for a downstream event either prior to or during vesicle docking and/or fusion. Inhibition of targeting and fusion could also be due to the inability of ER to Golgi carrier vesicles to fuse with the cis Golgi compartment due to an unusual accumulation of coat proteins on the acceptor Golgi compartment in the presence of the ARF1(Q71L) mutant. Given the ability of the 2 Ala substitution to prevent ARF1(Q71L) inhibition, myristoylation is clearly essential for association of ARF1(GTP) with the membrane in vivo.

**T31N Inhibits Export of VSV-G from the ER and Prevents Coat Assembly**—The substitution of N for T at position 31 is analogous to the growth inhibitory S17N mutant of H-ras (Feig and Cooper, 1988). This ras mutant shows preferential affinity for GDP over GTP and is thought to be preferentially contained to the GDP-bound inactive form. This mutant has been proposed to antagonize normal H-ras function by sequencing the endogenous pool of ras-GEF (Farnsworth et al., 1991). Analysis of GTP binding to ARF1(T31N) suggests a similar biochemical phenotype. Given the ras paradigm, we propose that the ARF1(T31N) similarly prevents the endogenous pool of ARF1 in the cell from interacting with an ARF1-GEF. Such a mutant would be anticipated to efficiently prevent coat assembly and export of VSV-G from the ER (Fig. 8). Although this mutant has a preferential affinity for GDP, it, like H-ras (Feig and Cooper, 1988), can still bind GTP, suggesting that even a low level of exchange is insufficient to maintain function of the vesicular traffic pathway.

In contrast to the effect of the ARF1(Q71L) mutation on Golgi structure or the distribution of β-COP, the ARF1(T31N) mutant triggered collapse of the Golgi into the ER, redistribution of β-COP to the cytosol, and promoted processing of VSV-G to the early Golgi-like forms (Fig. 8). Since the predicted phenotype of ARF1(T31N) is to "inactivate" an ARF-specific GEF, our results are consistent with the recent suggestion that BFA may also inactivate either directly and indirectly an ARF-specific GEF (Donaldson et al., 1992b; Helms and Rothman, 1992), and emphasize an essential role for ARF and ARF-GEF in the dynamic turnover of β-COP on the ER and the Golgi compartments. Since the 2 Ala substitution also neutralized ARF1(T31N) function, myristoylation appears to be essential for interaction between ARF and its specific GEF in vivo. Moreover, these results suggest that either the retrograde traffic pathway does not involve an ARF/β-COP-mediated pathway or that the BFA-induced phenotype (GEF inhibition) is not necessarily serving to identify the physiological pathway involved in ER to Golgi recycling. Since coat components may normally mask the activity of receptors and fusion factors involved in the anterograde pathway, an artificial unmasking of these components by disrupting ARF function may release such constraints, leading to the fusion of companion compartments normally only communicating via vesicular carriers.

**N126I Inhibits Either Stable Recruitment or Recycling of β-COP**—Both the N126I and D129N substitutions are localized to the third GTP-binding domain and result in proteins which do not have detectable levels of GTP binding (Table I). Consistent with this result, substitutions in the same domain of H-ras lead to an exceptionally high guanine nucleotide-exchange rate and generate dominant transforming mutants (Der et al., 1988). Analogous substitutions in members of the YPT1/SEC4/rab family are dominantly lethal (Schmitt et al., 1986) or are defective in transport between exocytic compartments in vivo (Walworth et al., 1991; Tisdale et al., 1992); and in vitro. Although the ARF1(N126I) mutant protein exhibited dominant negative activity, its biochemical and morphological phenotypes were not like those observed for the Q71L mutant, but were similar to those observed for the T31N mutant leading to retention of VSV-G in the ER, collapse of the Golgi, and partial processing of VSV-G to the R form. However, ARF1(N126I) differed from ARF1(T31N) in causing the accumulation of β-COP in small dots which were distributed throughout the cytoplasm and which may represent insoluble, β-COP-containing aggregates.

There are a number of explanations currently being entertained to account for the phenotype of this mutant. One possibility is that it is recruited to membranes via an ARF-GEF. However, due to its high exchange rate, it may inhibit GEF function and may be unable to establish or maintain a stable association between β-COP and other transport components on the membrane surface. As a consequence, coats fail to assemble and a BFA-like effect ensues. Alternatively, the mutant could bind directly to the soluble pool of β-COP, leading to aggregation and creating a β-COP "null" phenotype.

**Multiple GTP-binging Proteins Are Required for Export from the ER**—The combined results from the above experiments suggest that ARF1 plays a critical role in the recruitment of β-COP-containing coat complexes from the cytosol in vivo which are involved in initiating vesicular traffic from the ER and the maintenance of Golgi function and structure. These results are consistent with our recent observations that antibodies specific for β-COP also inhibit the recruitment of a β-COP/rab1 containing pre-coat complex and block vesicle budding from the ER (Peter et al., 1993). In addition, we now have evidence that a homologue to yeast SAR1 (Nakano and Muramatsu, 1989; Oka et al., 1991; d'Enfert et al., 1991; Barlowe et al., 1993) is also required for vesicle budding from the ER in mammalian cells. Thus, at least 3 GTP-binding proteins, ARF1, rab1, and SAR1 are required for export from the ER. The individual functions of these three molecular switches must be ultimately integrated to choreograph the recruitment and concentration of cargo during export from the ER and/or assembly/disassembly during subsequent downstream budding, targeting, and fusion events. Experiments to elucidate their sequential roles in each of these processes are currently in progress.

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**REFERENCES**

Balch, W. E., Kahn, R. A., and Schwaninger, R. (1992) J. Biol. Chem. 267, 13053-13061

Barbedic, M. (1997) Annu. Rev. Biochem. 66, 779-872

Barlowe, C., d'Enfert, C., and Scheekman, R. (1993) J. Biol. Chem. 268, 879-879

Beckers, C. J. M., Keller, D. S., and Balch, W. E. (1988) Cell 50, 523-534

Beckers, C. J. M., Plutner, H., Davidson, H. W., and Balch, W. E. (1990) J. Biol. Chem. 265, 19296-19310

Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) Nature 348, 125-132

Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) Nature 349, 117-127

Bucci, C., Parton, R. G., Matther, I. H., Stennenberg, H., Simon,a, K., Hoflack, B., and Zerial, M. (1992) Cell 70, 75-78

d'Enfert, C., Wuestehube, L. J., Lila, T., and Scheekman, R. (1991) J. Cell Biol. 114, 863-870

Davidson, H. W., and Balch, W. E. (1993) J. Biol. Chem. 268, 4216-4226

Der, C. J., and Cox, A. D. (1991) Cancer Cells 3, 331-340

Der, C. J., Finkel, T., and Cooper, G. (1986) Cell 46, 167-176

Der, C. J., Weissman, E., and McDonald, M. J. (1988) Oncogene 3, 100-112

Dors, R. W., Russ, G., and Yewdell, J. W. (1989) J. Cell Biol. 109, 61-72

Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E., and Knauss, R. D. (1990) J. Cell Biol. 111, 2295-2306

Donaldson, J., Lippincott-Schwartz, J., and Knauss, R. D. (1991) J. Cell Biol. 118, 579-588

4 C. Dascher and W. E. Balch, unpublished.

8 O. Kuge, C. Dascher, M. Amherdt, H. Pluntsar, M. Ravazzola, L. Orci, J. E. Rothman, and W. E. Balch, submitted for publication.
