ADP-ribosylation factor 1 (ARF1) was originally found as a cofactor in CT-catalyzed ADP-ribosylation of Gαs but is now known to participate in vesicle trafficking. We asked whether ARF1 function in vesicular trafficking is necessary for CT-induced morphological changes in Chinese hamster ovary (CHO) cells, which result from increased intracellular cAMP. Brefeldin A treatment of cells suppressed CT action, confirming a requirement for Golgi integrity. Overexpression of a GFP-ARF1 fusion protein did not affect the morphological changes induced by CT, but changes were reduced in cells overexpressing guanine nucleotide exchange-defective ARF1(T31N) or GFP-ARF6(Q71L) mutants. In cells expressing these mutants, 8-bromo-cAMP-induced changes similar to those seen in cells transfected with ARF1 or vector. Inhibition of CT action was specific for mutants of ARF1 and not reproduced by analogous mutants of ARF5 or ARF6. GFP-ARF1(T31N) was mostly colocalized with βCOP, but ARF5(Q71L) less so. GFP-ARF6(Q67L) did not colocalize with βCOP and was partially associated with the plasma membrane. These data are consistent with the conclusion that ARF1 influenced CT action in cells by its specific function in the vesicular transport pathway used by CT to travel from plasma membrane to Golgi to ER.

Vibrio cholerae colonizes the surfaces of epithelial cells of the small intestine and produces cholera toxin (CT),1 causing severe diarrhea. CT comprises five identical B-subunits, which bind specifically to GM1 ganglioside on the cell membrane, and one A-subunit (CTA) that has enzymatic activity. CTA is cleaved into amino-terminal A1 and carboxyl-terminal A2 peptides by a bacterial protease acting at arginine 192. A1 and A2 are linked by a disulfide bridge between cysteine 199 and cysteine 187, reduction of which releases enzymatically active A1-subunit capable of ADP-ribosylating Gαs, a stimulatory regulator of adenyllyl cyclase, leading to persistent activation of adenyllyl cyclase with accumulation of cAMP (1–3).

According to the current view, after CT binds to GM1 ganglioside in the cell membrane, it is internalized and delivered to the Golgi and ER by vesicular transport (4). This concept was initially derived from reports that brefeldin A (BFA), which reversibly disrupts the Golgi architecture (5), suppressed CT action (6, 7), suggesting that CT action required Golgi function. Thereafter, immunofluorescence microscopy and subcellular fractionation studies demonstrated that both CTA and CTB are transported to Golgi, followed by CTA transfer to the ER (8) where the enzymatically active A1-subunit is likely to be generated (9, 10). This retrograde toxin transport is consistent with the presence of KDEL sequences near the carboxyl terminus of CTA (11, 12). These sequences serve as retrieval signals for soluble resident proteins of the ER and facilitate the retrograde transport of toxin proteins from Golgi to ER.

COP1-coated vesicles participate in retrograde traffic from Golgi to ER. Antibodies against βCOP, which is one of the components of COP1 coats, suppressed transport of CT from Golgi to ER (13). The budding of COP1-coated vesicles requires ARF1, a 20-kDa guanine nucleotide binding protein, which recruits COP1 coats to the membrane (14). ARF1 was discovered as an activator of CT-catalyzed ADP-ribosylation of Gαs (15). ARF1s belong to a multigene family, related to the Ras GTPase superfamily. Like other GTPases, ARF with GDP-bound is inactive, whereas the GTP-bound form is active. ARF1 activation is regulated by specific guanine nucleotide-exchange proteins (GEPs) (16, 17), which accelerate the binding of GTP to ARF1, and thereby ARF1-GTP binding to membranes with subsequent recruitment of COP1 coat proteins from the cytosol.

ARF1-GTP is inactivated by an ARF1 GTPase-activating protein (GAP) (18), one of which is recruited from cytosol to the transmembrane KDEL receptor (Er2d). The interaction of Er2d and GAP is enhanced by KDEL protein (19). Inactivated ARF1-GDP dissociates from the vesicle along with bound COP proteins. This uncoating is necessary for vesicle fusion with a target membrane. Thus, cycling of ARF1 between GDP and GTP forms is necessary for effective vesicular trafficking.

Six mammalian ARFs are now known. They are divided into three classes, with ARF1, 2, and 3 in class I, ARF4 and 5 in class II, and ARF6 in class III. ARF6 is most structurally different among the ARFs. It is localized in the plasma membrane and endosomes, where it participates in endocytosis and recycling of the plasma membrane (20, 21). Unlike ARF6, class I and II ARFs are associated with Golgi and ER, as well as endosomes, suggesting overlapping functions. There are reports showing the differences among ARFs in their regulatory proteins (22) and effectors (23).

To investigate the role of ARF1 in CT action in intact cells, ARF1 mutants, inactive ARF1(T31N), “a GDP mutant,” and...
Cholera Toxin Transport Requires ARF1 Cycling

MATERIALS AND METHODS

Reagents—8-Bromo cAMP (8Br-cAMP), brefeldin A (BFA), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma. CT was from List Biological Laboratories. FuGENE6 transfection reagent was from Roche Molecular Biochemicals. Monoclonal antibody against GFP was from Roche Molecular Biochemicals. Polyclonal rabbit anti-βCOP and anti-HA (Y-11) antibodies were from Affinity Bioreagents and Santa Cruz Biotechnology, respectively. cy3-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG were from Sigma. FITC-conjugated anti-mouse IgG was from ICN Pharmaceutical. The cAMP assay kit was from Cayman Chemical.

Cell cultures—CHO cells were grown at 37 °C in Eagle's MEM with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), in humidified air with 5% CO₂.

Assay of CT-induced Morphological Change in CHO Cells—Exponentially growing CHO cells were harvested and transferred to 6-well plates (3 × 10⁴ cells/well). Eagle's MEM supplemented with 1% FCS (2 ml/well) was added with or without CT. After incubation for 4–8 h in a CO₂ incubator, cells were fixed and inspected microscopically to determine the percentage of cells with morphological changes. More than 100 cells were counted in each plate. Cells overexpressing ARF were transferred to 6-cm plates (8 × 10⁵ cells/well) and incubated overnight at 37 °C in Eagle's MEM with 10% FCS. Medium was changed and BFA was added. After incubation for 30 min, 0.5 mM IBMX was added, followed 30 min later by 10 nM CT. 2 h later, cells were washed with PBS, and cAMP assay was performed.

Construction of ARF1 and ARF1 Mutants—Sequences of all plasmid clones were confirmed by sequencing. cDNAs for ARF1 and ARF1 mutants were constructed in pcDNA3.1+ (Invitrogen). To ligate ARF1 or mutant DNA in this vector, an Nhel site was introduced upstream of the initiation codon and a BamHI site was inserted after the termination codon. These sites were used for ligation of all constructs into pcDNA3.1+. For ARF1, PCR was performed with forward primer F3 (5′-CTCGCGTGCATCAAGGGAACATCTTCGCAAC-3′) and reverse primer R5 (5′-GGGAAGGTGCTATCCAAAATCTTG-3′). Both PCR products were gel-purified before use as templates for the second PCR using F3 and R5 to synthesize full-length ARF1(Q71L), which was ligated into pcDNA 3.1+ vector. All ARF1, ARF1(Q71L), and ARF1(Q71L) constructs were transformed into DH5α competent cells.

EGFP fusion ARF1 and ARF1 mutants (referred to subsequently as, e.g. GFP-ARF1) were prepared by PCR-based amplification from ARF1 and mutant cDNA in pcDNA3.1+. Forward primer was 5′-CCTGAGCCACCATGGGAACATCCTTCGCAAC-3′ (boldface is the Nhel site; initiator ATG is underlined) and reverse primer 5′-CAGCTCCGGAAACGAAAGCTTCC-3′ (boldface is a BamHI site). PCR product was ligated into pEGFP-N1 (CLONTECH).

HA epitope-tagged ARF1 and ARF1 mutants were produced by PCR-based amplification of ARF1 and mutant cDNA in pcDNA3.1+ vector. Forward primer was the same used to prepare GFP fusion ARF1 and reverse primer was 5′-AAGGGGATCCATGGGAACATCCTTCGCAAC-3′. Amplified products were ligated into pcDNA3.1+ vector that had been digested with Nhel and BamHI and ligated into pcDNA3.1+ vector that had been digested with Nhel and BamHI.

Construction of ARF5 and ARF5 Mutants—GFP-ARF5 and mutant fusion constructs were produced by PCR-based amplification from ARF5 cDNA. For GFP-ARF5, forward primer 5′-TCTGCTAGGCCACCATGGGAACATCCTTCGCAAC-3′ and reverse primer 5′-AGGGGATCCATGGGAACATCCTTCGCAAC-3′ were used. Boldface sequences in forward and reverse primers are Nhel and BamHI sites, respectively. Amplified products were digested with Nhel and BamHI and ligated into pEGFP-N1 vector that had been digested with the same enzymes, which was then transformed into DH5α competent cells. To construct HA-tagged ARF5, reverse primer was 5′-AAGGGGATCCATGGGAACATCCTTCGCAAC-3′ and reverse primer 5′-AGGGGATCCATGGGAACATCCTTCGCAAC-3′ were used. Boldface sequences in forward and reverse primers are Nhel and BamHI sites, respectively. Amplified products were digested with Nhel and BamHI and ligated into pcDNA3.1+ vector that had been digested with Nhel and BamHI.

Construction of ARF6 and ARF6 Mutants—For HA-tag constructs, ARF6 cDNA was amplified from ARF6pET7 (26) by PCR with forward primer 5′-CCGGAATTCATGGGAACATCCTTCGCAAC-3′ and reverse primer 5′-GGCGAACTTTTAAAGGGAACATCCTTCGCAAC-3′. Boldface sequences in forward and reverse primers are EcoRI and XbaI restriction sites, respectively. HA-tag sequence is underlined, and italics represent the starting and termination codons, respectively. The PCR product was purified and ligated in-frame to the EcoRI- and XbaI-digested pXES expression vector. For GFP fusion construct, ARF6 cDNA was amplified from ARF6pET7 by PCR with forward primer 5′-TATCTGCAAGTGGAAGGTGCTCAGTACCCAAATCTCC-3′ and reverse primer 5′-ATAATGCAACATTCTTTAGTATGAGTTAC-3′. Boldface sequences are XhoI and SalI sites, respectively. Italic is the starting codon. The PCR product was purified and ligated in-frame to pcDNA3.1+ vector. For ARF1(Q71L), two PCR products, one with forward primer F3 and reverse primer R4 (5′-CAGGGGGCGATCGTTGTACCGGCCACCATCTCCGCAAC-3′) and the other with forward primer F5 (5′-GGCGGAAGCTTGGTGCTCAGTACCCAAATCTCC-3′) and reverse primer R5 were prepared. Mutated bases are represented as italic in F3 and T5 in F6. Both PCR products were gel-purified before use as templates for the second PCR with primers F3 and R5 to synthesize full-length ARF1(Q71L). The PCR product was ligated into pcDNA 3.1+ vector. For ARF1(Q71L), two PCR products, were prepared. Mutated bases are represented as italic in F3 and T5 in F6. Both PCR products were gel-purified before use as templates for the second PCR with primers F3 and R5 to synthesize full-length ARF1(Q71L). The PCR product was ligated into pcDNA 3.1+ vector.
the XhoI- and SalI-digested pEGFP-N1 expression vector. ARF6(T27N) and ARF6(Q67L) mutant were generated by site-specific mutagenesis using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. ARF6(T27N) was generated by PCR with forward primer 5'-GACGCGGCCGGCAAGACTACAATCTGTTGACAA-3' and reverse primer 5'-CTTGTACAGGATTGGTG-3' and reverse primer 5'-CGGCCGATTCGTCGAGAGCCGACATCACC-3'. Italized sequences indicate mutated bases.

**Indirect Immunofluorescence**—Cells were fixed with 3% paraformaldehyde in PBS for 20 min, incubated with 50 mM NH4Cl for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 4 min. Then cells were treated with 3% bovine serum albumin in PBS for 1 h for blocking. To identify the HA epitope, cells were incubated with polyclonal anti-HA antibody followed by cy3-conjugated sheep anti-rabbit IgG antibody. For double staining of HA-tagged ARF and βCOP, cells were incubated with monoclonal anti-HA antibody (1:400) as a primary antibody and then FITC-conjugated goat anti-mouse IgG (1:3000) for 1 h as a secondary antibody. To identify βCOP, polyclonal rabbit anti-βCOP antibody (1:2000) was the primary antibody and cy3-conjugated sheep anti-rabbit IgG (1:3000) was used as the secondary antibody. All procedures used were performed at room temperature, and, following each step, cells were washed with PBS three times.

**RESULTS**

**CT-induced Morphological Changes in CHO Cells**—When CHO cells, which had been detached with trypsin, were incubated with 10 nM CT, 60–80% of the cells became bi-polar in shape or less frequently showed polymorphic shape changes. Most of the untreated cells were round even after overnight culture (usually fewer than 5% of cells changed their morphology) (Fig. 1). Similar morphological changes were induced by

**Fig. 2. Effect of CT and 8Br-cAMP on CHO cell morphology.** Cells were incubated in the presence of 10 nM CT (open square) or 0.5 mM 8Br-cAMP (closed square). At the indicated time, cells were fixed and morphology was evaluated microscopically. More than 100 cells were examined at each time to calculate percentage of morphologically changed cells. The data are average of two plates.

**Fig. 3. Effect of BFA on CT action on CHO cells.** A, freshly trypsinized CHO cells were plated as in Fig. 1 and incubated with the indicated concentration of BFA for 30 min before addition of 10 nM CT, at which time, the cells were still floating. After mixing well and incubation for 7 h more, cells were fixed and the morphology was examined as in Fig. 2. B, CHO cells (2 × 10⁵) were incubated in 3-cm plates overnight, then for 30 min with BFA at the indicated concentration, followed by addition of 50 mM IBMX for another 30 min. 10 nM CT was then added, and 2 h later, cells were washed twice with PBS and extracted with 500 μl of 6% trichloroacetic acid for assay of cAMP. Data are means of values from duplicate samples.

**Fig. 4. Expression of wild-type ARFs and ARF mutants.** CHO cells were transfected overnight with pEGFP vector containing ARF, GDP-bound ARF, or GTP-bound ARF construct or pcDNA3.1 vector containing HA epitope-tagged ARF and its mutant constructs. The cells were collected from the plates and washed once with PBS and disrupted with sonication. Samples (20 μg) of the homogenate protein were used for SDS-polyacrylamide gel electrophoresis in a 14% gel, followed by transfer to nitrocellulose filters. Expression of GFP fusion proteins or HA-tagged proteins was quantified with HA epitope-tagged ARF and its mutant constructs. The cells were collected from the plates and washed once with PBS and disrupted with sonication. Samples (20 μg) of the homogenate protein were used for SDS-polyacrylamide gel electrophoresis in a 14% gel, followed by transfer to nitrocellulose filters. Expression of GFP fusion proteins or HA-tagged proteins was quantified with HA epitope-tagged ARF and its mutant constructs. The cells were collected from the plates and washed once with PBS and disrupted with sonication. Samples (20 μg) of the homogenate protein were used for SDS-polyacrylamide gel electrophoresis in a 14% gel, followed by transfer to nitrocellulose filters. Expression of GFP fusion proteins or HA-tagged proteins was quantified with HA epitope-tagged ARF and its mutant constructs.
ARF1 mutants were expressed in CHO cells and CT action was evaluated. Percentage of morphologically changed cells is reported. Values are means of duplicate samples.

The localization of ARF1 and ARF1 mutants in CHO cells was examined by fluorescence microscopy. ARF1 localized to the cytosol and Golgi region, as expected if ARF-GTP were membrane-associated (data not shown). On the other hand, ARF1(Q71L) (GTP-bound) was chiefly cytosolic. Overexpression of ARF1 and ARF1(Q71L) mutants did not suppress CT-induced morphological changes. This experiment was performed as described for Fig. 6A. Data are representative of four similar experiments.

These data suggested that ARF1 activation is necessary for CT action on CHO cells. To confirm this, we examined the effects of overexpression of ARF1 dominant negative and constitutively active mutants on the CT-induced morphological changes in CHO cells.

Transient Expression of Wild Type ARF1 and ARF1 Mutant Proteins in CHO Cells—CHO cells were transfected with pEGFP expression vector and GFP fusion wild type or mutant ARF1 constructs. Expression was observed initially at 8 h, and the percentage of cells expressing the GFP fusion proteins gradually increased to a maximum at 37 h, where it remained for at least 50 h (data not shown). Transfection efficiency was 2–5% in CHO cells. Western blotting of cell homogenate proteins demonstrated expression of ARF1 and ARF1 mutants using anti-GFP antibody (Fig. 4). The localization of ARF1 and ARF1 mutants in CHO cells was examined by fluorescence microscopy. ARF1 localized to the cytosol and Golgi region whereas ARF1(T31N) (GDP-bound) was chiefly cytosolic. On the other hand, ARF1(Q71L) (GTP-bound) was concentrated in the Golgi region, as expected if ARF-GTP were membrane-associated (data not shown).

Time Course of CT-induced Morphological Changes in ARF1-expressing Cells—Effects of GFP-ARF1 on CT-induced morphological changes were examined. After CT addition, cells were fixed at the indicated times, and morphology was evaluated by fluorescence microscopy for comparison with that of control cells (FuGENE6-treated). As shown in Fig. 5, ARF1 overexpression did not change the rate of appearance of CT-induced morphological changes.

CT-induced Morphological Changes in Cells Expressing ARF1 Mutants—GFP fusion ARF1 mutants were transfected to evaluate the importance of ARF1 function in CT action. CT-induced changes in morphology of ARF1-expressing cells were similar to control cells (FuGENE6 6-treated) and to cells transfected with vector alone (Fig. 6A). In cells expressing ARF1(T31N) or ARF1(Q71L) mutants, CT-induced morphological changes were significantly suppressed. To confirm that this effect was not related to GFP, HA epitope-tagged ARF1 and ARF1 mutants were expressed in CHO cells and CT action was determined. In this experiment, expression was detected by treatment with dibutyryl cAMP, 8Br-cAMP, or forskolin, a direct activator of adenyl cyclase (data not shown). Appearance of the morphological changes was about 1 h later with CT than with 8Br-cAMP (Fig. 2), corresponding to the time needed for CT internalization and activation (27).

BFA Suppressed CT-induced Morphological Changes in CHO Cells—BFA was used to determine whether an intact Golgi complex was necessary for CT action on CHO cells. BFA is a fungal metabolite that reversibly disrupts Golgi structure by stabilizing an ARF1GEP-ARF1-GDP complex, thereby preventing ARF1 activation (28). In the presence of BFA, CT-induced morphological changes were reduced (Fig. 3A). BFA treatment also suppressed the accumulation of cAMP (Fig. 3B). These data suggested that ARF1 activation is necessary for CT action on CHO cells. To confirm this, we examined the effects of overexpression of ARF1 dominant negative and constitutively active mutants on the CT-induced morphological changes in CHO cells.

Transient Expression of Wild Type ARF1 and ARF1 Mutant Proteins in CHO Cells—CHO cells were transfected with pEGFP expression vector and GFP fusion wild type or mutant ARF1 constructs. Expression was observed initially at 8 h, and the percentage of cells expressing the GFP fusion proteins gradually increased to a maximum at 37 h, where it remained for at least 50 h (data not shown). Transfection efficiency was 2–5% in CHO cells. Western blotting of cell homogenate proteins demonstrated expression of ARF1 and ARF1 mutants using anti-GFP antibody (Fig. 4). The localization of ARF1 and ARF1 mutants in CHO cells was examined by fluorescence microscopy. ARF1 localized to the cytosol and Golgi region whereas ARF1(T31N) (GDP-bound) was chiefly cytosolic. On the other hand, ARF1(Q71L) (GTP-bound) was concentrated in the Golgi region, as expected if ARF-GTP were membrane-associated (data not shown).

Time Course of CT-induced Morphological Changes in ARF1-expressing Cells—Effects of GFP-ARF1 on CT-induced morphological changes were examined. After CT addition, cells were fixed at the indicated times, and morphology was evaluated by fluorescence microscopy for comparison with that of control cells (FuGENE6 6-treated). As shown in Fig. 5, ARF1 overexpression did not change the rate of appearance of CT-induced morphological changes.

CT-induced Morphological Changes in Cells Expressing ARF1 Mutants—GFP fusion ARF1 mutants were transfected to evaluate the importance of ARF1 function in CT action. CT-induced changes in morphology of ARF1-expressing cells were similar to control cells (FuGENE6 6-treated) and to cells transfected with vector alone (Fig. 6A). In cells expressing ARF1(T31N) or ARF1(Q71L) mutants, CT-induced morphological changes were significantly suppressed. To confirm that this effect was not related to GFP, HA epitope-tagged ARF1 and ARF1 mutants were expressed in CHO cells and CT action was determined. In this experiment, expression was detected by
indirect immunostaining method using anti-HA rabbit antibody and then cy3-conjugated anti-rabbit IgG antibody. The localization of expressed proteins was similar to that of GFP-ARF fusion proteins. The rate of CT-induced morphological changes in cells expressing GFP-ARF6 fusion protein was similar to that of cells expressing vector (Fig. 7A). No effect of ARF6(Q67L) was observed. Furthermore, the ARF6(T27N) mutant did not suppress CT action on CHO cells, although participate in Golgi to ER trafficking. Therefore, ARF6 seems not to be involved in CT action. To confirm this, the effects of wild type ARF6, ARF6(Q67L)(GTP-bound), and ARF6(T27N)-(GDP-bound) mutants were evaluated. ARF6 and ARF6(T27N) and ARF6(Q67L) mutants were expressed as GFP fusion proteins and HA-tagged proteins. The rate of CT-induced morphological changes in cells expressing GFP-ARF6 fusion protein was similar to that of cells expressing vector (Fig. 7A). No effect of ARF6(Q67L) was observed. Furthermore, the ARF6(T27N) mutant did not suppress CT action on CHO cells, although
clear suppression was observed in ARF1 mutants expressing cells (Fig. 7B). HA-tagged ARF6 was also used to examine the CT effect, and a similar result was obtained (data not shown). These data suggested that ARF6 did not participate in CT action on CHO cells.

Effect of ARF5 on the Morphological Changes Induced by CT—To examine whether ARF5 affected CT action in CHO cells, GFP fusion and HA-tagged wild type ARF5 constructs, and ARF5(T31N)(GDP-bound) and ARF5(Q71L)(GTP-bound) mutants were investigated. Expression was confirmed by Western blotting and shown in Fig. 4. Cellular localization of GFP fusion ARF5 protein was compared with GFP fusion ARF1 and HA epitope-tagged ARF6 (Fig. 8). ARF5 localized to perinuclear region and cytosol similar to ARF1. Some ARF5 colocalized with βCOP, Q71L mutants of ARF1 and ARF5 were localized to the perinuclear region, and ARF1(Q71L) was mostly colocalized with βCOP, but ARF5(Q71L) less so (Fig. 9). HA-tagged ARF6 and ARF6(Q67L) did not colocalize with βCOP and were in part associated with plasma membrane. ARF5(T31N) was more sparsely distributed throughout cells than was wild type ARF5 (data not shown).

Morphological changes induced by CT in cells expressing GFP-ARF5(T31N) were slightly suppressed, but less so than with ARF1(T31N) (Fig. 10). No suppression was observed in ARF5(Q71L) mutant-expressing cells, although clear suppression was observed in ARF1(Q71L)-expressing cells. These data were similar to those obtained with HA epitope-tagged ARF5 (data not shown).

8Br-cAMP Induced Morphological Changes in Cells Expressing ARF1 Mutants—To determine whether the ARF1 effect on CT-induced morphological changes was specific for CT action and was not effected by cAMP, the effect of 8Br-cAMP was determined (data not shown).

DISCUSSION

To determine the effect of ARF1 function in CT action in vivo, we used the dominant negative and constitutively active mutants of ARF1, ARF1(T31N) and ARF1(Q71L). Overexpression of wild-type ARF1 did not accelerate the appearance of CT-induced morphological changes. Expression of either mutant of ARF1 suppressed morphological changes induced in CHO cells by CT more than did vector alone or wild type ARF1. Thus ARF1 cycling is necessary for CT action. The suppression, however, was not complete and some percentage of cells always showed changes in morphology, with a different percentage in each experiment. This may be the result of differences in levels of expression in different cells, with some cells not expressing mutant protein sufficient to suppress endogenous ARF1 function.

Suppressive effects of class II and class III ARF mutants were not found. Reports of the functional overlap of class I and II ARFs include the inhibition of ER to Golgi transport by aminoterminal peptides of ARF1 and ARF4 (29) and rescue of ARF-deleted yeast by expression of ARF1 and ARF4 (30). The peptides in the former study, however, are probably not specific inhibitors of ARF function (31, 32). Mutants of ARF5, which like ARF4 belongs to class II, did not clearly suppress CT action, suggesting that ARF5 cycling is not necessary in CT action. Indirect immunofluorescence microscopy did show differences between the GTP forms of ARF1 and ARF5 in localization with βCOP, consistent with their participation different pathways of vesicular transport. ARF6 (class III) did not affect CT action.

ARFs are activators of CT-catalyzed ADP-ribosylation of Goα. In vitro, all ARFs are effective in this reaction. It is not known which ARF, or whether any ARF, is necessary for CT activation in intact cells. ADP-ribosylation of Goα by CT in vitro requires GTP and phosphatidylcholine (33), suggesting roles for ARF activation and a membrane environment. If ADP-ribosylation of Goα occurs at the cytoplasmic face of the plasma membrane, near its effector adenyl cyclase, ARF6 might be a good candidate because of its localization. Other ARFs are not localized at the plasma membrane. In our experiment, the GTP-bound form of ARF6 did not affect CT action, suggesting that it did not participate in activation of the toxin.

It appears that all available data are most consistent with the notion that ARF1 influences CT action in cells by its critical physiological function in the required transport of CT from plasma membrane through the Golgi and ER.

Acknowledgments—We thank Dr. M. Vaughan for critical review of this paper. We also thank Drs. N. Toyoda and M. Komiyama for assistance with the confocal microscope.

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