The Escherichia coli Heat Labile Toxin Binds to Golgi Membranes and Alters Golgi and Cell Morphologies Using ADP-ribosylation Factor-dependent Processes*

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The fate of the catalytic subunit of the Escherichia coli heat labile toxin (LTA) was studied after expression in mammalian cells to assess the requirement for ADP-ribosylation factor (ARF) binding to localization and toxicity and ability to compete with endogenous ARF effectors. A progression in LTA localization from cytosol to binding Golgi stacks to condensation of Golgi membranes was found to correlate with the time and level of LTA expression. At the highest levels of LTA expression the staining of LTA and both extrinsic and luminal Golgi markers all became diffuse, in a fashion reminiscent of the actions of brefeldin A. Thus, LTA binds to the Golgi and can alter its morphology in two distinct ways. However, point mutants of LTA that are defective in the ability to bind activated ARF were also unable to bind Golgi membranes or modify Golgi morphology. Co-expression of mutants of ARF3 that regained binding to these same mutant LTA proteins restored the localization and activities of the toxin. Thus, binding to ARF is required both for the localization of the toxin to the Golgi and for effects on Golgi membranes. A correlation was also seen between the ability of LTA mutants to bind ARF and the increase in cellular cAMP levels. These results demonstrate the importance of ARF binding to the toxicity and cellular effects of the ADP-ribosylating bacterial toxin and reveal that mutants defective in binding ARF retain basal ADP-ribosylation activity but are the least toxic LTA mutants yet described, making them the best candidates for development as mucosal adjuvants.

Escherichia coli heat labile (LT) and choleragen (CT) toxins are heatstabilized proteins that complex with host enzymes, ADP-ribosyltransferase activity, and >80% primary sequence identity. During intoxication of mammalian cells, these enzymes catalyze the covalent modification of the regulatory, stimulatory subunit of adenyl cyclase, Gs, resulting in persistent activation of the cyclase (1–4). The high levels of cAMP generated in gut epithelial cells promote the secretion of chloride and water into the gut lumen and the resulting diarrhea that can be fatal if not treated. Neither toxin is very specific in the choice of substrate and can transfer the ADP-ribose moiety from NAD onto almost any arginine acceptor or even water (NADase activity). Both the enzymatic activity and specificity for Gs of each toxin can be shown in vitro to be increased markedly (>50-fold) by the addition to the in vitro reaction of the protein co-factor, ADP-ribosylation factor (ARF) (5, 6).

ARFs were later found to be ubiquitous and essential guanine nucleotide-binding proteins with a diverse array of functions and activities in eucaryote, including the regulation of membrane traffic (7, 8), phospholipase D (9, 10), and phosphatidylinositol 4-phosphate, 5-kinase activities (11, 12). The use of specific host proteins by pathogenic organisms is unlikely to be arbitrary and has led us to propose additional functional interactions between ARFs and these bacterial toxins. For example, in a recent study we identified a short sequence in the A2 subunit of LT with sequence homology to the effector binding switch 2 domain of ARF that has weak ARF mimetic activity (13). However, it has not yet been shown whether ARFs play any role in the actions of these bacterial toxins in live cells.

LT and CT are AB₅ type toxins in which the five identical B subunits bind to receptors on target cells. The structure of the holotoxin has been solved (14, 15) and reveals that the A subunit lies in the center of a hole created by a ring of the five B subunits. The A subunit is made up of the catalytic A₁ and linker A₂ chains. Although it was originally thought that the B subunit doughnut promoted penetration of the A subunits across the plasma membrane and access to the Gs and adenyl cyclase at the cell surface, it was later found that the toxins must transit the entire secretory pathway in reverse, probably making use of the endoplasmic reticulum-retrieval (KDEL) sequence at the C terminus of the A₂ subunit (16–19). Access to the cytotoxic A₂ subunit is achieved only after traffic to the Golgi and then endoplasmic reticulum (17, 20, 21). It was assumed that the toxin then finds its way to the plasma membrane where it activates the Gs and cyclase. The levels of toxin achieved in cytosol are normally quite low because toxicity prevents continued entry. This has limited the ability to localize the A subunit in intact cells.

In this report we made use of a collection of recently described (13) mutations in LTA and human ARF3 that are altered in their ability to bind each other, to test the importance of ARF binding to toxin action in intact cells. Our results provide the first experimental evidence for a required role for ARFs in the actions of these toxins.

EXPERIMENTAL PROCEDURES

Materials—The FuGene6 transfection reagent was obtained from Roche Molecular Biochemicals. Ham’s F-12 medium was purchased from Life Technologies, Inc. Other reagents and chemicals were obtained from Sigma.

ARF and LTA Mutants—Each of the mutants used in this study were
FIG. 1. The level of LTA1 expression in CHO cells increased with time between 12 and 48 h. CHO cells were transiently transfected with Myc-His6-LTA1, for different periods of time and labeled with antibodies to the Myc epitope or Mann II, as described under “Experimental Procedures.” The levels of expression of LTA1 were determined by visual inspection of the intensity of anti-Myc staining and were scored 12–48 h after transfection as weak, medium, or strong.

Results

LTA1 Expressed in Mammalian Cells Binds to Golgi Membranes—To study the fate of the catalytic subunit of LT, LTA1, once in the cytosol, and assess the importance of ARF binding to cell toxicity and the ability of LTA1 to compete with endogenous ARF effectors, we transiently transfected a plasmid (pCDNA3-LTA1-Myc-His6), carrying C-terminally Myc- and His6-tagged LTA1 under control of the strong, constitutive cytomegalovirus promoter, into CHO cells. The location of LTA1 within transfected cells was determined by indirect immunofluorescence using a monoclonal antibody directed against the Myc tag or polyclonal antibody to the His6 epitope. Fluorescence intensity was used as a measure of the level of protein expression and was classified as low, intermediate, or high. As expected, the level of LTA1 expression in CHO cells was time-dependent (Fig. 1). 12 h after transient transfection, we found 57% of expressing cells exhibiting low level expression, 31% had intermediate expression, and 11% high expression of LTA1 (Fig. 1). At later times (e.g. 24 h), the number of cells with low level of expression of LTA1 decreased and almost disappeared, whereas the number of cells with medium or strong expression of LTA1 increased from 31 and 11% to 49 and 48%, respectively (Fig. 1). This temporal progression is important to document both as an indication that the expressed protein is stable in these cells and because later analyses of phenotypes depend on similar temporal progressions and correlations.

When LTA1 can first be detected (low level) we observed a diffuse signal in cytosol (Fig. 2A) and markers of the Golgi apparatus reveal the normal tight, perinuclear staining, as visualized by immunostaining with Mann II (Fig. 2B), β-COP, and AP-1 antibodies were used as markers of the medial, cis-, and trans-Golgi compartments, respectively. Texas Red-conjugated anti-mouse IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG (Cappel) were the secondary antibodies used in immunofluorescence studies.

Data Collection—The images were obtained using an Olympus BX-60 with 100× objective and B-max filter cubes. Images were processed using Image Pro software. Fluorescent cell staining studies were each performed at least three times with similar results. The levels of protein expression were compared by using the same integration time within each experiment.

Intracellular cAMP Determinations—Intracellular cAMP was determined using the nonacetylation enzyme immunoassay procedure, as provided by Amersham Pharmacia Biotech, according to the manufacturer’s directions. CHO cells were grown and transfected as described above. 18 h post-transfection the cells were trypsinized and washed twice with Ham's F-12 medium, and cell density was determined. Cells (2.5 x 10⁶) were added to wells of a standard 96-well microtiter plate and spun at 1500 x g for 5 min, and the supernatant was discarded. The cell pellets were resuspended in 200 µl of lysis reagent and agitated for 10 min. Samples (100 µl) and standards (100 µl; 12.5-3200 fmol/well) were incubated at 4°C for 2 h with 100 µl of rabbit anti-cAMP antisera before the addition of 50 µl of cAMP-peroxidase conjugate, and the reaction was allowed to proceed for an additional hour. The peroxidase substrate (3,3',5,5' -tetramethylbenzidine) was added at room temperature, and the colored product was allowed to accumulate for 1 h before stopping with 1 M sulfuric acid. The optical density was determined in a plate reader at 450 nm within 30 min. The standard curve was plotted and filtered by the exponential decay equation y = y₀ + A e⁻ˣ⁻ˣ₀, where y₀ stands for offset and A stands for amplitude, using Origin 4.0 software.
diffuse staining of Mann II in related with the time and level of LTA 1 expression. The staining of the same cells for Mann II are shown in the panels on the right. Note the co-localization of LTA 1 with Man II in B–F and the diffuse staining of Mann II in H, seen at high levels of LTA 1 expression.

**Fig. 2. Patterns of localization of LTA 1 in CHO cells.** CHO cells were prepared for indirect immunofluorescence 16 h after transient transfection with Myc-His 6-LTA 1, as described under “Experimental Procedures.” Mouse monoclonal Myc and rabbit polyclonal Mann II antibodies were used as primary antibodies to identify the LTA 1 and Golgi compartments, respectively. Weak, medium, and strong levels of expression of LTA 1 are shown in A, C, E, and G, respectively. The staining of the same cells for Mann II are shown in the panels on the right. Note the co-localization of LTA 1, with Man II in B–F and the diffuse staining of Mann II in H, seen at high levels of LTA 1 expression.

was a progression in LTA 1 localization from cytosol to Golgi stacks to condensation of Golgi membranes into one area to a dispersion of both LTA 1 and the Golgi compartments that correlated with the time and level of LTA 1 expression.

**ARF Was Recruited to the Golgi by LTA 1—** We have previously reported (24) that overexpression of the ARF-binding protein GGA1 caused increased accumulation of ARF at Golgi membranes through a novel mechanism of feed forward activation. To begin to examine the relationship between LTA 1 and ARF binding to Golgi membranes we first asked whether the presence of LTA 1 at Golgi membranes altered the staining of ARF on those same membranes. As seen in Fig. 3, low to intermediate levels of LTA 1 consistently produced increases in the intensity of staining of ARF at the Golgi, as visualized with the use of the monoclonal ARF antibody, 1D9 (Fig. 3B). Note in this panel that the surrounding cells not expressing LTA 1 have lower intensity staining for ARF at the Golgi. When the expression of LTA 1 was higher (Fig. 3C), the ARF staining behaved just like the other markers of the Golgi, and only the diffuse staining was observed throughout the cytosol (Fig. 3D). Thus, like increased expression of GGA1, the presence of LTA 1 in cells can influence the activation status of the ARF in the cell.

**The Presence of LTA 1 at Golgi Membranes Delays the Response to Brefeldin A—** A central tenet in models of ARF action is its ability to recruit soluble proteins onto membranes in concert with its own translocation that occurs upon activation (GTP binding). To test the requirement for ARF binding to LTA 1 localization, we examined the sensitivity to brefeldin A. CHO cells expressing LTA 1 were treated with brefeldin A for different times, then fixed, and stained with anti-Myc or anti-His 6 antibodies against epitopes on the expressed LTA 1 proteins (Fig. 4, A and C) or with Mann II (Fig. 4B) b-COP (Fig. 4D) or ARF antibodies (data not shown). Untransfected cells in the same field served as controls for the action of brefeldin A. The dissociation of COP-I, visualized with the antibody against the β subunit, and ARF are rapid, typically within 1–3 min, whereas that of lumenal Golgi markers, e.g. Mann II, occurs later (typically 5–30 min). The tight perinuclear staining can be seen in control cells that were not exposed to the drug (Fig. 4, A and B). After 2 min of brefeldin A (10 µM) treatment, the staining of Mann II in untransfected or transfected cells were unaffected and remained in the condensed, perinuclear pattern typical for these cells (data not shown). The staining of β-COP or ARF in untransfected cells became diffuse within 2–3 min. In contrast, in those cells that had expressed LTA 1 and had a condensed Golgi the staining of β-COP (Fig. 4D), ARF, and LTA 1 (not shown) were still evident at the Golgi. After 5 min or more of brefeldin A treatment, the staining of all three proteins, as well as LTA 1, was diffuse (data not shown). Thus, the presence of LTA 1 and induction of a condensed Golgi confers a degree of resistance to brefeldin A at concentrations that were sufficient to cause the dissociation of ARF and COP-I and (later) dissolution of the compartment. The finding that this partial resistance extends to ARF and β-COP suggests that the toxin may be acting through a stabilization or promotion of the active conformation of ARF (24). The changes in Golgi morphology and actions of the toxin to increase activated ARF while the brefeldin A acts in the opposite direction makes clear-cut conclusions from this approach difficult, so a more definitive test of the role of ARF binding in toxin action was sought.

**LTA 1 Mutants Failed to Localize to the Golgi—** We have recently described (13) a series of LTA 1 mutants, both point and deletion mutations, that have lost the ability to bind activated ARF3. We made use of these mutants to ask whether LTA 1 proteins with decreased affinity for ARF could still bind...
to Golgi membranes. Each of the deletion and point mutants of LTA1 that are used below were previously shown (13) to be expressed as soluble, folded proteins capable of forming specific protein-protein interactions, at least in yeast cells. One deletion, (1–172)LTA1, and three point mutants of LTA1, D43G, N93I, and W179R, were cloned into the pCDNA3-Myc-His6-based plasmid for expression in mammalian cells. CHO cells were transiently transfected with plasmids directing expression of LTA1 mutants, and the cells were examined after 16 h. We observed levels of fluorescent intensity that were comparable with those of low, medium, or high expressors, as described above (data not shown). In each case only cytosolic staining was observed when cells were probed with antibodies to the Myc tag on the LTA1 proteins. In addition, markers of the Golgi compartment were indistinguishable in transfected and untransfected cells, revealing the typical, punctate, perinuclear Golgi staining (data not shown). Thus, the loss of ARF binding correlated precisely with the loss of Golgi staining in intact cells in four of four mutants tested.

A further test of the importance of ARF to LTA1 localization at the Golgi was provided by three point mutants of LTA1. A flexible loop in LTA1 contains two residues, Phe31 and Arg33 that make extensive contacts with LTA2 in the holotoxin and are involved in binding ARF (13, 15). In contrast, the intervening residue, Asp32, faces away from the protein binding surface, and thus mutations at this residue did not alter ARF binding (13). We tested the ability of three mutants in the flexible loop, F31L, D32H, and R33A, to influence LTA1 localization in CHO cells and found that the two mutants that cannot bind ARF (F31L and R33A) show only cytosolic staining (Fig. 5, top left and bottom left panels) and did not produce any changes in Golgi morphology, as indicated by staining for Mann II (Fig. 5, right panels). In contrast, the mutation with unaltered ARF binding (D32H)LTA1 behaved like the wild type or (E112D)LTA1 proteins with regard to Golgi localization and disruption of Golgi structures when expressed at higher levels (Fig. 5, middle panels). These data further support the conclusion that the interaction between LTA1 and ARF is required for LTA1 to bind to Golgi membranes and subsequently alter the morphology of the Golgi.

Gain of Interaction LTA1 Mutants Relocalized to Golgi in the Presence of the Corresponding ARF Mutants—In the study of the binding sites for the ARF-LTA1 interaction (13), we identified mutations in ARF3 that resulted in a loss of binding and also used two of these mutants to screen for mutations in LTA1 that had regained the ability to bind to the mutated ARF3. It turned out that these mutants of LTA1 bound the mutated ARF3 but not wild type ARF3. Such pairs of mutated binding partners allowed a more rigorous test of the importance of ARF binding to LTA actions in cells.

The Q71L mutation in ARF proteins produces a GTPase-deficient protein that is more persistently active in cells. The second site mutants, (V53M,Q71L)ARF3 and (I74S,Q71L)ARF3, are deficient in binding or activation of LTA1 but retain interactions with at least some other ARF effectors and also bind both GDP and GTP (13). (Y145H)LTA1 and (Y149C)LTA1 were identified in yeast two-hybrid screens as mutants that bind to (V53M,Q71L)ARF3 and (I74S,Q71L)ARF3, respectively, but neither LTA1 double mutant binds to the parental (Q71L)ARF3 (13). We expressed each of these LTA1 mutants, either alone or in combination with the corresponding ARF3 mutant to which it binds. As expected for toxins that do not bind ARF, (Y145H)LTA1 and (Y149C)LTA1 alone were found in

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**Fig. 4.** Localization of LTA1 at Golgi is brefeldin A-resistant. Transfected and control cells were either untreated (A and B) or exposed to 10 μM brefeldin A for 2 min (C and D) and then immediately fixed and indirect immunofluorescence with primary antibodies to Myc (A and C), Mann II (B), or β-COP (D). Note the persistence of staining of LTA1 and β-COP at the perinuclear region in contrast to untransfected cells in the same field.

**Fig. 5.** Mutants of LTA1 that are defective in ARF binding fail to bind to the Golgi. CHO cells were transiently transfected with plasmids directing the expression of (F31L)LTA1 (top panels), (D32H)LTA1 (middle panels), or (R33A)LTA1 (bottom panels). The cells were doubly labeled with Myc (left panels) and Mann II (right panels) antibodies. Note that the two mutants defective in binding active ARF (F31L and R33A) are present throughout the cytosol, but the mutant that retained ARF binding (D32H) behaves like the wild type protein and co-localized with Mann II.
cytosol as diffuse staining (Fig. 6, A and C, respectively) and failed to co-localize with or alter the location of Golgi markers (Fig. 6, B and D). However, the co-expression of (Y145H)LTA1 with (I74S,Q71L)ARF3 (Fig. 6E) or (Y149C)LTA1 with (V53M,Q71L)ARF3 (Fig. 6G) resulted in the re-establishment of the Golgi localization of each LTA1 protein and condensation of the Golgi compartment (Fig. 6, F and H), comparable with that seen with wild type or (E112D)LTA1. Because expression of (V53M,Q71L)ARF3, (I74S,Q71L)ARF3, or (Q71L)ARF3 caused Golgi expansion, we used this change in Golgi morphology as an indicator of cells expressing the mutant ARF. These observations provide strong evidence that LTA1 binds to ARF directly and in so doing is directed to and concentrated at the surface of Golgi membranes and that subsequent to this interaction the morphology of the Golgi and its contents are first altered into a compressed area and later the integrity is compromised in a fashion that resembles treatment with brefeldin A. Thus, each of the activities described for LTA1 in cells was found to be dependent on its ability to bind ARF.

Other consequence of medium or high levels of LTA1 but not low level expression was a change in cell morphology from the flattened cuboidal shape, typical of CHO cells, into elongated, spindle-shaped cells (Fig. 7, C and D). This same response has been noted previously for externally applied LT or CT toxins and has been shown to result from increased production of cAMP (25). This explanation is likely and supported by the observation that at similar levels of expression the wild type LTA1 yielded a higher percentage of cells that displayed the spindle shaped morphology than did the (E112D)LTA1 mutant, which has decreased ability to ADP-ribosylate Gs thereby activate adenylyl cyclase (26), although see below. These results also indicate that the E112D mutation, although clearly decreased in ADP-ribosyltransferase activity (26, 27), is still active in live cells.

When co-transfected with both LTA1 and (Q71L)ARF3, cells became much more asymmetric and dendritic in appearance as they sent out processes that extend to twice the diameter of the cell body or more (Fig. 8, A, D, and F), even in cells expressing low levels of LTA1 and (Q71L)ARF3 (Fig. 8, A–C). Although elongated, spindle shaped cells were prevalent with expression of LTA1, and any mutant that bound ARF, the dendritic processes were only observed with the expression of both LTA1 and an activated ARF that bound the toxin. Note that the intense staining of LTA1 seen in Fig. 8D is the result of overexposure in capturing the image to visualize the processes and is not the result of gross overexpression of the protein, in contrast to other images shown.

The cells co-expressing both LTA1 gain of interaction mutants and their corresponding ARF3 double mutants also showed dendritic-like morphology, whereas the cells expressing these LTA1 mutants alone did not (Fig. 9). Although the expression of (Q71L)ARF3 alone had dramatic effects on the morphology of the Golgi, it did not change the cell morphology (Fig. 9). The percentage of spindle-like cells was increased from 0 to 2% in cells expressing LTA1, mutants that lost ARF binding, to 91.7% of cells expressing LTA1 alone (Fig. 9). Similarly, the percentage of dendritic-like cells changed from 8.3% with expression of LTA1 alone, to 87% of cells expressing both toxin and (Q71L)ARF3 (Fig. 9). Again, we found that we could at least partially restore this effect by pairing the LTA1 and ARF3...
ARF Binding Is Required for Activities of E. coli Toxin

Plasmids directing expression of LTA and/or ARF3 were transfected into CHO cells, and cellular cAMP content was determined 18 h later, as described in “Experimental Procedures.” Note that both LTA and (E112D)LTA produce very similar increases in intracellular cAMP. In contrast, some of the mutants defective in ARF binding have lost the ability to increase cAMP in these cells. The results shown are the averages of duplicates (all differences less than 5% from one experiment. Each condition was repeated at least twice with essentially identical results, although absolute values vary between experiments. The readings below that of the lowest point in the standard curve (12.5 fmol) are indicated as <12.5.

![Image](51x567 to 296x729)

**FIG. 8.** CHO cells extend dendritic-like processes after co-expression of LTA and (Q71L)ARF3. CHO cells were transfected with both LTA and (Q71L)ARF3 and 16 h later were fixed for doubly labeled indirect immunofluorescence to visualize LTA (A and D) and Mann II (B and E). C and F are the same fields viewed by phase contrast. The level of LTA in the cells shown in D is comparable with that shown in A, but the image was overexposed to reveal the processes extending from the cell body.

![Image](71x369 to 276x485)

**FIG. 9.** Cells expressing LTA become elongated, but cells expressing both ARF and LTA, extend dendritic-like processes. CHO cells were transfected with plasmid(s) directing expression of the indicated proteins and prepared for indirect immunofluorescence after 16 h, as described under “Experimental Procedures.” The percentage of cells displaying spindly or dendritic-like morphologies were then determined by counting 100–200 cells. Spindly cells were defined as those with one axis at least twice the length of the other (and typically much more than twice the length). Dendritic-like cells were those with a thin process that extended to a length at least equal to the diameter of the cell body. Cells expressing (Y145H,E112D)LTA1, (Y149C,E112D)LTA1, and (E112D)LTA1 with or without the corresponding ARF mutants to which they bind ((I74S,Q71L)ARF3, (V53M,Q71L)ARF3, and (Q71L)ARF3, respectively) were separated. Those conditions with no bars evident had no spindly or dendritic-like cells. This experiment was repeated twice, and the data from one set are shown.

**TABLE I**

| Expressed protein(s)       | cAMP (fmol/well) |
|---------------------------|-----------------|
| pCDNA3 (empty vector)     | <12.5           |
| LTA1                      | 414.9           |
| (E112D)LTA1               | 383.0           |
| (E112D,F31L)LTA1          | 38.1            |
| (E112D,R53A)LTA1          | <12.5           |
| (E112D,1–172)LTA1         | 172.2           |
| (E112D,Y145H)LTA1         | <12.5           |
| (Q71L)ARF3                | 44.4            |
| LTA1 + (Q71L)ARF3         | 459.6           |
| (E112D)LTA1 + (Q71L)ARF3  | 498.0           |
| (Q71L)V53M5ARF3           | <12.5           |
| (E112D,Y145H)LTA1 + (Y145H,V53M5ARF3) | 25.3 |

**DISCUSSION**

In this study we have expressed wild type and mutants of LTA within the cytoplasm and followed the events that then unfold. More toxin is likely expressed than would enter a eucaryotic cell during pathophysiological conditions, thus creating an artificial situation. However, the events described can clearly be linked to specific protein-protein interactions and offer several insights into the events that occur during intoxication of eucaryotic cells. The first experimental evidence for the requirement of ARF proteins in the actions of LT in live cells is provided. Through its binding to ARF the LTA becomes concentrated at Golgi membranes where it can first alter the morphology of the Golgi into a more condensed structure and later disperse the Golgi stacks in a fashion highly reminiscent of the actions of brefeldin A (28, 29). Finally, we identified point mutants of LTA1 with reduced binding to ARF that resulted in proteins that retained basal catalytic (ADP-ribosyltransferase) activity but lacked all evidence of toxicity to mammalian cells. Such mutants make attractive nontoxic alternatives to the native toxins and NAD-binding site mutants for development, should they retain mucosal adjuvant properties (30–33).

A required role for ARF proteins in the intoxication and toxicity of LT in mammalian cells is evident first from the finding that LTA mutants that are deficient in binding ARF no longer 1) localize to the Golgi, 2) cause the condensation or dissociation of Golgi elements, 3) promote changes in cell morphology to either elongated spindles or dendritic-like outgrowths, or 4) increase intracellular cAMP levels. Compelling evidence that the defects in LTA activity were a direct result of the loss of ARF binding was provided by the finding that each

The volumes of cell extracts were held constant within each experiment to prevent cell debris from interfering with the assay. When the volume of the extracts assayed was increased (data not shown), the values for the empty vector and these LTA1 mutants were within the standard curve, allowing accurate values to be determined, and still there were no differences.
of these effects could be restored by the co-expression of LTA mutants with mutants of ARF3 that bind to the mutant LTA proteins. Thus, we conclude that the binding of LTA to ARF is required for localization and activation of adenyl cyclase in mammalian cells.

The complex routing of LT and CT along the (reverse) secretory path leaves open the site within cells where the toxins catalyze the covalent modification of Gα, although the presence of the latter at the plasma membrane still makes this the most likely site of action. Early studies of CT and LT action included the assumption that the toxins acted at the plasma membrane, where the receptors for the toxins on the outside of cells and the Gα and adenyl cyclase targets were found on the cytosolic side. However, the finding that once inside cells LTA concentrates at Golgi membranes offers an alternative site of action. Although the expressed LTA accumulates at the Golgi, it is clear that it is a soluble protein that can also migrate to other sites in the cell but the dependence on ARF for cell toxicity (see below) suggests that co-localization with an ARF is required for activity. No major differences have been described in the specific activities of the six mammalian ARF isoforms as co-factors for CT or LT in vitro assays so they appear equally likely to act with the toxins in cells. Even though the bulk of the expressed toxin is seen at the Golgi, where ARF1–5 can be found, there is likely sufficient toxin in cytosol or at the plasma membrane, where ARF6 predominates, to fully activate the Gα and cyclase there.

From examination of cells at different times after transfection with plasmids directing expression of the toxin we have concluded that there is a progression from 1) synthesis in the cytosol to 2) association with a morphologically normal appearing Golgi to 3) induction of a highly condensed Golgi (that contains all the extrinsic and luminal markers of this organelle) to (4) dispersion of the Golgi throughout the cytosol, similar in appearance to the effects of brefeldin A. As described above, we found that the association to the Golgi was ARF-dependent and likely limited only by the diffusion of the toxin to the site where activated ARF is most abundant. But what is the explanation for the effects on Golgi morphologies?

The dispersion of Golgi is seen at later times and correlates with higher levels of LTA expression. As indicated already the effect is very similar to that described previously for the actions of brefeldin A that result from the inhibition of ARF exchange factors and consequent deficiency in activated ARF (34–36). We believe a similar scheme can explain the dispersion of Golgi by LTA. But instead of decreasing the available activated ARF by inhibiting the activity of ARF exchange factor(s), the presence of high levels of LTA is predicted to effectively compete with endogenous effectors that are required for maintenance of Golgi integrity by simple mass action. The result would thus be the same as that of brefeldin A, an insufficient amount of activated ARF to bind and activate the key effectors that maintain the normal Golgi morphology.

The condensation of the Golgi is a more difficult observation to explain. At least three different things may influence or cause this effect; increased expression of ARF effectors has been shown to increase the amount of ARF at the Golgi (24), the presence of LTA and ARF could activate Gα and adenyl cyclase either at other sites or potentially locally (see above), and the LTA may be competing with endogenous effectors important to Golgi morphology (see above). The increase in cAMP levels in these cells will activate protein kinase A and may increase the binding of ARF to Golgi membranes, as described by Martin et al. (37). Thus, two of these responses likely produce a more activated ARF on Golgi membranes. An excess of activated ARF, by expression of the activating mutant (Q71L)ARF1, has been shown to result in vesiculation of the Golgi (23), so it is possible that the combination of increased ARF and PKA activities modulate the effects on Golgi structure to this condensed structure. The presence of eight or more ARF-binding partners on Golgi membranes (38) makes it risky to ascribe this phenomenon to any one mechanism.

The effects of CT and LT on mammalian cells have been described for over 25 years, and to date all of the toxic effects on cells or the organism result from the increased production of cAMP. Although some cultured cells are killed by increases in cAMP, others respond with changes in cell shape or not at all. Changes in cell morphology, e.g. in CHO cells, have been used throughout as sensitive indicators of toxin action at the cellular level (25). The finding that LTA mutants with reduced affinity for ARF have also lost this activity is remarkable, given the higher levels of the LTA proteins in cells induced to express the toxin, compared with those in which the toxin must enter through the endocytic pathway. We conclude that the loss of ARF binding has a more marked effect on the intracellular activities of LTA than do mutations that cripple the catalytic site, e.g. E112D (26, 27, 39) or cleavage of the A1-A2 subunits (40).

CT and LT are the most potent mucosal adjuvants known, but toxicity to mammals has slowed their clinical development (41, 42). Although controversial, some studies have demonstrated the retention of potent mucosal adjuvancy with mutants that are deficient in ADP-ribosyltransferase activity (40, 43). However, toxicity of at least some of these toxins is still problematic (31). The E112D mutant of LTA has previously been shown to retain only 5–10% the in vitro ADP-ribosyltransferase activity of the wild type protein (26), so we were surprised to see only slight decreases in toxicities on our cells and cAMP levels similar to those produced by the wild type toxin. We concluded that the higher levels of toxin achieved in cytosol in our system overcame the decrease in enzymatic activity and resulted in full activation of adenyl cyclase. Thus, the lack of effects of our mutants, e.g. F31L, R33A, Y145H, or Y149C, which retain ADP-ribosyltransferase activity but have lost the ARF-dependent activity, is even more remarkable. We are currently testing for retention of adjuvant properties of these mutants and should one or more retain that activity they should immediately become the most promising modified toxins for the development of mucosal adjuvant therapies.

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REFERENCES

1. Merritt, E. A., and Hol, W. G. (1995) Curr. Opin. Struct. Biol. 5, 165–171
2. Cassel, D., and Pfeiffer, T. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2669–2673
3. Moss, J., and Vaughan, M. (1977) J. Biol. Chem. 252, 2455–2457
4. Gill, D. M., and Richardson, S. H. (1980) J. Infect. Dis. 141, 64–70
5. Kahn, R. A., and Gilman, A. G. (1984) J. Biol. Chem. 259, 6228–6234
6. Kahn, R. A., and Gilman, A. G. (1986) J. Biol. Chem. 261, 7960–7961
7. 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
8. Donaldson, J. G., Cassel, D., Kahn, R. A., and Klausner, R. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6408–6412
9. Brown, H. A., Guteowski, S., Somavat, S. C., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1317–1324
10. Cockcroft, S., Thomas, G. M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hild, I., Totty, N. F., Truong, O., and Hsu, J. A. (1994) Science 263, 523–526
11. Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Iurisi, C., Luini, A., Corda, D., and De Matteis, M. A. (1999) Nat. Cell Biol. 1, 280–287
12. Honda, A., Nagami, M., Yokoeiki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanaho, Y. (1999) Cell 99, 521–532
13. Zhu, X., Kim, E., Boman, A. L., and Kahn, R. A. (2001) Biochemistry 40, 4560–4568
14. Sixma, T. K., Pronk, S. E., Kalk, K. H., Wartna, E. S., van Zanten, B. A., Witholt, B., and Hol, W. G. (1991) Nature 351, 371–377
15. Sixma, T. K., Kalk, K. H., van Zanten, B. A., Dauter, Z., Kingma, J., Witholt, B., and Hol, W. G. (1993) J. Mol. Biol. 230, 890–918
16. Majoul, I., Sohn, K., Wieland, F. T., Pepperkok, R., Pizza, M., Hilleman, J., and Soling, H. D. (1998) J. Cell Biol. 143, 601–612
17. Majoul, I. V., Bastiaens, P. I., and Soling, H. D. (1996) J. Cell Biol. 133, 777–789
18. Rodighiero, C., Aman, A. T., Kenny, M. J., Moss, J., Lencer, W. I., and Hirst, T. R. (1999) J. Biol. Chem. 274, 3962–3969
19. Lencer, W. I., Constable, C., Moe, S., Jobling, M. G., Webb, H. M., Ruston, S., Madaia, J. L., Hirst, T. R., and Holmes, R. K. (1995) J. Cell Biol. 131, 951–962
20. Lencer, W. I., Hirst, T. R., and Holmes, R. K. (1999) Biochim. Biophys. Acta 1450, 177–190
21. Bastiaens, P. I., Majoul, I. V., Verveer, P. J., Soling, H. D., and Jovin, T. M. (1996) EMBO J. 15, 4246–4253
22. Cavenagh, M. M., Whitney, J. A., Carroll, K., Zhang, C., Boman, A. L., Rosenwald, A. G., Whitney, J. A., Carroll, K., Zhang, C., Boman, A. L., Rosenwald, A. G., Mellman, I., and Kahn, R. A. (1996) J. Biol. Chem. 271, 21767–21774
23. Zhang, C. J., Rosenwald, A. G., Willingham, M. C., Skunte, S., Clark, J., and Kahn, R. A. (1994) J. Cell Biol. 124, 289–300
24. Zhu, X., Boman, A. L., Kuai, J., Cieplak, W., and Kahn, R. A. (2000) J. Biol. Chem. 275, 13465–13475
25. Guillant, R. L., Brunton, L. L., Schnaitman, T. C., Rehun, L. I., and Gilman, A. G. (1974) Infect. Immun. 10, 320–327
26. Moss, J., Stanley, S. J., Vaughan, M., and Tsuji, T. (1993) J. Biol. Chem. 268, 6383–6387
27. Cieplak, W., Jr., Mead, D. J., Messer, R. J., and Grant, C. C. (1995) J. Biol. Chem. 270, 30545–30550
28. Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) J. Cell Biol. 116, 1071–1080
29. Pelham, H. R. (1991) Cell 67, 449–451
30. Snider, D. P. (1995) Crit. Rev. Immunol. 15, 317–348
31. Freytag, L. C., and Clements, J. D. (1999) Curr. Top. Microbiol. Immunol. 236, 215–236
32. Williams, N. A., Hirst, T. R., and Nashar, T. O. (1999) Immunol. Today 20, 95–101
33. Rappuoli, R., Pizza, M., Deuce, G., and Dougan, G. (1999) Immunol. Today 20, 493–500
34. Peyroche, A., Paris, S., and Jackson, C. L. (1996) Nature 384, 479–481
35. Peyroche, A., Antonny, B., Robineau, S., Acker, J., Cherfils, J., and Jackson, C. L. (1999) Mol. Cell 3, 275–285
36. Togawa, A., Morinaga, N., Ogashawara, M., Moss, J., and Vaughan, M. (1999) J. Biol. Chem. 274, 12308–12315
37. Martin, M. E., Hidalgo, J., Rosa, J. L., Crottet, P., and Velasco, A. (2000) J. Biol. Chem. 275, 19650–19659
38. Kuai, J., Boman, A., Arnold, R., Zhu, X., and Kahn, R. (2000) J. Biol. Chem. 275, 4022–4032
39. Stevens, L. A., Moss, J., Vaughan, M., Pizza, M., and Rappuoli, R. (1999) Infect Immun 67, 259–265
40. Cheng, E., Cardenas-Freytag, L., and Clements, J. D. (1999) Vaccine 18, 38–49
41. Elson, C. O., and Ealding, W. (1984) J. Immunol. 132, 2736–2741
42. Clements, J. D., Hartwig, N. M., and Lyon, F. L. (1988) Vaccine 6, 269–277
43. Nashar, T. O., Amin, T., Marcello, A., and Hirst, T. R. (1993) Vaccine 11, 235–240