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An M_r = 52,000 Peptide Can Mediate Effects of Cholera Toxin on Adenylate Cyclase in Intact Cells

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SUMMARY

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Cholera toxin acts by ADP-ribosylating a membrane protein, variously termed N, G, or G/F, that is a guanine nucleotide-binding regulatory component of adenylate cyclase. Membranes of several mammalian cells contain two peptides, of M_r = 42,000 and 52,000, which are radiolabeled by incubation with cholera toxin and [^{32}P]NAD. Toxin activation of N in avian and human erythrocytes leads to significant radiolabeling only of the M_r = 42,000 peptide. Thus ADP-ribosylation of the M_r = 42,000 peptide appears to suffice for stimulation of adenylate cyclase, and no biological function has yet been assigned to the M_r = 52,000 peptide. We have found that incubation with toxin and [^{32}P]NAD significantly radiolabels an M_r = 52,000 peptide, but *not* an M_r = 42,000 peptide in particulate preparations of A9 mouse and 380-6 hamster transformed lung fibroblasts. Treatment of intact A9 or 380-6 cells with cholera toxin increases their cyclic AMP content and blocks subsequent toxin-catalyzed incorporation of ^{32}P from NAD into the M_r = 52,000 peptide of particulate extracts from the toxin-treated cells. Treating A9 and 380-6 particulate extracts with toxin and NAD increases their N activity in the presence of GTP, assayed using N-deficient membranes of the S49 cyc$^-$ mutant. We conclude that (1) a functionally active form of the N protein can contain the M_r = 52,000 peptide and (2) ADP-ribosylation of the M_r = 52,000 peptide subunit of N can mediate the action of cholera toxin.

INTRODUCTION

Hormone-sensitive adenylate cyclase consists of at least three separable components: Hormone receptors (R), a catalytic subunit (C) and a regulatory component, termed N[3] in this report, but also referred to as G/F (1) or G (2). Functionally, N couples R and C and mediates activation of adenylate cyclase by guanine nucleotides, NaF, and cholera toxin (1, 3). N activity can be assayed by virtue of its ability to complement N-deficient membranes of the S49 cyc$^-$ mutant (4). In detergent extracts of human erythrocyte membranes, N is an oligomer of over-all M_r = 126,000; one peptide subunit, M_r = 42,000 (42K), of the erythrocyte N protein is specifically radiolabeled by incubations of membranes with [^{32}P]NAD and cholera toxin (5).

Substantial evidence indicates that ADP-ribosylation of the 42K N peptide by cholera toxin is responsible for the toxin's effects (2, 5–9). For example, the 42K peptide is the only peptide significantly labeled in human or pigeon erythrocyte membranes by incubation with toxin and [^{32}P]NAD (2, 5, 8). The toxin ADP-ribosylates additional peptides in membranes of other cells, including S49 mouse lymphoma (8, 9), HTC-1 (9), and thymus (10). The following evidence indicates that one of these peptides of M_r = 52,000 (52K), is clearly related to the 42K peptide. First, cholera toxin fails to catalyze radiolabeling of both the 42K and 52K peptides in N-deficient S49 cyc$^-$ mutant cells, although both peptides are present in the parental (wild-type) lines (9). Second, exposure of intact cells to cholera toxin prior to membrane preparation specifically blocks the labeling of both of these peptides during a subsequent incubation with cholera toxin and [^{32}P]NAD (9); this result suggests that toxin transfers

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[3] The abbreviations used are: N, guanine nucleotide-binding regulatory component of adenylate cyclase; K, × 1000 (e.g., 42K = 42,000), SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS; Hepes, 4-(2-hydroxyethyl)-1-piperazinesthanesulfonic acid; TCA, trichoroacetic acid; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate).

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ADP-ribose from endogenous NAD specifically to these two peptides in intact cells, and saturates the available ADP-ribosylation sites. Third, both the 42K and 52K peptides exhibit acidic charge shifts in the S49 unc variant (11), which contains an N protein that is unable to couple with R (12, 13). Fourth, the fragments generated by partial proteolysis of the two radiolabeled peptides show almost identical patterns in SDS-PAGE (14). Fifth, preparations of N, purified more than 2000-fold, contain both 42K and 52K peptides that can be radiolabeled by cholera toxin and [^{32}P]NAD (6).

Despite these results, no biological function has been assigned to the 52K peptide. In the present report, we present data which support the hypothesis that this peptide can mediate effects of cholera toxin on adenylate cyclase in intact cells.

METHODS

The sources of reagents and cell lines and the conditions of culture have been described (5, 15, 16).

Particulate extracts of A9 and 380-6 cells were prepared as follows. Cells were released from three or four 75-cm^2 culture flasks with trypsin, diluted with 150 ml of either 5 mM sodium-Hepes (pH 8) plus Trasylol (10^2 U/ml) or media containing 10% fetal calf serum, and pelleted by centrifugation. The pelleted cells were resuspended in 5 ml of 25 mM sodium-Hepes (pH 8), 1 mM EDTA, Trasylol (10^3 U/ml), and 2 mM β-mercaptoethanol, and broken by Dounce homogenization with 20 strokes using a tight-fitting pestle. The preparation was centrifuged (27,000 $\times$ g, 30 min) and the pellet was resuspended in approximately 0.5 ml of the same buffer. The suspension was dispersed by forcing it through a 27-gauge needle. Aliquots were frozen and stored at −70°.

N activity was assayed by the following modification of our adaptation (8) of the method of Ross and Gilman (4). Particulate preparations, 1 mg of protein per milliliter, were solubilized with 0.2% Lubrol (w/v) on ice overnight and centrifuged for 5 min at 7000 $\times$ g. The Lubrol extract (10 μl) was combined with 20 μg of S49 cyc$^-$ membrane protein in 30 μl of a reaction cocktail, as described (8), except that the ATP concentration in the 40-μl volume was 0.5 mM. After a 20-min incubation at 30°, 10 μl of [α-^{32}P]ATP and ^{3}H-labeled cyclic AMP were added and the 50-μl volume was incubated an additional 40 min at 30°C, and the ^{32}P-cAMP purified (17).

Radiolabeling of N with cholera toxin and [^{32}P]NAD$^+$ (2–20 Ci/mmole) was carried out as described (5). Particulate extracts (1–3 mg of protein per milliliter) were incubated with cholera toxin (50 μg/ml) and [^{32}P]NAD$^+$ (20–100 μM) for 30 min at 30° in a cocktail containing 10 mM thymidine, 12 mM arginine-HCl, 1 mM GTP, 0.4 mM ATP, and 200 mM sodium potassium phosphate (pH 6.8). After the incubation, the particulate preparation was diluted at least 10-fold with water, collected by centrifugation, and solubilized for electrophoresis. One-dimensional SDS-PAGE was carried out as described by Laemmli (18), and equilibrium two-dimensional gel electrophoresis utilized the following modification of the method of O'Farrell (19).

Our first attempts to isoelectrofocus the ^{32}P-labeled subunit of N were frustrated by the failure of the label to enter completely the first-dimension gel and by streaking of the label that did enter. Boiling samples in 1% SDS, as suggested (11), did not completely eliminate these problems, and appeared to increase the charge heterogeneity of the ^{32}P-labeled peptides. These problems appeared to be unique for the cholera toxin-^{32}P-labeled peptides, since the Coomassie blue staining patterns of most other proteins were relatively insensitive to changes in sample preparation. We found that increasing the urea concentration, both in the sample preparation buffer and in the gel itself, improved entry and resolution of the labeled peptide. Increasing the urea concentration made it necessary to keep the samples and the gel warm. Thus, solutions were kept at 37°, and gels were run under a heat lamp. The composition of the solutions is given in Table 1. In all other respects, the gels were handled as previously described (19).

TABLE 1

Solutions used for isoelectric focusing in polyacrylamide gels

Substance	Gel solution	Application buffer
Urea	6.25 g	6.5 g
Acrylamide solution[a]	1100 μl	—
NP-40,[b] 10% w/w	3000 μl	3000 μl
Water	800 μl	1400 μl
Ampholytes,[c] pH 5–7	475 μl	—
Ampholytes,[c] pH 3.5–10	25 μl	500 μl
β-Mercaptoethanol	—	500 μl
Ammonium persulfate, 10% w/v	10 μl	—
TEMED[d]	5 μl	—

[a] Acrylamide, 28.4%, and *N,N'*-methylenebis[acrylamide], 1.62%, w/v; Bio-Rad Laboratories, Richmond, Calif.

[b] Almega Corporation, Bensenville, Ill.

[c] LKB, 40% w/v.

[d] *N,N,N',N'*-tetramethylenediamine; Bio-Rad Laboratories.

Two methods of sample preparation were found to give equivalent results. Pellets of labeled membranes (10–50 μg of protein) previously washed with water were solubilized in 10 μl of 5% (v/v) 2-mercaptoethanol and 7.5% (w/v) glycerol, containing either 1% SDS (w/v) or 3% NP-40 (w/w). The SDS-containing solution effectively dissolved membranes within 30 min at 37°. The NP-40 solution required heating (10 min at 70°) for complete solubilization. After solubilization, 100 μl of application buffer were added, and the samples were incubated at 37° for approximately 30 min before applying to the first-dimension gel. It should be noted that, despite repeated attempts, this method failed to resolve the ^{32}P-labeled 42K peptide of human erythrocytes. In addition, with S49 extracts, recovery of the 42K peptide appeared to be diminished relative to the 52K peptide.

Samples of cyclic AMP were derived from 4 $\times$ 10^5 cells plated in 6-cm^2 dishes; 12 hr after plating the medium was aspirated, and 3 ml of serum-free Dulbecco's modified Eagle's medium, with or without cholera toxin (1 μg/ml), were added to each dish. The dishes were incubated at 37° for various times and the media were aspirated and replaced with cold 10% TCA. The TCA-soluble extracts were extracted with ether, lyophilized and assayed for cyclic AMP by radioimmunoassay (20). Assay reagents and methods were obtained from New England

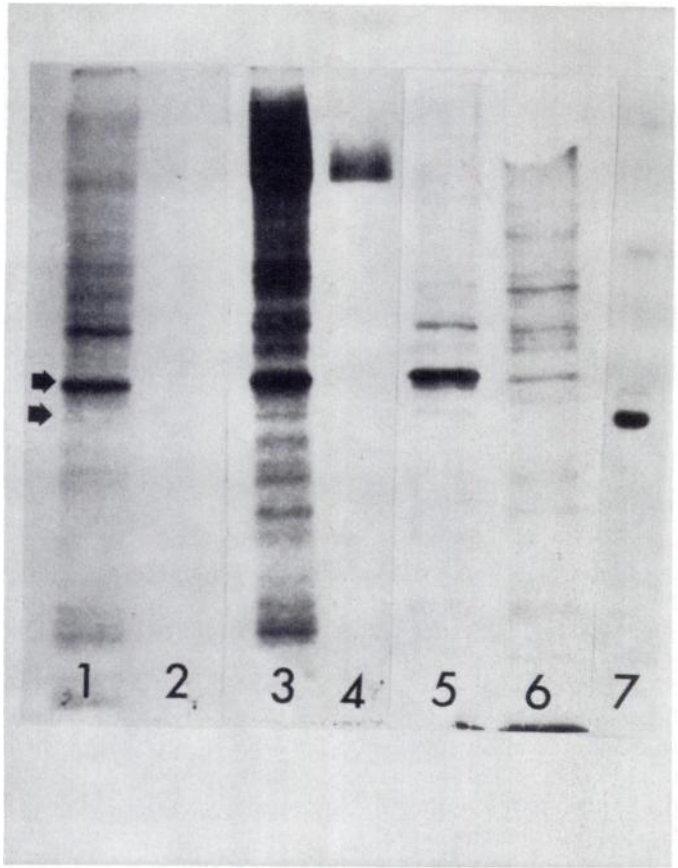

FIG. 1. *Autoradiograms of particulate cell extracts incubated with [^{32}P]NAD$^+$ and subjected to SDS-PAGE*

Experiment 1 (lanes *1–4*): extracts from A9 (lanes *1* and *2*) and 380-6 (lanes *3* and *4*) incubated with (lanes *1* and *3*) or without (lanes *2* and *4*) cholera toxin. Experiment 2 (lanes *5–7*): 380-6 cells in culture were treated for 16 hr without (lane *5*) or with (lane *6*) cholera toxin, 1 μg/ml. Particulate extracts of these cells were then incubated with cholera toxin and [^{32}P]NAD$^+$. Lane 7 shows the labeling pattern of human erythrocyte membranes treated with toxin and [^{32}P]NAD$^+$. The *upper* and *lower arrows* designate M_r = 52,000 and 42,000 respectively.

Nuclear Corporation, Boston, Mass. Protein was determined by treating the TCA-extracted dishes with 1 N NaOH and assaying the solution by the method of Lowry *et al.* (21).

RESULTS

Cholera toxin dramatically increased incorporation of ^{32}P from [^{32}P]NAD$^+$ into particulate extracts of both A9 and 380-6 cells (Fig. 1, lanes *1* and *3*): Toxin-dependent radiolabel was most prominent in the 52K peptide,[4] but little or none was detected in the region of SDS gels corresponding to the 42K peptide labeled in human erythrocyte membranes (Fig. 1, lane *7*).[5] The following data suggest that the toxin-dependent ADP-ribosylation of the 52K peptide mediates the toxin's activation of adenylate cyclase in A9 and 380-6 cells.

First, treatment of either cell type with cholera toxin increased intracellular cyclic AMP for a prolonged period (Table 2). Second, treatment of the cells with cholera toxin blocked labeling of the 52K peptide during subsequent incubation of particulate preparations with toxin and [^{32}P]NAD (Fig. 1, lanes *5* and *6*). Third, treatment of particulate preparations with toxin and NAD modified N activity in a fashion characteristic of cholera toxin: in mixtures containing S49 cyc$^-$ membranes and N from toxin-treated A9 or 380-6 membranes, adenylate cyclase activity in the presence of GTP approached the activity observed in the presence of isoproterenol and GTPγS, which can be considered an approximation of the V_{max} of the enzyme (Table 3).

Equilibrium 2-dimensional gels (Fig. 2) show that the 52K toxin substrate peptides of A9 and 380-6 cells migrate to the same position as does the corresponding peptide of wild-type S49 cells. Thus, the 52K peptide from these cells has the same size and charge. This structural similarity between the 52K peptides of S49 cells and those of both lung fibroblast lines suggests that this larger polypeptide can partake in the function of active N oligomers

TABLE 2

Cyclic AMP content of A9 and 380-6 cells incubated in the absence and presence of cholera toxin

Time of exposure	Cyclic AMP			
	A9		380-6	
	Control	+ Toxin[a]	Control	+ Toxin
hr	*pmoles/mg cell protein*			
1	3	27	4	41
2	6	40	2	140
6	6	50	4	580

[a] Concentration = 1 μg/ml.

TABLE 3

N activity in control or cholera toxin-treated particulate preparations

N activity is expressed as picomoles of ^{32}P-labeled cyclic AMP generated per minute per milligram of S49 cyc$^-$ membrane protein, as described (8). Isoproterenol (ISO) and GTPγS were present at 100 μM concentrations, and NaF at 10 mM. Adenylate cyclase activity in the absence of either Lubrol extracts or cyc$^-$ membranes was less than 10% of the activity observed in the complete assay system. Particulate preparations were incubated in 200 mM potassium phosphate (pH 6.8) with 20 μM NAD$^+$, 0.4 mM ATP, 1 mM GTP, 12 mM arginine HCl, and 10 mM thymidine in either the absence (control) or presence (+ toxin) of dithiothreitol-treated (5, 8) cholera toxin (50 μg/ml) for 30 min at 30°. The particulates were diluted with water, collected by centrifugation, resuspended in 0.2% Lubrol in 20 mM sodium-Hepes (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM 2-mercaptoethanol, and N was assayed (5, 8). The N activities of these preparations and human erythrocytes had similar specific activities on a protein basis.

Source of N	Treatment	N activity in presence of		
		GTP	ISO/GTPγS	NaF
A9 cells	Control	2	164	171
	+ Toxin	138	195	132
380-6 cells	Control	4	142	166
	+ Toxin	127	180	124

[4] Other peptides ADP-ribosylated by cholera toxin in these particulate preparations will be identified in a subsequent report. Because cholera toxin does not detectably ADP-ribosylate these peptides in intact cells, they do not appear to be involved in regulation of adenylate cyclase by the toxin (H. Kaslow, in preparation).

[5] In the autoradiograms shown, an exposure was used that led to an intensity of the 52K band (lanes *1*, *3*, and *5*) that was well beyond the linear range of the film. This long exposure was used in order to detect a labeled 42K peptide. The amount of label in the 42K region of the gel appears to be orders of magnitude less than that detected for the 52K peptide.

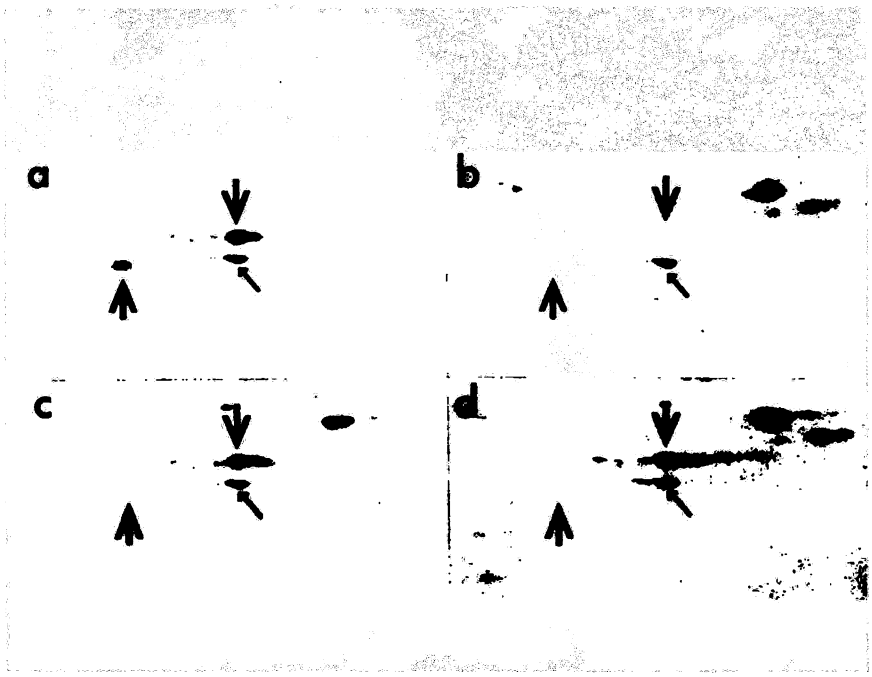

FIG. 2. *Autoradiograms of particulate cell extracts incubated with [^{32}]NAD$^+$ and cholera toxin, and then subjected to equilibrium 2-dimensional gel electrophoresis*

Extracts were from S49 wild-type (*a*), S49 cyc$^-$ (*b*), A9 (*c*), and 380-6 (*d*) cells. In each panel, the first (isoelectric focusing) dimension runs from basic (*left*) to acidic (*right*), while the second dimension shows migration (from *top* to *bottom*) in SDS-PAGE. Only a portion of each gel is shown. Spots corresponding to the peptides specifically ^{32}P-labeled by cholera toxin are indicated by *large arrows* pointing downward (52K peptide) or upward (42K peptide). Extracts from cells metabolically labeled with [^{35}S]methionine were added to each sample before electrophoresis, to serve as markers. The *small upward-pointing arrows* indicate ^{35}S-labeled actin (M_r = 45,000).

in S49 cells as well as in A9 and 380-6. In addition, the 42K peptide, which migrates to a more basic position in wild-type S49 extracts, was not detected in 2-dimensional gels of either lung fibroblast line. As reported previously (9), neither the 42K nor the 52K peptide is labeled in S49 cyc$^-$.

DISCUSSION

The fact that erythrocyte N proteins contain only the 42K peptide substrate of cholera toxin shows that this peptide can mediate the toxin's stimulation of adenylate cyclase (2, 5, 7, 8). Similarly, the present observations with A9 and 380-6 cells indicate that another form of N, containing the 52K but not the 42K toxin substrate, can also mediate the action of cholera toxin.

These results raise questions regarding the intramolecular arrangement and functional significance of toxin substrates in cells, such as wild-type S49 (9) and human fibroblasts,[6] whose membranes contain both the 42K and 52K toxin substrates. For example, the molecular size of N [M_r = 126,000–130,000 (5, 22)] could allow it to accommodate at least two toxin substrate peptides.[7] If so, three different subunit structures are possible for N in S49 cells, containing either two 42K peptides, two 52K peptides, or one of each.

Finally, do these differences in structure cause differences in function? Although each of the forms of N apparently can mediate the actions of cholera toxin, they may differ in capacity to mediate stimulation of adenylate cyclase by hormones or in ability to modulate agonist-induced down-regulation of receptor number (23, 24). Northup *et al.* (6) have reported that purified N from liver contains both the 42K and 52K peptides, but they were unable to resolve forms of N containing only one of these two peptides. If cell types that express only one of these two toxin substrates provide a source for purification of N consisting of only one subunit structure, then they may prove useful in answering this question.

[6] Human skin fibroblasts contain 52K and 42K peptides that are specifically radiolabeled by incubation with [^{32}P]NAD$^+$ and cholera toxin (H. Kaslow, unpublished results).

[7] Presumably these N oligomers contain, in addition to toxin substrates, a subunit corresponding to the M_r = 35,000 peptide, not susceptible to ADP-ribosylation by cholera toxin, that comigrates with the N protein purified from rat liver (6). If so, an oligomer of M_r = 126,000–130,000 probably could not accommodate more than two toxin substrate peptides.

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