Interaction of ADP-ribosylation Factor with *Escherichia coli* Enterotoxin That Contains an Inactivating Lysine 112 Substitution*

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Cholera toxin and *Escherichia coli* heat-labile enterotoxin (LT) exert their effects on cells through ADP-ribosylation of guanine nucleotide-binding proteins. Both toxins consist of one A subunit, which is an ADP-ribosyltransferase, and five B (or binding) subunits. Their enzymatic activities are latent; activation requires reduction and proteolysis, resulting in a catalytically active A protein and a much smaller A protein. These ADP-ribosyltransferases are activated by GTP-dependent 20-kDa ADP-ribosylation factors or ARFs. To determine if proteolysis plus reduction is required for appearance of the ARF allosteric site as well as for catalytic activity, an inactive mutant of LT, LT(E112K), with replacement of glutamate by lysine at position 112 of its A subunit, was utilized as a competitor in cholera toxin ADP-ribosyltransferase assays containing limiting amounts of ARF. LT(E112K) required trypsinization and reduction to become a potent, concentration-dependent inhibitor. Inhibition was reversed by increasing concentrations of ARF. Reduction or trypsinization alone did not generate an inhibitory form of LT(E112K). These studies are consistent with the conclusion that the ARF site is not expressed in the latent toxin. Both trypsinization and reduction are required for expression of a functional ARF binding site as well as for catalytic activity.

Some bacterial toxins (e.g. pertussis toxin, cholera toxin, *Escherichia coli* heat-labile enterotoxin) exert their effects on mammalian cells through the ADP-ribosylation of guanine nucleotide-binding proteins that couple cell surface receptors to their intracellular effectors (for review, see Moss and Vaughan, 1988). Toxin-catalyzed ADP-ribosylation alters the activity of guanine nucleotide-binding proteins and thus agonist-initiated signal transduction to the regulated effector. Cholera toxin (CT)* is an oligomeric protein composed of five B or binding subunits linked to an A subunit (CTA) that possesses latent ADP-ribosyltransferase expression. Expression of transferase activity requires proteolysis near the carboxyl terminus and reduction of the single disulfide linking the two proteolytic fragments, resulting in the generation of an ~23-kDa enzymatically active A protein (CTA,) and a 6-kDa A protein (CTA) derived from the carboxyl terminus (Mekalanos et al., 1979, 1983). *E. coli* heat-labile enterotoxin (LT), like cholera toxin, possesses A and B subunits and has similar enzymatic, immunological, and structural properties (Moss and Vaughan, 1988).

The A subunits of cholera toxin and LT and the S1 subunit of pertussis toxin share considerable amino acid identity (Rappuoli and Pizza, 1991). Similarities in the functional amino acids have been noted with other bacterial ADP-ribosyltransferases (Rappuoli and Pizza, 1991). For example, a glutamate residue participates in the pertussis toxin-, diphtheria toxin-, and *Pseudomonas* exotoxin A-catalyzed reactions and is covalently linked to the nicotinamide residue of NAD following irradiation (Carroll and Collier, 1984, 1987; Carroll et al., 1985; Barbieri et al., 1989; Cocke, 1989). Mutant forms of the toxins with replacements of the respective glutamate residues are inactive (Tweten et al., 1985; Barbieri et al., 1989; Tsuji et al., 1990). The critical glutamate in LT is located at position 112, corresponding to 148 in diphteria toxin, 533 in *Pseudomonas* exotoxin A, and 129 in pertussis toxin (Carroll and Collier, 1984, 1987; Barbieri et al., 1989; Tsuji et al., 1990). ADP-ribosylation by cholera toxin and *E. coli* enterotoxin is stimulated in vitro by a multigene family of ~20-kDa guanine nucleotide-binding proteins, known as ADP-ribosylation factors or ARFs (for review, see Serventi et al. (1992)). ARFs are highly conserved proteins and have been found in all eukaryotic cells, from *Giardia* to mammals (Kahn et al., 1988; Price et al., 1988; Sewell and Kahn, 1988; Tsuji et al., 1991a; Tsuji et al., 1991; Murtagh et al., 1992). In the presence of GTP or its analogues, but not GDP, ARFs stimulate the ADP-ribosyltransferase activity of cholera toxin and *E. coli* heat-labile enterotoxin in a reaction enhanced by certain phospholipids and detergents (Babak et al., 1990; Noda et al., 1990; Lee et al., 1991; Price et al., 1992). At least six mammalian ARFs, which have been identified by cDNA cloning, fall into three classes based on similarities in deduced amino acid sequence, size, and gene structure (Price et al., 1988; Sewell and Kahn, 1988; Babak et al., 1989; Monaco et al., 1990; Tsai et al., 1991b; Tsujiya et al., 1991; Murtagh et al., 1992). ARFs from all three classes, when expressed as recombinant proteins in *E. coli* or insect cells, stimulate cholera toxin activity (Weiss et al., 1986; Kunz et al., 1990; Price et al., 1992).

ARFs are allosteric activators of the active CTA protein (Noda et al., 1989, 1990). It is not known whether the ARF interaction site is present in the latent, inactive CTA or LTA.
species. In the studies reported here, we used an enzymatically inactive *E. coli* heat-labile enterotoxin containing a lysine for glutamate replacement at position 112 to determine whether latent forms of toxin were capable of interaction with ARF, specifically examining the effects of proteolysis and reduction on generation of an ARF binding site.

**EXPERIMENTAL PROCEDURES**

*Materials*

CT and CTA were purchased from List Biological Laboratories; 
[adenine-U-14C]NAD (250–300 mCi/mmol) and [carbonyl-14C]NAD (~50 mCi/mmol) were purchased from Amersham Corp.; diithio- 
reitol was from ICN; magnesium chloride was from Mallinkrodt; 
ovalbumin, NAD, agmatine, bovine serum albumin, GTP, dimyristoyl-
phosphatidylcholine, trypsin, trypsin inhibitor, and cholate were 
molecular weight standards were from Gibco/BRL; and [32P]NAD 
(10–50 Ci/mmol) was from Du Pont-New England Nuclear.

*Methods*

NAD:Agmatine ADP-riboosyltransferase Assay—Standard assays 
(final volume of 0.3 ml) contained 50 mM potassium phosphate, pH 
7.5, 100 μM [adenine-U-14C]NAD (50,000 cpm), 10 mM agmatine, 20 
mm diithiothreitol, 10 mM MgCl2, 100 μM GTP, 3 mM dimyristoyl-
phosphatidylcholine, 0.2% cholate, ovalbumin (0.1 mg/ml), and, as 
indicated, rARF6 (1 μg). Reaction was initiated with CTA (0.25 μg), 
and, after 90 min at 30 °C, two 0.1-ml samples were transferred to 
columns of AG 1-X2 (Bio-Rad) for isolation of [adenine-14C]ADP-
riboagmatine as described (Moss and Stanley, 1981; Price et al., 
1992). All assays were performed in duplicate.

Protein Purification—LT and LT(E112K) were purified as de-
scribed (Tsuji et al., 1990). rARF6 (recombinant human ARF 6) was 
synthesized in *E. coli* and purified as described (Price et al., 
1992).

**RESULTS AND DISCUSSION**

Trypsinization of LT increased basal and ARF-stimulated 
NAD:agmatine ADP-riboosyltransferase activity (Table I). As 
noted previously, glutamate 112 of LTA appears to be neces-
sary for activity (Tsuji et al., 1990). Substitution of lysine for 
glutamate LT(E112K) resulted in a loss of transferase activ-
ity. As shown with LT and CT, trypsinization and reduction 
of CTA in this experiment was 3.2 and 25.8 pmol. min⁻¹ without 
and with rARF6, respectively. LT(E112K) was inactive without 
and with rARF6. ND, not determined.

| Table I | Effect of trypsinization of LT and LT(E112K) on basal and rARF6-stimulated ADP-riboosyltransferase activity |
|---------|----------------------------------------------------------------------------------------------------------|
| LT (10 μg) or LT(E112K) (60.8 μg) was incubated in 50 mM glycine (pH 8.0), 20 mM diithiothreitol for 30 min at 30 °C with or without trypsin (6 μg) (final volume of 258 μl) before addition of 15 μl of water or trypsin inhibitor (30 μg). Bovine serum albumin (BSA) and ovalbumin (OVAL) (96 μg each) were similarly treated. Samples (20 μl) were assayed with CT and CTA, with or without ARF, ARF6, T, trypsin, TI, trypsin inhibitor. Activity of trypsinized LT was 7.4 and 37.0 pmol.min⁻¹, without and with rARF6, respectively. ND, not determined. |
| Trypsin | ADP-riboosyltransferase activity |
|---------|---------------------------------|
| LT(E112K) | CTA plus LT(E112K) |
| Minus A6 | Plus A6 | Minus A6 | Plus A6 |
| μg | pmol.min⁻¹ |
| None | 0.0 | 0.0 | 3.3 | 26.1 |
| 1 | 2.4 | 16.0 | ND | ND |
| 2 | 3.0 | 21.7 | 2.7 | 8.1 |
| 4 | 3.5 | 22.9 | 1.9 | 7.6 |
| 8 | 2.9 | 19.1 | 2.3 | 5.4 |
| 16 | ND | ND | 2.1 | 6.4 |

**TABLE II**

Effect of (r)ARF6 on inhibition of rCTA-catalyzed ADP-riboosyltransferase activity

LT (30 μg) or LT(E112K) (91.2 μg) was incubated in 50 mM glycine (pH 8.0), 20 mM diithiothreitol for 30 min at 30 °C with or without trypsin (6 μg) (final volume of 258 μl) before addition of 15 μl of water or trypsin inhibitor (30 μg). Bovine serum albumin (BSA) and ovalbumin (OVAL) (96 μg each) were similarly treated. Samples (20 μl) were assayed with LT without and with ARF, ARF6, T, trypsin, TI, trypsin inhibitor. Activity of trypsinized LT was 7.4 and 37.0 pmol.min⁻¹, without and with rARF6, respectively. ND, not determined.

| Additions | CTA ADP-riboosyltransferase activity |
|-----------|------------------------------------|
| Control | +T | +TI | +TI/T |
| −ARF6 | +ARF6 | −ARF6 | +ARF6 | −ARF6 | +ARF6 |
| pmol.min⁻¹ |
| None | 4.4 | 30.8 | 4.6 | 24.9 | 4.2 | 26.8 | 3.5 | 26.8 |
| LT(E112K) | 4.2 | 25.2 | 3.5 | 7.1 | 4.4 | 24.2 | 3.2 | 6.2 |
| BSA | 4.1 | 23.7 | ND | ND | ND | 4.2 | 23.5 |
| OVAL | 3.9 | 25.0 | ND | ND | ND | 3.3 | 23.1 |

**FIG. 1.** Effect of trypsinization on generation of LTA, and LTA(E112K). LT (20 μg) and LT(E112K) (60.8 μg) were incubated in 50 mM glycine (pH 8.0), 20 mM diithiothreitol for 30 min at 30 °C with or without trypsin (4 μg) (final volume of 200 μl) followed by addition of trypsin inhibitor (20 μg). Samples (50 μl) were then mixed with an equal volume of SDS sample mix (62.5 mM Tris (pH 6.8), 3% SDS, 10% glycerol, 5% mercaptoethanol, 0.01% bromphenol blue) and subjected to electrophoresis in 18% polyacrylamide gels, which were stained with Coomassie Blue (Laemmli, 1970). To determine whether the trypsinization was effective and the toxins were active, all samples were assayed with and without rARF6. Activity in pmol.min⁻¹ is in parentheses, that without rARF6 in the numerator and that with rARF6 in the denominator. In lanes A–D, LT was present in all samples. A, no additions (1.2/0.9); B, plus trypsin (2.7/11.4); C, plus trypsin inhibitor (0.7/0.9); D, plus trypsin and trypsin inhibitor (2.6/10.5). Activities of LT(E112K) (lanes E–H) were assessed as inhibition of the ARF effect on activity of CTA. Activity of CTA without additions was 4.5/20.3. E, no additions (4.7/18.4); F, plus trypsin (4.0/5.7); G, plus trypsin inhibitor (4.8/19.8); H, plus trypsin and trypsin inhibitor (3.7/4.5). Molecular mass standards were myo-
sin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglob-
ulin (18.4 kDa), and lysisozyme (14.3 kDa). LT-B, the B subunit of LT.
Fig. 2. Effect of rARF6 and LT(E112K) on cholera toxin-catalyzed ADP-ribosylation. LT(E112K) (182.4 µg) was incubated in 50 mM glycine (pH 8.0), 20 mM dithiothreitol for 30 min at 30°C with trypsin (12 µg) (final volume of 570 µl) before addition of 30 µl of trypsin inhibitor (60 µg) or with trypsin inhibitor alone (A, Δ). The indicated amounts of LT(E112K) were assayed as described under "Experimental Procedures" with (C, Δ) or without (C, Δ) rARF6.

**TABLE III**

| Additions to incubation | CTA ADP-ribosyltransferase activity |
|------------------------|-------------------------------------|
|                        | Minus A6 | Plus A6 |
|                        | pmol min⁻¹ |
| ---                    | ---      | ---     |
| -^a                    | 3.9      | 21.1    |
| T                      | 3.0      | 6.1     |
| -^T                    | 4.2      | 22.9    |
| T                      | 3.0      | 7.7     |
| T^T                    | 3.8      | 20.5    |

^a No additions.

proteins (e.g. bovine serum albumin and ovalbumin) when substituted for LT(E112K) did not block rARF6-stimulated activity (Table II) nor did cholera toxin B subunit when subjected to the same trypsin/trypsin inhibitor incubation procedure (data not shown). No inhibition was observed when the order of addition of trypsin and trypsin inhibitor was reversed (Table III). Inhibition of ADP-ribosyltransferase activity by LT(E112K) was observed whether [adenine-U-¹⁴C]NAD or [carbonyl-¹⁴C]NAD served as substrate (data not shown).

Inhibition by trypsinsized LT(E112K) of rARF6-stimulated and basal activities was concentration-dependent (Fig. 2). At equal protein concentrations, the inhibitory effects on rARF6-stimulated activity were much more marked than those on basal activity; at concentrations of trypsinsized LT(E112K) that completely blocked rARF6-stimulated activity, basal activity was slightly inhibited (Fig. 2). Increasing the concentrations of rARF6 overcame inhibition by trypsinsized LT(E112K) (Fig. 3), as might be expected if the inhibitory effect resulted from competition between CTA and LT(E112K) for limiting amounts of rARF6.

CT (Fig. 4, II-IV) and LT (Fig. 4f) also catalyzed the auto-ADP-ribosylation of their respective A1 catalytic units as well as the ADP-ribosylation of other proteins. CT notably labeled LT(E112K) A and B subunits (Fig. 4, II and IV). These reactions were stimulated by ARF and were inhibited by trypsinsized LT(E112K) (Fig. 4II-IV).

Activation of LT requires both trypsinsization and reduction and leads to the generation of LTA1; similar treatment of the inactive mutant resulted in the formation of LTA1(E112K), visualized after electrophoresis in sodium dodecyl sulfate-polyacrylamide gels run under reducing conditions (Fig. 1). These data suggest that trypsinsization and reduction generate an LTA1(E112K) protein, which although enzymatically inactive due to replacement of a critical glutamate residue, still possesses a rARF6 binding site. Since thiol was present during trypsinsization and in the control preparation, reduction of LT(E112K) by thiol was alone insufficient to generate an inhibitory molecule, consistent with the conclusion that proteolysis is necessary. Similarly, activation of LTA requires proteolysis and reduction.

To examine the thiol requirements, trypsinsization of LT(E112K) was conducted with and without dithiothreitol (Table IV). Reactions were terminated with trypsins inhibitor, and the ability of the products to inhibit rARF6-stimulated CTA ADP-ribosyltransferase activity was assessed (Table...
A

B

C

D

E

F

G

H

I

LT

LT(E112K)

Interaction of ADP-ribosylation Factors with Enterotoxins

FIG. 4. Effect of trypsinization of LT(E112K) on inhibition of cholera toxin-catalyzed ADP-ribosylation. LT (20 µg) and LT(E112K) (60.8 µg) were incubated in 50 mM glycine (pH 8.0), 20 mM dithiothreitol with or without trypsin (4 µg), trypsin inhibitor (20 µg), or no additions for 30 min at 30 °C (total volume of 190 µl) before addition of 10 µl of trypsin (4 µg) or trypsin inhibitor (20 µg) as indicated. Panel I, LT; panels II–IV, LT(E112K). A, no additions; B, trypsin; C, trypsin inhibitor following incubation; D, trypsin during incubation followed by trypsin inhibitor; E, no additions; F, trypsin inhibitor following incubation; G, trypsin during incubation: H, trypsin during incubation followed by trypsin inhibitor; I, trypsin inhibitor during incubation followed by trypsin. Samples (10 µl of LT and 20 µl of LT(E112K)) were assayed in the standard NAD:agmatine ADP-ribosyltransferase assay to verify that trypsinolysis had occurred (i.e. that LT was activated and LT(E112K) was inhibitory of ARF) and also to monitor the ADP-ribosylation of proteins. For the latter, assays (total volume of 300 µl) contained 50 mM potassium phosphate (pH 7.5), 10 mM MgCl₂, 0.1 mg/ml ovalbumin, 100 µM [³²P]NAD (2 µCi), 100 µM GTP, 20 mM dithiothreitol, and 0.003% SDS with CTA (0.25 µg) and/or rARF6 (1 µg), as indicated in the figure. After 90 min at 30 °C, 0.7 ml of 10.7% trichloroacetic acid was added, and the samples were refrigerated overnight. After centrifugation (1 h, –2000 × g), the supernatant was removed, and the pellet was dispersed in 30 µl of water plus 30 µl of SDS sample mix (see legend to Fig. 1) and heated (75 °C, 10 min). A sample (50 µl) was subjected to electrophoresis in 18% polyacrylamide gels. Gels were stained in Coomassie Blue and exposed to x-ray film. Positions of subunits are indicated on the left. LTA, migrates like CTA,. Activities (pmol. min⁻¹) in the NAD:agmatine ADP-ribosyltransferase assay (±rARF6) were: A, 1.6/0.5; B, 2.6/13.5; C, 0/0.8; D, 2.0/13.5; E, 5.0/31.3; F, 1.6/1.7; G, 9.7 (0.2); H, 3.9/9.5; I, 3.7/9.3. Activity of CTA alone was 4.7/34.3. LTB, the B subunit of LT.

TABLE IV

Effect of dithiothreitol on activation of LT(E112K) by trypsin

LT(E112K) (242.8 µg, sample A; 121.4 µg, sample B) was incubated for 30 min at 30 °C in 50 mM glycine, pH 8.0, with or without 20 mM dithiothreitol and trypsin (6 µg, B; 12 µg, A, as indicated, in a total volume of 580 µl (A) or 290 µl (B). Reaction was stopped with trypsin inhibitor (30 µg in 10 µl or 60 µg in 20 µl for B and A, respectively). Control incubations were performed with trypsin and/or trypsin inhibitor and dithiothreitol (DTT) without LT(E112K). Samples (60 µl) were assayed in duplicate with or without dithiothreitol; activities without are in parentheses. Assays differed due to the presence of 1 µg of CTA in the LT(E112K) reactions and 3 µg of rARF6 (A6) where indicated. Trypsin increased LT activity and response to rARF6 as expected. A6, rARF6; T, trypsin; TI, trypsin inhibitor.

| Additions to incubation | CTA ADP-ribosyltransferase activity |
|------------------------|-----------------------------------|
|                        | Minus A6 | Plus A6 | pmol.min⁻¹ |
| LT(E112K)              |          |        |            |
| LT(E112K)/DTT          | 20.0 (0) | 65.1 (3.4) |
| LT(E112K)/T/TI         | 22.3     |        |            |
| LT(E112K)/DTT/T/TI     | 9.7      | 10.1 (3.0) |
| None                   | 21.5 (0) | 71.7 (3.2) |
| DTT                    | 21.4     |        |            |
| T/TI                   | 22.8 (0) | 71.0 (3.6) |
| DTT/T/TI               | 24.0     |        | 70.8       |

IV). Thiol appeared not to be necessary for trypsinization of LT(E112K) (Table IV). Trypsinized LT(E112K) interfered with activation of CTA by rARF6 but appeared also to inhibit basal activity, an effect that was not solely the result of trypsin and trypsin inhibitor in the preparation (Table IV). In these studies, the CTA assays were conducted with and without dithiothreitol to determine whether there was an endogenous source of thiol responsible for activation. A low level of activity was observed in the presence of rARF6 (Table IV), consistent with a source of thiol in that preparation but an amount insufficient to activate CTA significantly. LT activated by trypsin in the presence of chol had slightly higher activity than LT activated in its absence (35.9 pmol.min⁻¹ versus 28.2 pmol.min⁻¹), but, again, thiol was not necessary for trypsinization under these conditions, consistent with the above result.

To determine whether the unreduced but nicked LT(E112K) containing the intrachain disulfide was active and possessed a rARF6 binding site, LT ADP-ribosyltransferase activity was assayed under conditions in which it was not dependent on thiol. Commercially available, nicked CTA was reduced and alkylated with iodoacetamide; the alkylated CTA was purified and assayed. LT(E112K) was trypsinized in the absence of dithiothreitol, under conditions similar to those shown in Table IV. In contrast to nicked CTA, which required thiol for activity, presumably to reduce the intrachain disulfide, alkylated CTA was thiol-independent and was still stimulated by rARF6 in a thiol-independent fashion (Table V). Dithiothreitol appeared to slightly inhibit ADP-ribosyltransferase activity. LT(E112K) that was trypsinized but not reduced did not inhibit rARF6-stimulated activity (Table V), consistent with the view that trypsinization and reduction are required to generate an ARF binding site. The requirements for inhibition of rARF6-stimulated activity by LT(E112K) are thus identical to those needed to generate an active A₆ protein, i.e. the appearance of an ARF binding site appears to coincide with formation of an active catalytic site (proteolysis and reduction of the single disulfide).

Without trypsinization, the toxin thus seems to be inactive, whether or not it is reduced, when assayed both catalytically and by its ability to interact with ARF. Proteolysis in the absence of reduction is insufficient to create a functional ARF-binding protein. Both proteolysis and reduction are
therefore necessary for activation and to generate an ARF binding site. The fact that basal ADP-ribosylagmatine for-
tion of basal ADP-ribosyltransferase activity.

TABLE V
Effect of dithiothreitol on inhibition of rARF6 stimulation of cholera toxin ADP-ribosyltransferase activity

| Additions to assay | ADP-ribosyltransferase activity |
|--------------------|---------------------------------|
|                    | CTA1                            |
|                    | Minus E/K | Plus E/K | Minus E/K | Plus E/K |
| None               | 4.1       | 3.3      |           |          |
| A6                 | 20.8      | 20.1     | 20.5      | 2.8      |
| DTT                | 75.0      | 12.0     |           |          |
| DTT, A6            | 14.8      | 3.4      | 20.5      | 8.8      |

Additions to assay

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