The Amino Terminus of ADP-ribosylation Factor (ARF) Is a Critical Determinant of ARF Activities and Is a Potent and Specific Inhibitor of Protein Transport*

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Deletion of the amino-terminal 17 residues from human ADP-ribosylation factor (ARF) resulted in a protein ([AI-17]mARFlp) devoid of ARF activity but which retained the ability to bind guanine nucleotides with high affinity. Unlike the wild type, the binding of guanine nucleotides to this deletion mutant was found to be independent of added phospholipids. A chimeric protein was produced, consisting of 10% (the amino-terminal 17 amino acids) human ARFlp and 90% ARLlp, an ARF-like protein (55% identical protein sequence) from Drosophila. This chimera was found to have ARF activity, lacking in the parental ARLlp protein. Thus, the amino terminus of ARFlp was shown to be a critical component of ARF activity. A synthetic peptide, derived from the amino terminus of ARFlp, has no ARF activity. Rather, the peptide was found to be a specific inhibitor of ARF activities. This peptide was also found to be a potent and specific inhibitor of both an in situ intra-Golgi transport assay and the guanosine 5'-3-O-(thio)triphosphate-stimulated accumulation of coated vesicles and buds from Golgi preparations. We conclude that ARF is required for the budding of coated vesicles from the Golgi stacks and serves a regulatory role in protein secretion through the Golgi in eukaryotic cells.

The ADP-ribosylation factors (ARFs)* and structurally related proteins comprise one of the five subfamilies of the RAS superfamily of small (=20-kDa) regulatory GTP-binding proteins (for recent reviews see 1, 2). Although originally identified and characterized (3–5) as the protein cofactor required for the cholera toxin-catalyzed ADP-ribosylation of Gt, the stimulatory regulatory component of adenylate cyclase, more recent genetic and immunocytochemical data have provided evidence for an essential role for ARF proteins in protein secretion. Specifically, these results include a secretion-defective phenotype associated with ARF mutants in the yeast Saccharomyces cerevisiae (6), synthetic lethality observed when viable ARF mutations are combined with a specific subset of SEC genes (sec7–1, sec21–1, ypt1–1, and bet2–1) (6), and localization of ARF on the Golgi (6) and Golgi-derived structures (7) of mammalian cells. As ARFs are extremely well conserved both structurally (8) and functionally (9) in evolution, it is likely that the essential function of ARF proteins in yeast has been conserved in man.

Comparison of ARF with other members of the RAS superfamily reveals a number of biochemical and structural differences. Although all members of the RAS superfamily bind guanine nucleotides with high affinity, ARFs are unusual in having a much greater affinity for GDP than GTP (10). In addition, ARFs are unique in having a near absolute requirement for phospholipids to allow nucleotide exchange (5). Optimal conditions for nucleotide exchange and ARF activity assays include 3 mM L-a-dimyristoyl phosphatidylcholine (DMPC) and 0.1% sodium cholate (5). Although ARF proteins have low levels of intrinsic GTPase activity, mammalian ARFs and RAS proteins share only the conserved GTP binding consensus sequences, thus accounting for the 20–30% sequence identity. Although processing of the carboxyl termini of the RAS proteins is essential to their function(s) in vivo, ARFs do not undergo such carboxyl-terminal processing. Rather, all ARFs have a conserved glycine at residue 2, the site of attachment of the 14-carbon fatty acid, myristate (11). Myristoylation of ARF is required for its function in vivo, as the [G2A]ARFl protein is inactive in yeast cells but is equipotent with wild-type yeast ARF in the ARF assay.2 Alignments of ARF proteins to the RAS proteins revealed that ARFs have an amino-terminal extension of about 14–16 amino acids. Thus, the critical Gly2 residue of RAS is analogous to Asp26 in ARF proteins. With these differences between ARFs and RAS proteins it is likely that major differences in the mechanism of regulation and topology of functional domains of these proteins will emerge.

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The abbreviations used are: ARF(s), ADP-ribosylation factor(s); [G2A]ARFl, mutation resulting in a change from a glycine at position 2 to an alanine; DMPC, L-a-dimyristoyl phosphatidylcholine; GTP•S, guanosine 5'-3-O-(thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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2 R. A. Kahn, J. Clark, and C. Rulka, unpublished observation.
Incubation of Golgi membranes with cytosol and GTP\textsubscript{7}S results in the accumulation of small (75-nm) vesicles with a coat, visible by electron microscopy (12–15). These Golgi-derived, non-clathrin-coated vesicles are proposed to mediate the flow of proteins and lipids between stacks of the Golgi. Purification of these vesicles has allowed the identification of a number of coat proteins (16). ARF proteins have recently been shown to be present in at least stoichiometric amounts with other coat proteins and are thus among the most abundant proteins found associated with the coat (7) when vesicles are produced in the presence of GTP\textsubscript{7}S. For these reasons, we tested the consequences of addition of an amino-terminal ARF1 peptide (P-13) on the formation and stability of coated vesicles and buds emerging from the Golgi membranes.

Complete understanding of the role and mechanism of regulation of a specific cellular process by a specific GTP-binding protein will ultimately require a combination of structural, biochemical, and genetic data. The results presented below define a domain of an ARF protein that is essential for function as well as evidence that this domain exists in an ordered, \(\alpha\) helical structure stabilized by the covalent modification of the amino terminus, N-myristoylation. Those results led to the synthesis of a specific peptide inhibitor of ARF functions which provide compelling evidence for the essential role of ARF in protein transport through the Golgi stacks.

**MATERIALS AND METHODS**

**Expression and Purification of Recombinant Proteins—**A single bacterial expression system (17) was used to express each of the proteins used in these studies in large amounts. The individual coding regions were subcloned into the pET3C vector (18) after amplification by the inverse chain reaction to add an NdeI at the initiating methionine and a BamHI site 6 base pairs downstream of the stop codon. In each case induction of BL21(DE3) cells carrying the appropriate pET3C-derived plasmid with 1 mM isopropyl \(\beta\)-thiogalactopyranoside for 2-3 h resulted in the production of the recombinant protein at levels of 5-10% of total cell protein. In each case purification was achieved by affinity in lysosome and Triton X-100 followed by sequential purification of the soluble fraction on ion exchange (DEAE-Sephadex) and gel filtration (AcA 54) chromatographies in 20 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 2 mM MgCl\(_2\) (HENDM), as described previously for mARFlp (10), yARFlp (9), and dARLlp (19). Attempts to rescue the lethal double mutant arf1-arf2 after overexpression of [Al-17]mARFlp in yeast were performed as described in Kahn et al. (9).

**Biochemical Assays—**The binding of \([\text{S}]\text{GTP}\text{S}\) to ARF and related proteins was performed at 30 °C as described previously (5). GTPase (5), GDP off-rates (10), and ARF (2) assays were all performed at 30 °C and have been described elsewhere. The ARF assay measures the ARF-dependent, ADP-ribosylation of recombinant G\(_{\text{m}}\) (2.2 \(\mu\)M) with cholera toxin (40 \(\mu\)g/ml) in the presence of 20 \(\mu\)M GTP\textsubscript{7}S, 5 \(\mu\)M [\text{35S}]NAD (20,000 cpm/pmol), and the indicated concentration of ARF. This reaction is dependent on the presence of DMPC (80%) and sodium cholate (0.1%) in the reaction.

**Circular Dichroism Measurements—**CD spectra were recorded from 260–195 nm on a Jasco model J-500A/DP501N CD spectropolarimeter in Helima QH cells with a path length of 1 mm. Final peptide concentrations in solution were determined by amino acid composition determination after the addition of ninhydrin as internal standard. The mean residue ellipticity (\(\Theta\)) was calculated from the formula (20): 
\[
\Theta = 100 \times \Theta(\text{deg})/(\text{M} \times \text{cm} \times \text{dm})
\]

where \(\Theta\) is the ellipticity at 222 nm measured in millidegrees, M is the mean residue molarity, and I is the cell path length measured in cm. Fractional helical content for each peptide was calculated according to the assumption (21) that for a 100% helix and 16 peptide bonds, \(\Theta_{222} = 39,500 \times (1 - 2.6/16)\).

**In Vitro Protein Transport Assay—**Intra-Golgi transport was measured in vitro using the incorporation of \([\text{3}^3\text{H}]\text{acetylglucosamine into vesicles budding from recombinant G protein, as described in Balch et al. (22).}\

Incorporations included 25 mM HEPES, pH 7.4, 2.5 mM magnesium acetate, 25 mM potassium chloride, 50 \(\mu\)M ATP, 250 \(\mu\)M UTP, 5 mM creatine phosphate, 0.8 unit creatine kinase, 0.2 M sucrose, 100 \(\mu\)M dithiothreitol, 5.0 \(\mu\)g of donor membranes, 5.0 \(\mu\)g of acceptor membranes, 200 \(\mu\)g of bovine brain cytosol, and the indicated additions in a total volume of 50 \(\mu\)l.

**Electron Microscopy—**In vitro incubations for electron microscopy were performed as above, and the membranes were collected by centrifugation and fixed with glutaraldehyde. Membrane pellets were processed for electron microscopy and quantitated as described previously (23). All quantitations were performed in a double-blind fashion using a code that was not broken until the quantitation had been completed.

**Miscellaneous—**Peptides were synthesized and purified, by reverse phase or countercurrent distribution chromatographies, by Peptide Technologies (Washington, D.C.). Recombinant G\(_{\text{m}}\) was purified from bacteria as described in Graziano et al. (24). G\(_{\text{n}}\), was purified from bovine brain as described in Sternweis and Robishaw (25). Cholera toxin was purchased from Calbiochem and was preactivated by prior incubation at 37 °C in 25 mM potassium phosphate, pH 7.5, and 20 mM dithiothreitol for at least 15 min.

**RESULTS**

**An Amino-terminal Deletion Mutant of mARFlp Results in Loss of Function—**An amino-terminal deletion mutation was created in mARFl by deleting the first 51 base pairs of coding region and using the first internal methionine (Met\textsuperscript{19}) as the initiating methionine. Expression of this protein in bacteria resulted in a protein, termed [Al-17]mARFlp, with a predicted molecular mass of 18,800 daltons. Purification of this shorter, recombinant ARF protein was achieved using the same purification procedure used to obtain mARFlp in large amounts (10). The [Al-17]mARFlp migrated on sodium dodecyl sulfate gels with an apparent molecular mass of 18–19 kDa. The recombinant protein was found to bind \([\text{S}]\text{GTP}\text{S}\) with much higher stoichiometry than the wild-type protein (e.g. see Table I), reaching steady state of about 0.5 mol of GTP\textsubscript{7}S bound per mol of protein within 30 min. This is likely caused by the more rapid release of bound GDP from [Al-17]mARFlp \((t_{1/2} = 3 \text{ min at } 30 \degree C)\) than from the wild-type protein \((t_{1/2} = 20 \text{ min})\). Thus, the mutant protein maintained the ability to bind guanine nucleotides, although apparently with reduced affinity for GDP. Surprisingly, the mutant protein has lost the requirement for phospholipids and cholate to allow nucleotide exchange which has been described previously for ARF protein purified from mammalian tissues (5). The lack of a phospholipid requirement for nucleotide exchange cannot be explained by the lack of myristate at the amino terminus as recombinant ARF proteins purified from bacteria, which are also not myristoylated, retain the requirement for DMPC and cholate.

We next examined other activities of the mutant protein.

| Protein added | ADP-ribosylation sites | GTP\textsubscript{7}S binding sites | Specific activity* |
|---------------|------------------------|----------------------------------|-------------------|
| mARFlp        | 1.8                    | 240                              | 0.013             | 18,500          |
| [Al-17]mARFlp | 75                     | 29                               | 31.5              | 0.9             |
| dARLlp        | 150                    | 104                              | 62.9              | 1.7             |
| M              | 50                      | 1                                | 1.15              | 0               |
| M/dACp        | 100                    | 210                              | 2.34              | 0               |

*Specific activity is defined as fmol G\(_{\text{m}}\) ADP-ribosylated (ADP-rib) pmol of activated ARF (determined as the number of GTP\textsubscript{7}S binding sites under identical conditions) in a 20-min assay at 30 °C under standard conditions (2).
and compared it with the wild-type protein. The [Δ1-17] mARFlp was found to be essentially devoid of any activity (see Table I) as cofactor in the cholera toxin catalyzed ADP-riboseylation of G. Specific activities were about 10,000-fold lower than those for mARFlp. Very little activity was detected even at very high concentrations of the protein or GTP and in the presence or absence of DMPC and cholate. The [Δ1-17]mARFlp also did not inhibit the activity of mARFlp in the ARF assay. Further, we have been unable to rescue yeast cells having the lethal double mutation arfl-1 arf2-2, by expression of the shorter protein. In contrast, full-length mARFl and HARF4 do possess this ability (9).

The intrinsic GTP hydrolytic rate was determined for both wild-type and mutant proteins as the rate of release of \(^{32}\)P from \([\gamma ^{32}\text{P}]\text{GTP}\). No GTPase activity was detected for either the amino terminus of mARFlp to ARF activity we compared it with the wild-type protein. The [Δ1-17]mARFlp and the shorter protein. In contrast, full-length mARFl also did not inhibit the activity of mARFlp in the ARF assay an upper limit of GTPase activity was calculated to be 0.05 min\(^{-1}\) for mARFlp and 0.005 min\(^{-1}\) for [Δ1-17] mARFlp. The difference in the upper limit of activity results from the higher binding by the mutant. The upper limit for mARFlp has been determined previously to be no more than 0.005 min\(^{-1}\) (10).

It appears from the above results that the amino terminus of mARFlp is essential for ARF activity and may act both as an inhibitor of nucleotide release as well as being an important component of interactions between the ARF and other protein(s) and phospholipids.

**The Amino Terminus of mARFlp Is Capable of Bestowing ARF Activity on a Protein Otherwise Devoid of Such Activity—** dARLlp is the product of the essential Drosophila melanogaster ARl1 gene and shares 55% amino acid identity with both mammalian and yeast ARF proteins. In spite of the high degree of conservation this protein has been shown (19) to lack ARF activity in vitro and is proposed to have a distinct function from the ARF proteins. To test the importance of the amino terminus of mARFlp to ARF activity we constructed a chimeric protein in which the amino terminus of mARFlp was switched with that of dARLlp. The result is a protein, termed m/dACp (ARF chimeric protein) with over 90% originating from dARLlp and less than 10%, the amino-terminal 17 amino acids, from mARFlp.

Expression of m/dACp in bacteria was achieved using the same expression system as that used previously to express mARFlp (10), yeast ARF1p (9), dARLlp (19), and [Δ1-17] mARFlp (see above). The recombinant protein was purified using the same procedure that has been used for purified ARF proteins previously (10). The purified chimeric protein was examined in nucleotide exchange assays, the ARF assay, and in GTPase assays in the presence and absence of DMPC and sodium cholate.

The binding of GTP\(^\gamma\)S to the chimera was found to be identical to that of dARL1p in the presence of DMPC and cholate but was reduced by 87% when assayed in the absence of DMPC/cholate (see Fig. 1). Unlike ARF proteins which have less than 1% of the maximal amount of guanine nucleotide binding in the absence of DMPC/cholate, the binding of GTP\(^\gamma\)S to dARL1p is about 40-50% of the maximum in the absence of DMPC/cholate (19; Fig. 1). Thus, the effect of switching amino termini was to reduce significantly the amount of binding of GTP\(^\gamma\)S to the chimera when assayed in the absence of DMPC/cholate, making it more like the mammalian ARF proteins. This further supports the hypothesis (see above) that the amino terminus of mARFlp may play an important role in the protein-phospholipid interaction.

We then tested the activity of the chimera in the ARF assay as an indication of the importance of the amino terminus in protein-protein interactions. As seen in Table I, the chimeric protein has acquired ARF activity as determined by its ability to serve as cofactor in the ADP-riboseylation reaction. The specific activity of d/mACp was calculated to be about 100-fold lower than that of mARFlp. The actual intrinsic activity of the chimera in this assay is almost certainly higher than this, however, and may even be comparable to the mARFlp. This conclusion is based on the finding that the chimeric protein has a much higher rate of GTP hydrolysis (1.7 min\(^{-1}\)) than either the parent proteins (0.05 min\(^{-1}\) for dARL1p and < 0.005 min\(^{-1}\) for mARFlp). The GTPase rate

![Fig. 1. Time course of [\( ^{32}\text{P}\)]GTP\(^\gamma\)S binding to dARL1p and m/dACp in the presence and absence of DMPC and cholate. Binding of [\( ^{32}\text{P}\)]GTP\(^\gamma\)S to dARL1p (O, □) or m/dACp (■, △) was determined as described under "Materials and Methods" for varying times at 30 °C in the presence (O, □) or absence (■, △) of 3 mM DMPC and 0.1% sodium cholate. Values were then normalized relative to the maximum binding observed for each protein in the 60-min assay and are reported as the percentage of this maximum.](image-url)
no activity at concentrations as high as 300 μM. However, addition of the peptide to assays that contained mARFlp revealed an inhibitory activity of the peptide. The peptide was found to inhibit the ARF assay completely at 200 μM and above, and inhibition was half-maximal at 75 μM. Slightly greater sensitivity was consistently found when a 5–15-min preincubation was performed at 30 °C including all reactants, prior to the addition of cholera toxin. Under these conditions the half-maximal inhibition was observed at 40 μM peptide. Nine other peptides from other regions of ARF (see Table II) were also tested for inhibitory activity in the ARF assay. P-14 corresponds to amino acids 46–61 of mARFlp which includes most of the loop between the first and second consensus GTP binding domains. The corresponding region in p21 ras is termed the effector domain as it is thought to interact with the downstream effector. This peptide showed no inhibitory activity at concentrations as high as 300 μM. Two other peptides which correspond to residues 23–36 (P-2, includes the first consensus GTP binding domain) and 169–181 (P-16, the carboxyl terminus) showed no dose-dependent inhibition of the ARF assay, although in these cases there was lower ARF activity above 200 μM peptide.

A series of peptides (P-27 through P-31), each 2 residues shorter from the amino terminus, was also tested for inhibition of ARF activity. The 14-mer (P-27) appears to be as active an inhibitor as the 16-mer (P-13). The 12-mer (P-28) and 10-mer (P-29) were less active but retain some inhibitory activity (see Fig. 2). The two shorter peptides of 8 (P-30) or 6 (P-31) residues are each without activity as inhibitors of the ARF assay. The most divergent mammalian ARF known to date is hARF4p, which is 80% identical in sequence to hARFlp and has activities in both the in vitro ARF assay and the ability to rescue the lethal double mutant arf1"arf2" in yeast. In fact, the activity of hARF4p appears somewhat greater than that of mARFlp in each case. The amino-terminal 16-mer peptide (P-26) from hARF4p was synthesized and tested for anti-ARF activity and found to be at least as potent as P-13 (see Fig. 2). These two peptides are only 50% identical, sharing the conserved Leu-Phe repeats and charged lysines at the termini of ARF proteins and may require retention of at least 12 amino-terminal residues to retain inhibitory activity. The extent of inhibition of a fixed concentration of peptide can be lowered by increasing the concentration of ARF, suggesting that the peptide and ARF may indeed be competing for a similar site. However, at high concentrations of peptide (200 μM) the extent of the ADP-ribosylation of G, is clearly suppressed (not shown). Thus, the data indicate that the peptide is a mixed type of inhibitor of the ARF assay.

**The Amino Terminus of mARFlp Likely Forms an Amphipathic α Helix in Solution**—Examination of the sequence of the amino terminus of mARFlp reveals a repeat of hydrophobic residues with regular spacing; Ile-Phe-X-X-Leu-Phe-X-Leu-Phe. When plotted on an α helical wheel it is clear that the hydrophobic residues all align on one face with the opposite face containing the 2 asparagines and the lysine. To test the hypothesis that the amino terminus forms an α helical structure, CD spectra of P-13 and myristoylated P-13 were obtained under several conditions. As seen in Table III and Fig. 3, P-13 in aqueous solution (Fig. 3, dashed line with dots) has very little α helical tendency (2.2 and 0% at 22 and 4 °C, respectively). However, the addition of either 50% trifluoroethanol or 3 mM sonicated DMPC vesicles with 0.1% sodium cholate (Fig. 5, dashed line; optimal conditions for in vitro nucleotide exchange and ARF activities) dramatically alters the CD spectrum and indicates extensive α helix formation, as evidenced by the negative ellipticity maximum now apparent at 222 nm. The α helical content was calculated to be approximately 30% in each case (see Table III). This was increased to nearly 37% in 50% trifluoroethanol at 4 °C. No CD spectrum was obtained for DMPC-containing solutions below 27 °C because melting temperature of this phospholipid is around 23 °C. Thus, the amino-terminal peptide readily forms an α helix when exposed to a hydrophobic environment but not in water alone. Interestingly, when peptide 13 is myristoylated at the amino-terminal glycine (P-17), which is how the protein exists in vivo, we find the same maximal tendency toward an α helix even in an aqueous environment (Fig. 3, solid line). The addition of DMPC and cholate does not increase the α helical content (see Table III) of the myristoylated peptide. The presence of this acyl group on the peptide, and by extension on the whole protein, is likely to provide a microenvironment that stabilizes the amino terminus into an amphipathic α helix. When CD spectra of P-17 are generated either 1 day or 1 week after the solution is prepared a different result is obtained. The spectra, which exhibited a broad negative ellipticity maximum at 211 nm and with unchanged amplitude (data not shown), has no obvious interpretation but may result from micellar formation.

### Table II

**Peptides used in these studies**

| Name  | Sequence       | Amino acid residues | Inhibition of ARF assay | Inhibition of transport |
|-------|----------------|---------------------|-------------------------|------------------------|
| P-13  | GNIFANLFKGLFGKKE | 2-17 of mARFlp      | ++++                    | ++++                   |
| P-17  | myr-GNIFANLFKGLFGKKE | 2-17 of mARFlp      | ++++                    | ++++                   |
| P-27  | IFANLFKGLFGKKE | 4-17 of mARFlp      | ++++                    | +                      |
| P-28  | ANLFKGLFGK | 6-17 of mARFlp      | +                       | -                      |
| P-29  | LFKGLFGKKE | 8-17 of mARFlp      | +                       | -                      |
| P-30  | KGLFGKKE | 10-17 of mARFlp     | -                       | -                      |
| P-31  | LFKKE | 12-17 of mARFlp     | -                       | -                      |
| P-26  | GLTSSFLSRFLFGKKE | 2-17 of hARF4p      | +++                    | ++++                   |
| P-2   | CVGLDAAGKWYLYK | 23-36 of mARFlp     | -                       | -                      |
| P-14  | IPTGIFYNVETQVKNI | 46-61 of mARFlp     | -                       | -                      |
| P-16  | GLDWLSNQLRNQK | 169-181 of mARFlp   | -                       | -                      |
The Amino Terminus of ARF Is a Critical Domain

The cofactor activity in the cholera toxin reaction was determined, as described under "Materials and Methods," in the presence of 0.5 μM mARFlp and the indicated peptides. Peptide concentrations were 100 μM, except P-30 and P-31, which were 200 μM. Triplicate determinations were made and expressed as the activity relative to that in the absence of any added peptides. Vertical bars indicate the standard deviation.

Table III

| Peptide | Temperature °C | m" at 222 nm [θ] | %α Helix |
|---------|----------------|-----------------|---------|
| P-13    | 10 mM Tris, pH 7.4 | 23 1.3 761 2.2 |         |
| 50:50 10 mM Tris, pH 7.4, TFE | 4 0.0 0.0 0.0 |
| 10 mM Tris, pH 7.4, 3 mM DMPC, 0.1% cholate | 3 16.0 12,180 36.7 |
| P-17    | 10 mM Tris, pH 7.4 | 27 13.2 10,050 30.3 |
| 10 mM Tris, pH 7.4, 3 mM DMPC, 0.1% cholate | 27 10.4 9,366 28.2 |

or aggregation. When the myristoylated peptide was tested as inhibitor in in vitro assays of ARF function we also observed considerable variability with time; that is, freshly prepared solutions of P-17 inhibit the ARF assay at least as well as P-13, but with time, or freezing and thawing, the inhibitory activity becomes quite variable. Thus, this peptide was not used in further biochemical studies.

P-13 Is a Potent Inhibitor of in Vitro Protein Transport

A number of studies using different approaches all suggest that ARF proteins function in cells as a component of the protein secretory machinery. However, none of the antibodies raised against ARF proteins or peptides is neutralizing, and it has proven technically difficult to immunodeplete cell cytosol and membranes of ARF to test the role of ARF proteins in in vitro protein transport assays. The specific inhibitor of ARF activity in the cholera toxin-catalyzed reaction, peptide 13, was tested for potential inhibitory activity in the in vitro protein transport assay of Balch et al. (22). As seen in Fig. 4, P-13 is a potent inhibitor of protein transport with an IC50 of about 16 μM. The same peptides which had no effect on the ARF assay were tested and were also without effect on the protein transport assay. As seen in Fig. 4, loss of the 2 amino-terminal residues results in a 14-mer peptide, P-27, with reduced activity relative to P-13. The shortened peptides 12-mer, 10-mer, 8-mer, and 6-mer were all inactive in the protein transport assay (see Fig. 5). It is not clear whether these results indicate a specific sequence or length requirement for inhibitory activity. The peptides, listed in Table II, from other regions of mARFlp were also tested and found to be inactive as inhibitors of this assay. These results demonstrate that P-13 is a specific and potent inhibitor of this in vitro protein transport assay and is likely acting to inhibit the binding or activity of at least one ARF protein present in cytosol or on the Golgi.

Inasmuch as both mARFlp and hARF4p are active in the ARF assay and hARF4 has higher specific activity, we tested whether the peptide derived from the amino terminus of hARF4 (P-26) would also be inhibitory in the transport assay, and further whether it was as or more potent than P-13, derived from mARFlp. As seen in Fig. 4, P-26 is a potent inhibitor of the protein transport assay with an IC50 of 7 μM.
Although the absolute values vary between experiments performed on different days, we have found a consistent 2–5-fold greater potency for P-26 compared with P-13.

Peptide Inhibitors of ARF Activity Block the Formation of Golgi-derived Coated Vesicles—As seen in Table IV, line 1, and Fig. 6a, a 15-min incubation at 37 °C of Golgi membranes in the presence of cytosol and GTPyS results in a dramatic increase in the number of coated vesicles and buds. Inclusion of P-13 from the beginning of the incubation (Table IV, line 2; Fig. 6b) resulted in the loss of more than 96% of the coated vesicles and more than 95% of coated buds. If the coated vesicles are allowed to form prior to the addition of P-13, there is a much smaller effect of the peptide (compare Table IV, lines 3 and 4; see Fig. 6c), on the density of coated vesicles and coated buds; 56 and 27%, respectively, of the density of controls. The differences observed (lines 3 and 4) under these conditions may result from differences in the rate of production of coated vesicles during the 8-min (second) incubation with peptide or water, or some unknown effect of the peptide, but it is clear that the peptide does not cause wholesale loss of coats once formed. Rather, the peptide appears to block the formation of coats and, thus, prevents the formation of these 75-nm vesicles. Thus, the amino-terminal peptide is a potent inhibitor of GTPyS-stimulated formation or stabilization of coated vesicles and buds. Again, it is likely that the peptide is acting as an inhibitor of ARF proteins present in both cytosol and on the Golgi.

**DISCUSSION**

The results described above demonstrate the importance of the amino terminus of ARF proteins as a critical determinant of biochemical and physiological functions, including ARF activity in vitro, intrinsic GTPase activity, interaction with phospholipids and proteins, and the role of ARF in protein secretion. The data support the conclusion that the amino-terminal peptide, P-13, is a potent and specific inhibitor of ARF activities. This peptide has been used to demonstrate an absolute requirement for an ARF protein in the in vitro protein transport assay and in formation of coated vesicles and buds from Golgi stacks. Together with the secretion-defective phenotype of ARF mutants in yeast (6) and the localization of ARF proteins to Golgi stacks (6) and to non-clathrin-coated vesicles (7), these data provide compelling evidence of a role for ARF proteins as an essential component in the protein secretory pathway of eukaryotic cells. Specifically, ARF proteins are required for budding of vesicles, or possibly tubules, from the Golgi stacks and under some conditions are found to remain tightly associated with these Golgi-derived vesicles.

Peptides derived from specific regions of small GTP-binding proteins, including p21 ras (26) and rab (27) proteins, have been useful in identifying domains of these proteins which interact with specific effector proteins. In both cases cited, the effector binding domain of the RAS and RAB proteins appears to be in residues 32–40, between the first and second consensus sequences for nucleotide binding. X-ray crystallographic studies of RAS proteins have confirmed the amino terminus. Further experimentation, including three-dimensional structural studies, should provide an interesting comparison between ARF and RAS structures and are necessary to determine whether the structure of the putative effector domain at the amino terminus is sensitive to the type of guanine nucleotide occupying the site on ARF.

Examination of the data obtained from four recombinant proteins (mARFlp, dARLlp, [a1-17]mARFlp, and m/dACp) leads to several conclusions regarding the amino terminus of mARFlp. The amino-terminal 17 residues (i) are absolutely required for ARF activities in vitro and probably in vivo; (ii) act to constrain the release of bound GDP; and (iii) are likely to form an α helix, particularly after myristate addition. Finally, the GTPase activity of ARF, whether intrinsic or ARF GTPase-activating protein-stimulated, is proposed to lead to the alteration in the conformation of this highly

**Fig. 5.** The effect of peptides on in vitro intra-Golgi protein transport. N-[³H]Acetylglucosamine ([³H]GlcNAc) incorporation into vesicular stomatitis virus G protein was used to quantitate intra-Golgi protein transport as described under “Materials and Methods.” The concentration of peptides 13, 26, 27, 28, 29, 30, and 31 was 50 μM, and the values shown are the average percent of control ± the standard error for triplicate determinations.

**Table IV**

| Condition                  | Density of coated and uncoated vesicles plus coated buds | Density of coated vesicles | Density of coated buds* |
|----------------------------|--------------------------------------------------------|---------------------------|-------------------------|
| GTPyS (15 min)             | 34 ± 2                                                 | 24 ± 2                    | 8.8 ± 0.8               |
| GTPyS + P-13 (15 min)      | 4 ± 1                                                  | 0.8 ± 0.2                 | 0.4 ± 0.2               |
| GTPyS (15 min), then H₂O (8 min) | 22 ± 2                                              | 17 ± 2                    | 4.5 ± 0.7               |
| GTPyS (15 min), then P-13 (8 min) | 13 ± 1                                              | 9.6 ± 1                   | 1.2 ± 0.3               |

* Number/μm² of Golgi.
proteins. Specifically, the myristate may act to stabilize an amphipathic structure, an α helix, which is critical to the ARF (11), are found predominantly in the cytosol (e.g. 28).

The attachment of fatty acids to proteins is generally thought to provide a hydrophobic region which is necessary for attachment of proteins to membranes. In the case of myristoylated proteins, attachment to membranes is often dependent on myristoylation and dependent on GTP binding to the ARF. The attachment of fatty acids to proteins reveals some interesting similarities. Both P-13 and the wasp venom peptide, mastoparan, are small (16 versus 14 residues) peptides with activity toward myristoylated GTP-binding proteins. Both form α helices in the presence of phospholipid vesicles (29). Activities appear to target the amino terminus of the sensitive GTP-binding protein (31). However, whereas mastoparan can dramatically alter the dissociation rate of GDP from the sensitive G proteins, P-13 is without direct effect on the nucleotide binding properties of ARF. It remains an intriguing possibility that the amino terminus of ARF may have some mastoparan-like effects which could begin to explain the effect of ARF to modulate the sensitivity of G, to cholera toxin.

Arf proteins are myristoylated at the amino-terminal glycine (11), and this myristylation is required for function in vivo, inasmuch as the [G2A]Arf1 mutant is completely inactive in the yeast S. cerevisiae. 2 Association of ARF proteins with phospholipid vesicles or Golgi membranes is also dependent on addition of fatty acid to the amino terminus. 3 Thus, it is important to be able to discriminate clearly between direct effects of amino-terminal peptides on ARF activities from effects on the process of amino-terminal myristoylation. We consider the latter very unlikely because the required components of such a system, including the N-myristoyltransferase and the myristoylated CoA substrate, are not present in many of the in vitro assays used. That P-13 is not active as a result of inhibition of or competition for a site on N-myristoyltransferase can be further seen from the following

We favor the interpretation that the amino-terminal peptides block ARF activities through competition for specific ARF:effectector binding sites. Perhaps the strongest support for this conclusion comes from the ARF assay which is sensitive to inhibition by both amino-terminal peptides. We have found that the peptides do not block (phospholipid-dependent) guanine nucleotide exchange or stable association of activated ARF with the phospholipid vesicles. Thus, the most likely explanation is that the peptides interfere with the interaction of ARF with either G, or cholera toxin after association with the vesicles. However, conclusions regarding the mechanism of inhibition of ARF activities by the amino-terminal peptides are complicated by the absence of ideal controls. Controls performed in this and the accompanying papers (32, 33) remove concern of a gross disruption or detergent-like property of the peptides. However, it appears unlikely that there is a strict sequence requirement for inhibitory peptides (P-13 and P-26 are 50% identical). To address fully the contribution of specific residues in the peptide to inhibitory activities and structure, a series of peptides with point mutations is required (e.g. 26, 27, 29). Comparison of the effects of such isolated changes on structure versus activities of the peptide may address the role of α helix formation in inhibitory action. It is suggested that an amphipathic α helix with specific charge distribution acts at the interface between lipid bilayers and aqueous environments to disrupt specific protein (ARF):protein interactions, similar to what has been proposed for mastoparan (30).

A comparison of the above results with those described previously for the stimulatory effects of mastoparan on G proteins reveals some interesting similarities. Both P-13 and the amino terminus of the sensitive GTP-binding protein (31). Activities appear to target the amino terminus of the sensitive GTP-binding protein (31). However, whereas mastoparan can dramatically alter the dissociation rate of GDP from the sensitive G proteins, P-13 is without direct effect on the nucleotide binding properties of ARF. It remains an intriguing possibility that the amino terminus of ARF may have some mastoparan-like effects which could begin to explain the effect of ARF to modulate the sensitivity of G, to cholera toxin.

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three results. (i) The myristoylated peptide, P-17, is at least as active as the nonmyristoylated peptide, P-13. (ii) The 14-
mer peptide, P-27, which lacks the amino-terminal glycine, is active as an inhibitor of both the ARF and protein transport
assays. (iii) Myristoylated and nonmyristoylated forms of ARF are equipotent in the ARF assay, performed in the absence of any N-myristoyltransferase or myristoylated CoA. Thus, we have ruled out effects of peptides on potential N-
myristoylation as an explanation for the inhibitory (anti-
ARF) properties of the peptides.

Recent results have revealed the presence of at least eight new ARF or ARF-like cDNAs in human cells, in addition to the six already described. It is likely that a larger number of discrete activities will eventually emerge for subgroups or each individual protein in this family. Perhaps different ARFs target to different intracellular membranes to perform like functions. Although just one of many possibilities, recent results in several laboratories have provided evidence that ARF proteins may function to regulate membrane-membrane fusion events in a wide variety of in vitro assays including endoplasmic reticulum to Golgi transport (32), endosome-
endosome fusion (33), and nuclear membrane fusion. The results described above provide compelling evidence for the essential physiological role for (at least one) ARF in vesicle budding and protein transport through the Golgi stacks. It is hoped that the reagents described above will provide a beginning to unraveling the sites and mechanisms of action for ARF proteins in secretion and other cellular processes.

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