ADP-ribosylation Factor Is Required for Vesicular Trafficking between the Endoplasmic Reticulum and the cis-Golgi Compartment*

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We describe the potential role of ADP-ribosylation factor (ARF) in vesicular trafficking using an in vitro assay that efficiently reconstitutes transport between the endoplasmic reticulum (ER) and the cis-Golgi compartment in mammalian semi-intact cells, a population of cells in which the plasma membrane is physically perforated to reveal intact ER and Golgi compartments. We demonstrate that peptides identical to the amino-terminal domain of ARF, which inhibit ARF cofactor activity in choler toxin-catalyzed ADP-ribosylation of Gαs (Kahn, R. A., Randazzo, P., Serafini, T., Weiss, O., Rulka, C., Clark, J., Amherdt, M., Roller, P., Orci, L., and Rothman, J. E. (1992) J. Biol. Chem. 267, 13039–13046), inhibit transport of the vesicular stomatitis virus G protein between the ER and cis-Golgi compartment. Inhibition of transport was rapid (τ/2 = 30–60 s) and irreversible. Half-maximal inhibition was observed at concentrations of 15 and 22 mM with peptides identical to the amino-terminal domain of the human ARF4 (hARF4) protein and the human ARF1 protein, respectively. Kinetic analysis of vesicular stomatitis virus G protein transport suggested that the hARF4 peptide inhibits a late vesicle fusion step. In addition, incubation of semi-intact cells in the presence of the myristoylated form human ARF1 (hARF1myr) protein, but not the nonmyristoylated form of ARF1, inhibited transport. In contrast to peptide, the hARF1myr blocked an early transport step, similar to that observed with guanosine 5'-3-O-(thio)triphosphate. These results suggest that ARF and components facilitating ARF function play an important role in the cyclical fusion and fusion of transport vesicles mediating ER to Golgi trafficking.

Vesicular membrane trafficking between the endoplasmic reticulum (ER)† and the Golgi compartments of eukaryotic cells is now recognized to involve multiple small GTP-binding proteins including members of the rab, SAR, and ARF gene families. The rab family is a member of the ras superfamily (Valencia et al., 1991) and contains at least 20 related members (Zahraoui et al., 1989; Chavrier et al., 1990a, 1990b; for review see Balch, 1990; Goud and Mccaffrey, 1991; Pfeffer, 1992). Transport between the ER and cis-Golgi compartment requires the rab1 protein in mammalian cells (Plutner et al., 1990, 1991) and its homolog YPT1 in yeast (Schmitt et al., 1988; Segev et al., 1988; Bacon et al., 1989; Baker et al., 1990). A second class of small GTP-binding proteins characterized by SAR1 is required for ER to Golgi transport in yeast (Nakano et al., 1988; Nakano and Muramatsu, 1989; Nishikawa and Nakano, 1991). SAR1 is required for vesicle budding (fusion) and is functionally associated with the SEC12 protein, an integral membrane protein found in the ER which is essential for function of the exocytic pathway (Nakano et al., 1988; Oka et al., 1991; d’Enfert et al., 1991). Although SAR1 is distantly related to each of the subfamilies of the ras superfamily (26% identity), it is more closely related to the ARF subfamily (35% identity).

ARF (for ADP-ribosylation factor) has been detected in both yeast and mammalian cells and now comprises at least six known members (Kahn, 1991; Kahn et al., 1991). ARF was originally discovered as a cofactor for the efficient ADP-ribosylation of the stimulatory regulatory subunit (Gαs) of adenylyl cyclase by choler toxin (Kahn and Gilman, 1984, 1986). The role of ARF in this reaction has been characterized extensively (for review see Kahn, 1989). Recent evidence suggests that the physiological role of ARF in the cell may be in the regulation of vesicular trafficking through the exocytic pathway (Stearns et al., 1990, 1991; Orci et al., 1991). ARF is an abundant protein, comprising nearly 1% of soluble cytosolic protein in at least some neural tissues (Kahn and Gilman, 1986). In yeast, deletion of ARF1 (one of two ARF genes) results in impaired secretion through the secretory pathway. This defect can be corrected by expression of either human ARF1 and ARF4 gene products (Kahn et al., 1991) which are 80% identical to one another and 74% identical to yeast ARF (Stearns et al., 1990). Using indirect immunofluorescence and immunoelectron microscopy, ARF has been demonstrated to be physically associated with the Golgi apparatus (Stearns et al., 1990; Serafini et al., 1991b; Donaldson et al., 1991a, 1991b). Biochemical evidence has demonstrated that all stages of the endocytic and exocytic pathways which have been reconstituted in vitro can be inhibited by the nonhydrolyzable analog of GTP, GTPγS (Melancon et al., 1987; Beckers and Balch, 1988; Diaz et al., 1989; Schwanger et al., 1991; Toozee et al., 1992; Gravotta and Sabatini, 1990; Goda and Pfeffer, 1991; Miller and Moore, 1991; Gorvel et al., 1991). In the case of intra-Golgi transport, ARF has been identified as an abundant protein of Golgi-derived non-clath-
INBALLED vesicles, which accumulate in the presence of the GTP\(\gamma\)S (Serafini et al., 1991b), and is the inhibitory factor responsible for inhibition of intra-Golgi transport by GTP\(\gamma\)S (Taylor and Melancon, 1992). A direct functional role for ARF in transport vesicle formation or fusion is proposed in the accompanying paper (Kahn et al., 1992).

Given the multiple lines of evidence which suggest ARF involvement in vesicular transport through early compartments of the secretory pathway, we have now examined its role in the first stage, ER to Golgi transport. Taking advantage of a recent observation that synthetic peptides derived from the amino terminus of human ARF are potent inhibitors of its cofactor activity in ADP-ribosylation of the regulatory stimulatory subunit \(\mathrm{G}_\text{sa}\) of adenylate cyclase (Kahn et al., 1992), we examined the effect of these peptides using an assay which efficiently reconstitutes ER to Golgi trafficking in a cell-free system using perforated, semi-intact cells (Beckers et al., 1987, 1990; Plutner et al., 1990, 1991). These peptides were found to be potent inhibitors of vesicular transport. Moreover, the concentration of ARF protein in the assay was found to be important in regulating the extent of vesicular transport. Our results suggest that ARF and the molecular machinery facilitating its function are critical for regulation of organelle function in the transport of protein between the ER and the cis-Golgi compartment.

**EXPERIMENTAL PROCEDURES**

**Materials**—Semi-intact cells used for the analysis of ER to Golgi transport were prepared from wild-type or clone 15B cells infected with either the wild-type, or the ts045 strains of vesicular stomatitis virus (VSV) using the swelling method as described previously (Beckers et al., 1987). Trans\(^\text{A-}\)label, \([\text{35}S]\)methionine, and \([\text{35}S]\)cysteine (>1,000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA). Cytosol used in transport assays was prepared from uninfected Chinese hamster ovary wild-type cells or rat liver as described previously (Beckers et al., 1987; Beckers and Balch, 1989). Endoglycosidase D (endo D) and endoglycosidase H (endo H) were obtained from Boehringer Mannheim.

UDP-GlcNAc was added to a final concentration of 25 mM HEPES (K\(^+\)) (7.2) in 2.5 mg/ml stock solution (Kahn et al., 1992). Two forms of human ARF1 protein were tested for activity in vitro: recombinant human ARF1 protein purified from Escherichia coli, which lacks the myristoylation site, and a modification found in human ARF1 purified from bovine tissues (Kahn et al., 1988), and a form which has been myristoylated in bacteria by the coexpression of hARF1 and yeast N-myristoyltransferase (hARF1\(_\text{m1m2}\)) (Duronio et al., 1990). Both human ARF proteins (1.1 mg/ml) were dialyzed into 25 mM HEPES (K\(^+\)) (7.2), 125 mM KOAc prior to use and were approximately 60% pure based on SDS-gel electrophoresis.

**Incubation Conditions to Achieve Transport in Vitro and Analysis of Transport**—The ER to Golgi transport assays using clone 15B semi-intact cells infected with ts045 VSV were performed as described previously (Beckers et al., 1987; Beckers and Balch, 1989; Plutner et al., 1990). For assays using wild-type cells infected with wild-type VSV, cells were labeled as described previously (Beckers et al., 1987; Schwanger et al., 1991) except that the pulse of \([\text{35}S]\)methionine (150 \(\mu\)Ci) was reduced to 3 min at 37 °C prior to transfer of cells to ice and preparation of semi-intact cells. Briefly, transport incubations in vitro contained a final total volume of 40 \(\mu\)l (final concentration: 25 mM HEPES/KOH (pH 7.2), 27 mM KOAC (wild-type cells) or 88 mM KOAc (15B cells), 2.5 mg OAc, 5 mM EGTA, 1.8 mM CaCl\(_2\), 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase, 10–125 \(\mu\)g of cytosol (as indicated under "Results"), and 5 \(\mu\)l (25–30 \(\mu\)g of protein; 1–2 x 10\(^5\) cells) of semi-intact cells. UDP-GlcNAc was added to a final concentration of 1 mM which indicated under "Results" to detect the appearance of the endo H-resistant forms of VSV G protein. Assays were supplemented with additional reagents as indicated under "Results." Transport was terminated by transfer to 32 °C in the case of semi-intact cells containing ts045 VSV G protein, and 37 °C for semi-intact cells containing wild-type VSV. Where indicated under "Results," intact cells were incubated in labeling medium instead of the transport mixture to determine the extent of transport in vivo in the presence of peptide.

After termination of transport by transfer to ice, the membranes were pelleted by a brief (15 s) centrifugation in a microcentrifuge at top speed. For analysis of processing of VSV G protein to the Man\(_2\) form, the pellet was subsequently solubilized in an endo D digestion buffer and digested with endo D as described previously (Beckers et al., 1987). For endo H digestion, samples were pelleted and the material solubilized by the addition of 50 \(\mu\)l of 0.1% SDS in 100 mM NaOAc (pH 5.6) and boiling for 5 min. Samples were digested overnight at 37 °C in the presence of 1 milliunit of endo H. Endo D and endo H digestions were terminated by adding a 5 x concentrated gel sample buffer (Laemmli, 1970) and boiling for 5 min. The samples were analyzed by SDS-polyacrylamide gel electrophoresis using 7% acrylamide gels (Beckers et al., 1987), autoradiographed, and the fraction of VSV G protein processed to the endo-D-sensitive, or endo H resistant forms was determined by densitometry (Beckers et al., 1987; Beckers and Balch, 1989).

**RESULTS**

**Amino-terminal Termini of Human ARF Inhibit ER to Golgi Transport in Semi-intact Cells**—Peptides homologous to the amino-terminal domains of either human ARF1 (hARF1) or human ARF4 (hARF4) protein inhibit ARF cofactor activity in vitro (Kahn et al., 1992). To begin to explore the potential role of ARF in vesicular transport, synthetic peptides identical to the amino termini of human ARF1 and ARF4 proteins were tested in an in vitro assay which efficiently reconstitutes the transport of VSV G protein between the ER and the Golgi in semi-intact cells, a population of cells in which the plasma membrane has been perforated to expose intact intracellular organelles (Beckers et al., 1987; Beckers and Balch, 1989; Beckers et al., 1990). In this assay, transport of VSV G protein is detected by following the processing of the two asparagine-linked high mannose (Man\(_9\)) oligosaccharides acquired in the ER to the Man\(_9\) form as a consequence of delivery to the cis-Golgi compartment where they are trimmed by the resident enzyme \(\alpha\)-1,2 mannosidase I. In wild-type Chinese hamster ovary cells, the Man\(_9\) oligosaccharide form is a transient, intermediate as VSV G is rapidly processed to the complex structure containing additional GlcNAc, galactose, and sialic acid in the medial and trans-Golgi compartments. To focus specifically on ER to Golgi transport, our assay utilizes a mutant Chinese hamster ovary cell line, clone 15B, which is defective in processing of protein beyond the Man\(_9\) oligosaccharide form (Tabas and Kornfeld, 1979; Beckers et al., 1987). Although transport to the cell surface is normal in this cell line, VSV G protein accumulates in the Man\(_9\) oligosaccharide form which can be readily quantitated using SDS-gel electrophoresis (Beckers et al., 1987).

Peptides (Table I) were added to an assay mixture containing semi-intact cells, ATP and cytosol, and incubated for 90 min at 32 °C. As shown in Fig. 1A, the addition of increasing concentrations of hARF1 or hARF4 led to complete inhibition of transport of VSV G protein between the ER and the cis-Golgi compartment. Half-maximal inhibition of transport was observed in the presence of ~1 \(\mu\)g of the hARF4 peptide (15 \(\mu\)M) and 1.3 \(\mu\)g (22 \(\mu\)M) for the hARF1 peptide (Fig. 1, open and closed circles). Complete inhibition (>80–90%) was observed at 20–25 \(\mu\)M in the case of the hARF4 peptide. Precise

**Table I**

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<th>Peptide</th>
<th>hARF1</th>
<th>hARF4</th>
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<tr>
<td><strong>Nonconsensus</strong></td>
<td>GLTISSLFSRLFGKKQ</td>
<td>GLTISSLFSRLFGKKQ</td>
</tr>
<tr>
<td><strong>Consensus</strong></td>
<td>G**,**L.F</td>
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* Asterisk indicates a conserved substitution.
protein transport between the ER and the Golgi in intact cells. The barrier prevents access of these peptides to a component(s) of the transport machinery associated with the ER, Golgi, or cytosol which is only accessible in perforated semi-intact cells.

**Truncated Peptides Do Not Inhibit Transport**—To explore the contribution of the peptide sequence to inhibition of transport, the hARF1 peptide was truncated sequentially from the amino-terminal end (see Kahn et al., 1992). Removal of the first 2 amino-terminal amino acids (glycine and aspartic acid) to generate a 14-mer results in a peptide with reduced inhibitory activity (Fig. 1B, closed squares). This peptide shows half-maximal inhibition of transport at 50 μM, nearly a 4-fold decrease in sensitivity compared with the full-length (16-mer) peptide (Fig. 1B, open circles). Further truncation results in complete loss of inhibitory activity (Fig. 1B, open squares). These results parallel the effect of truncated peptides on inhibition of cholera-toxin catalyzed ADP-ribosylation of Gs subunit and intra-Golgi transport (Kahn et al., 1992).

**Peptides Inhibit ER to Golgi Transport Rapidly and Irreversibly**—To determine the kinetics of inhibition of transport by the hARF4 peptide, we first tested whether transport could be inactivated by a brief exposure to peptide or whether inhibition required the continuous presence of peptide throughout the 90-min incubation period. Semi-intact cells were preincubated in a first stage (Fig. 2, Stage 1) (in a complete assay mixture containing cytosol and ATP) with peptide on ice or at 32 °C for increasing time prior to pelleting of semi-intact cells, aspiration of the supernatant to remove soluble peptide, and reincubation of the semi-intact cells in a second stage (Fig. 2, Stage 2) for 90 min in fresh mixture lacking peptide. Under these conditions, if inhibition was rapid and irreversible, pelleting even after a short period of incubation would be expected to prevent further transport. As shown in Fig. 2, both the rate and extent of inhibition are greater at 32 °C than on ice. Preincubation at 32 °C in the presence of cytosol and ATP for only 2–3 min led to a rapid and nearly complete inhibition of transport ($t_{1/2} = \sim 30–60$ s) (Fig. 2, closed squares). These results suggest that inhibition values for inhibition vary for each preparation of semi-intact cells and cytosol (see below). However, the hARF4 peptide was consistently more potent (≈25–50%) than the hARF1 peptide.

Both peptides form amphipathic helices in solution (Kahn et al., 1992). It was therefore important to establish that inhibition of transport was not a result of nonspecific detergent effects of these peptides on the ER and/or Golgi membranes accessible in semi-intact cells. For this purpose, an identical concentration of intact cells transporting VSV G protein was incubated in the presence of peptide. Using intact cells, no inhibition was observed in the presence of up to 10 μg (150 μM) of the hARF4 peptide (Fig. 1, closed squares). In contrast, detergents such as Triton X-100 and β-octyl glucoside or the fatty acid palmitate (Na+) potently inhibit VSV G protein transport between the ER and the Golgi in intact cells at concentrations of 10–100 μM (data not shown). These results argue that the presence of an intact plasma membrane barrier prevents access of these peptides to a component(s) of the transport machinery associated with the ER, Golgi, or cytosol.
by the hARF4 peptide is rapid and irreversible or that the hARF peptide partitions in a temperature-dependent fashion into the membranes of semi-intact cells.

Inhibition by Peptide Requires the Presence of Semi-intact Cells—The hARF4 peptide could potentially inhibit a factor(s) associated with the ER and/or Golgi membranes present in semi-intact cells, transport components present in the soluble cytosolic fraction, or both. As a control to demonstrate that preincubation on ice does not enhance sensitivity of cytosol or semi-intact cells to peptide, cells were preincubated in the presence or absence of cytosol for 20 min on ice with increasing concentrations of peptide. The missing components were subsequently added, and the mixture was incubated for 90 min at 32 \(^\circ\)C in the presence of peptide. Pretreatment of semi-intact cells or cytosol alone did not result in an increased sensitivity to peptide compared with the complete mixture during subsequent incubation at 32 \(^\circ\)C (Fig. 3A). To test if semi-intact cells were a target for peptide, cells were incubated with increasing concentrations of peptide in the absence of cytosol for 20 min on ice, followed by pelleting, aspiration of the supernatant (to remove soluble peptide), and resuspension in a complete mixture containing untreated cytosol and ATP. Pretreatment of semi-intact cells alone in the presence of 1–1.5 \(\mu\)g (15–25 \(\mu\)M) of peptide resulted in complete inhibition of transport (Fig. 3B, open circles). Moreover, the sensitivity to peptide was enhanced markedly when semi-intact cells were preincubated at reduced concentrations of cytosol (Fig. 3B, compare open and closed circles). These results suggest that the peptide may partition with semi-intact cells membranes in an cytosol-sensitive fashion.

To provide additional evidence that the cytosol contained peptide-responsive component(s), semi-intact cells were preincubated in the presence of increasing concentrations of peptide with either a low (15 \(\mu\)g), rate-limiting concentration of cytosol or a high (125 \(\mu\)g) concentration of cytosol to promote maximal transport. In this experiment, it is evident that the concentration of cytosol markedly affected the ability of the peptide to inhibit transport (Fig. 3C). Although nearly 80% inhibition was observed in the presence of 0.75 \(\mu\)g (10 \(\mu\)M) of peptide and 15 \(\mu\)g of cytosol, less than 20% inhibition was observed in the presence 125 \(\mu\)g of cytosol. The addition of protease inhibitors had no effect on capacity of cytosol to reduce the effective potency of the hARF4 peptide (data not shown).

Peptide Inhibits a Late Step in Vesicular Transport—We have established that transport from the ER to the Golgi occurs through sequential intermediates in semi-intact cells (Beckers et al., 1990). An initial GTP-\(\gamma\)S-sensitive, ATP- and cytosol-dependent lag period of 20–30 min is involved in vesicle formation (budding) and targeting. During this time, VSV G protein largely remains in the high mannose (Man\(_9\)) form indicative of its pre-cis-Golgi localization. During the subsequent 60–75 min, transport vesicles containing VSV G protein fuse to the cis-Golgi compartment and are rapidly processed by \(\alpha\)-1,2-mannosidase I (Beckers et al., 1990). Fusion requires Ca\(^{2+}\) and is sensitive to a peptide homologous to the rab1 effector domain (Beckers and Balch, 1989; Beckers et al., 1990; Plutner et al., 1990, 1991).

To explore whether the hARF4 peptide inhibits an early vesicle fusion step, or a late vesicle fusion step, semi-intact cells were incubated in the presence of cytosol and ATP for increasing time in the absence of peptide to initiate transport. Subsequently, peptide was added to the assay and the incubation continued for a total of 90 min at 32 \(^\circ\)C. If peptide inhibits an early vesicle budding or targeting step, then the addition of peptide after 20–30 min should have no effect on subsequent transport of VSV G protein to the cis-Golgi compartment (Beckers et al., 1990). In this case, incubation for an additional 60 min will result in all of the VSV G protein being processed to the trimmed (Man\(_6\)) form. In contrast, if the hARF4 peptide inhibits a late vesicle fusion...
step, the addition of the peptide at any time point during the 90-min incubation will result in a rapid inhibition of transport given that the hARF4 peptide inhibits with a \( t_{1/2} \) of 30–60 s at 32 °C (see Fig. 3).

As shown in Fig. 4 (open circles), the addition of the hARF4 peptide to the assay resulted in the immediate cessation of processing, functionally equivalent to transferring cells to ice (Fig. 4, compare open and closed circles). This is in contrast to the effect of GTPyS, which inhibits an earlier transport step (Fig. 4, closed squares). In this case, the additional level of processing at each time point after addition of GTPyS measures the amount of VSV G transported past the early GTPyS-sensitive vesicle fission step prior to addition of inhibitor. In support of the interpretation that the hARF4 peptide inhibits a late step, when semi-intact cells, cytosol, and ATP were incubated in the presence of EGTA to accumulate VSV G protein in a late, presynthesis transport intermediate (Beckers and Balch, 1989; Beckers et al., 1990), transport was sensitive to the hARF4 peptide but insensitive to GTPyS (data not shown).

ARF Protein Inhibits ER to Golgi Transport—Although ARF is an abundant protein comprising nearly 1% of total soluble protein present in neural tissue (Kahn et al., 1988), overexpression of ARFp results a lethality in yeast (Stearns et al., 1991), suggesting that a critical concentration is important for ARF function in the secretory pathway. To test whether the concentration of hARF protein present in the incubation mixture is also critical for ER to Golgi transport in vitro, we examined the effect of the hARF1 recombinant protein in vitro. Two forms of hARF1 were tested: recombinant hARF1 protein purified from E. coli which lacks the \( N \)-myristoylation modification found in native hARF1 purified from bovine tissues (Kahn et al., 1988), and a form that has been myristoylated in bacteria by the coexpression of hARF1 and yeast \( N \)-myristoyltransferase (hARF1myr) (Duronio et al., 1990). Addition of up to 1 \( \mu \)g of recombinant hARF1 lacking myristic acid had a small effect on transport (Fig. 5A). Maximal inhibition (20%) of transport was observed in the presence of 1 \( \mu \)g of nonmyristoylated hARF1p. In contrast, complete (>90%) inhibition was observed in the presence of 1 \( \mu \)g of hARF1myr (1.25 \( \mu \)M), with half-maximal inhibition of transport being observed in the presence of 0.3 \( \mu \)g of hARF1myr. The concentration of hARF1myr required to inhibit transport was variable between different preparations of semi-intact cells over a 2-fold concentration range but was consistently sensitive to the concentration of cytosol used in the assay. Incubation in the presence of 15 \( \mu \)g of cytosol resulted in >
80% inhibition of transport in the presence of 1 μg of hARF1, compared with <20% inhibition in the presence of excess cytosol (125 μg) (Fig. 5B).

In contrast to the late acting hARF4 peptide, the presence of excess hARF1, inhibited an early transport step (Fig. 5C). After a 20–30-min incubation, a time point at which <20% of the VSV G protein has been processed to the Man5 form, the addition of 1 μg of hARF1, had a markedly reduced effect on transport. This result is similar to the effect of an antibody which neutralizes rab1 function (Plutner et al., 1991). In a separate experiment we found that >80% inhibition of transport was observed within 5 min after the addition of the hARF1, protein (data not shown), indicating that hARF1, like peptide which requires ~5 min to elicit full inhibition (Fig. 2). As the hARF4 peptide and added hARF1, appear to inhibit two different steps in transport, the combined inhibitory activities of each should be additive. As shown in Fig. 6 (closed circles), the addition of increasing concentrations of peptide to an assay containing 1 μg of hARF1, showed a significant increase in inhibition compared with the control which contained only peptide (Fig. 6, open circles). At the lowest concentration of peptide tested (0.5 μg), in which the ARF peptide alone demonstrated negligible inhibition (Fig. 7, open circles) and was present at a 5-fold molar excess of hARF1, transport was strongly inhibited. These results suggest that the peptide does not serve to neutralize ARF function per se.

hARF4 Peptide Inhibits both ER to Golgi and Intra-Golgi Trafficking in Semi-intact Cells—Semi-intact cells efficiently reconstitute the sequential transport of VSV G protein from the ER to both the cis- and medial Golgi compartments (Schwaninger et al., 1991). To follow transport through sequential Golgi compartments, semi-intact cells were prepared from a wild-type cell line which processes the two asparagine-linked oligosaccharides found on ts045 VSV G protein to the complex structures containing GlcNAc, galactose, and sialic acid (Schwaninger, 1991). In wild-type cells transport of ts045 VSV G protein from the ER to the cis-Golgi compartment can be detected by the processing of one of its two oligosaccharide chains to the GH1 form (Fig. 7A, open squares), which is resistant to endo H. This enzyme cleaves N-linked oligosaccharides from glycoproteins that have not been processed by the cis-medial enzymes GlcNAc Tr I and α1, 2-,mannosidase II (Schwaninger et al., 1991). Subsequently, VSV G protein undergoes a second round of vesicular transport from the cis-Golgi compartment to the medial Golgi compartment. In the medial Golgi compartment, the second oligosaccharide is processed to the endo H-resistant GH2 form (Fig. 7A, open circles) (Schwaninger et al., 1991). Sequential processing of the two VSV G protein oligosaccharides allows us to distinguish between components required for transport from the ER to cis-Golgi compartment from those required for cis- to medial Golgi transport.

hARF1 peptide was added to the assay after increasing times of incubation at 32°C similar to the experiment used to define the step sensitive to peptide in ER to cis-Golgi transport (see Fig. 4). As expected, the addition of peptide prior to incubation inhibited the appearance of either the GH1 or GH2 forms (Fig. 7B, t = 0, closed squares; 7C, t = 0, closed circles). The addition of peptide after an increasing time of incubation at 32°C detected two peptide-sensitive steps, one inhibiting processing of VSV G to the GH1 form with a t1/2 of

![Fig. 6. Peptide and myristoylated hARF are additive in inhibition of ER to Golgi transport.](image-url)

![Fig. 7. Peptide inhibits transport between the cis and medial Golgi compartments in semi-intact cells.](image-url)
30–40 min (Fig. 7B, closed squares), and a second inhibiting processing of VSV G to the G_{12} form with a t_{1/2} of 60–70 min (Fig. 7C, closed circles). Similar results were obtained for the hARF4 peptide (data not shown).

**DISCUSSION**

We have provided two lines of evidence that ARF is a component required for vesicular protein transport between the ER and the cis-Golgi compartment. The first line of evidence, while indirect, demonstrated that the addition of ARF peptides to semi-intact cells leads to complete inhibition of transport. Because inhibition was rapid and irreversible, we were able to demonstrate that peptide inhibited a step in transport diagnostic of a late vesicle fusion step. These results are similar to those observed for inhibition by the addition of EGTA (to chelate free Ca^{2+}) or by the addition of a synthetic peptide homologous to the rab1 effector domain. In the latter case, both genetic and biochemical evidence strongly support the role of rab1 and its yeast homolog, YPT1, in ER to Golgi transport (Haubruck et al., 1987, 1988; Segev et al., 1988; Schmitt et al., 1988; Bacon et al., 1989; Baker et al., 1990; Kaiser and Schekman, 1990; Segev, 1991; Becker et al., 1991; Plutner et al., 1990, 1991). Rab1 function, like other members of the ras superfamily of small GTP-binding proteins, is likely to be facilitated by both upstream and downstream components which are regulated by, or in turn regulate rab1 guanine nucleotide exchange and hydrolysis. The rab effector domain peptide is believed to irreversibly inhibit the function of a downstream component function involved in vesicle fusion (Plutner et al., 1990). By analogy, the current results suggest that the hARF peptides may disrupt the interaction of ARF with a component facilitating a late fusion-related step.

Although our results are completely consistent with the ability of hARF peptides to inhibit fusion between endosomes (Lenard et al., 1992), it contrasts with the apparent inhibition of vesicle formation and GTPγS-induced hARF accumulation on Golgi membranes in the intra-Golgi transport assay using purified Golgi membranes (Kahn et al., 1992; Orci et al., 1991). The capacity of the hARF peptides to inhibit both budding and fusion steps in membrane trafficking could have several interpretations. The first possibility is that the hARF peptides interact (inhibit) components with distinct functional roles in the endocytic and exocytic pathways. This possibility cannot be ruled out given that the target(s) for the peptides in the various assays is presently unknown. A second possibility is that ARF per se plays distinct roles at different stages of the exocytic and endocytic pathways. This may certainly be the case when comparing events occurring during exocytosis and endocytosis. This could also be reflected in the requirement for separate gene products at different stages of the exocytic pathway (ER to Golgi, intra-Golgi, and Golgi to cell surface) (Novick et al., 1980; Walworth et al., 1988; Kaiser and Schekman, 1990). A third possibility is that inhibition of a late step in transport (as detected biochemically in the ER to Golgi transport assay) may rapidly inhibit the recycling of components of the transport machinery required for vesicle formation, leading to an apparent inhibition of vesicle formation at the morphological level. These and other possible interpretations are currently under investigation.

A concern in our studies was whether the effects observed with the two full-length hARF peptides (16-mers) were related to their propensity to bind phospholipid vesicles and form amphipathic helices leading to potential detergent-like effects (Kahn et al., 1992). Given the low (50%) identity between the hARF1 and hARF4 peptides, sequential truncation leading to the loss of structure lends credence to the interpretation that their structure is critical for inhibition, although the issue of sequence specificity of inhibition remains to be examined carefully. We provided several lines of evidence that the amphipathic structure of the peptide does not lead to nonspecific detergent effects. First, incubation of intact cells in the presence of a 10-fold excess of peptide over that used to block transport of VSV G protein in vitro did not inhibit transport in vivo. The addition of low concentrations of detergents under identical conditions completely inhibits transport in vitro. In addition, these results are consistent with experiments which demonstrate that these peptides do not render membranes permeable to small (<300 Da) molecules. Thus, the peptides neither solubilize the plasma membrane nor form pores that are permeable to low molecular weight compounds. Only in semi-intact cells, which have a physically perforated plasma membrane, do the peptides gain direct access to cytosolic components of the transport machinery. Although these studies address the potential of the ARF peptides to permeabilize membranes, they do not rule out the possibility that binding or penetration of membranes by these amphipathic peptides is essential to eliciting inhibition. Although we have observed a strong cytosol effect on inhibition of transport by peptide, the contribution, if any, of specific cytosolic factors remains to be demonstrated. We have tested more than 30 peptides of varying sequence and amphipathicity (Plutner et al., 1990). One of these is a peptide comprising the putative ARF effector region (peptide P-14 identical to the putative ARF effector domain (Kahn et al., 1992)). It had no effect on transport in concentrations up to 150 µM. We conclude that these peptides provide new probes to begin to assess the role of proteins potentiating ARF function in the secretory pathway.

Evidence that ARF is directly involved in ER to Golgi transport came from the observation that addition of increasing concentrations of hARF_{1mv}, progressively inhibited transport. Specifically, as little as 1 µg of hARF_{1mv}, but not nonmyristoylated ARF1, conferred complete inhibition. These results are in keeping with the observation that overexpression of ARFp in yeast also leads to lethality, suggesting that an imbalance in the levels of ARF leads to disruption of the transport machinery and cell death (Stearns et al., 1990). One of these is a peptide comprising the putative ARF effector region (peptide P-14 identical to the putative ARF effector domain (Kahn et al., 1992)). It had no effect on transport in concentrations up to 150 µM. We conclude that these peptides provide new probes to begin to assess the role of proteins potentiating ARF function in the secretory pathway.

4 W. E. Balch, unpublished results.
5 R. Kahn, unpublished results.
It is noteworthy that the addition of hARF1ins to the assay inhibited an apparent early step in transport, diagnostic of vesicle formation. This is in direct contrast to the effect of the hARF peptides which inhibited a late, vesicle fusion step. One interpretation of these results would suggest that the ARF protein recruited during early vesicle formation steps may also be involved in the regulation of a late vesicle fusion step, thus being required throughout the dynamic life cycle of a transport vesicle. However, given the potential complexities of the interaction of ARF with both soluble and membrane-associated components, an answer to these results will require further investigation.

Although our experiments focused on the ARF1 and ARF4 peptides, the potential function of specific members of the ARF gene family in different stages of the exocytic pathway remains to be tested in vitro. Identification of the role of different members of the ARF gene family will require preparation of membranes and cytosol which are depleted of specific ARFs. Conditions are currently not available which render our in vitro assay ARF-dependent. Given the abundance of ARF and the multiple factors required for reconstitution of transport between the ER and the Golgi, this approach will require development of specific antibody reagents that can be used to immunodeplete or inhibit ARF function in vitro.

What is the common theme underlying ARF function? Although the answer to this question is largely conjecture, there is now a growing realization that ARF is only one of several families of small GTP-binding proteins which regulate the structure and function of organelles of both the endocytic and exocytic pathways. Genetic and biochemical evidence in yeast suggests that SAR1 is required for vesicle formation (d’Enfert et al., 1991; Oka et al., 1991), whereas YPT1 (Kaiser and Schekman, 1990; Segev; Becker et al., 1991) and SEC4 (Salminen and Novick, 1987; Walworth et al., 1989) are required for vesicle fusion. In mammalian cells, the YPT1 homolog, rab1, is apparently recruited at an early step (Plutner et al., 1991), but may function at a late step (Plutner et al., 1990). The requirement for rab1 at a postvesicle fusion step has been demonstrated for the yeast YPT1 protein (Kaiser and Schekman, 1990). ARF has been shown to have genetic interactions with yeast YPT1 and several other gene products essential for ER to Golgi transport in yeast (Stearns et al., 1990; Kaiser and Schekman, 1990). More recently, ARF has been proposed to be involved in the biochemical machinery forming the putative coat of non-clathrin-coated vesicles (Serafini et al., 1991a, 1991b). In the presence of GTPγS, ARF is found at a stoichiometry of 3 molecules of ARF to 1 molecule of β-coat protein in non-clathrin-coated vesicles. β-Coat proteins are molecules reminiscent of the adaptin components of clathrin-coated vesicles (Wilson et al., 1991; Duden et al., 1991). On the other hand, the association of both β-coat protein and ARF with the Golgi complex is blocked by the drug Brefeldin A (Donaldson et al., 1990, 1991a, 1991b). In the presence of Brefeldin A endosomes fuse with the trans-Golgi network (Wood et al., 1991; Lippincott-Schwartz et al., 1991), transcytosis is inhibited (Hunziker et al., 1991), Golgi compartments collapse into the ER in vitro (Lippincott-Schwartz et al., 1989, 1990), and Golgi compartments fuse in vitro (Orci et al., 1991) (for review see Pelham, 1991). Thus, in the absence of ARF atypical membrane fusion events dominant the function of both the exocytic and endocytic pathways, suggesting that members of the ARF gene family may have a more general role in the regulation of membrane structure.

The ARF gene family is highly conserved from yeast to mammalian cells and contains at least six known members and eight additional potential members (Kahn et al., 1991, 1992). Similar to the rapidly growing family of rab proteins which contains at least 20 members distributed between different compartments of the endocytic and exocytic pathways, the ARF gene family is likely to encompass a group of functionally related proteins. Given the multiple roles of ARF in ER to Golgi and intra-Golgi transport (Kahn et al., 1992), as well as their potential role endosome fusion (Lenard et al., 1992), these combined results suggest that ARF may function in a general capacity to regulate membrane recognition and fusion of intracellular organelles.

REFERENCES

ARF Regulation of ER to Golgi Transport