Cholera Toxin can Catalyze ADP-Ribosylation of Cytoskeletal Proteins

HARVEY R. KASLOW, VINCENT E. GROPPI, MARY E. ABOOD, and HENRY R. BOURNE
Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, and the Cardiovascular Research Institute and Department of Microbiology, University of California, San Francisco, California 94143

ABSTRACT Cholera toxin catalyzes transfer of radiolabel from [^{32}P]NAD$^+$ to several peptides in particulate preparations of human foreskin fibroblasts. Resolution of these peptides by two-dimensional gel electrophoresis allowed identification of two peptides of M_r = 42,000 and 52,000 as peptide subunits of a regulatory component of adenylate cyclase. The radiolabeling of another group of peptides (M_r = 50,000 to 65,000) suggested that cholera toxin could catalyze ADP-ribosylation of cytoskeletal proteins. This suggestion was confirmed by showing that incubation with cholera toxin and [^{32}P]NAD$^+$ caused radiolabeling of purified microtubule and intermediate filament proteins.

Cholera toxin activates synthesis of cyclic 3',5'-adenosine monophosphate (cAMP) by catalyzing ADP-ribosylation of a regulatory component of adenylate cyclase (1–3). This component, variously termed G/F (4), G (5), or N[1] (6), is required for regulation of the enzyme by hormones, guanine nucleotides, and cholera toxin. When [^{32}P]NAD$^+$ is used as the substrate for the toxin, ^{32}P-radiolabeled peptides can be separated by gel electrophoresis and detected by autoradiography. This technique has led to identification of two peptide subunits, of M_r = 42,000 and 52,000, of the N component(s) (1–3). In some preparations, however, the toxin causes ADP-ribosylation of other peptides, not known to be involved in regulation of adenylate cyclase (1–3, 7). The radiolabeling of these peptides by cholera toxin must involve some degree of specificity, because they are not radiolabeled in proportion to their relative abundance in tissue extracts. We have sought to identify some of these peptides.

MATERIALS AND METHODS

Human foreskin fibroblasts ($H_{27}F_{14}$), obtained from the Tissue Culture Facility, University of California, San Francisco, Calif., were maintained in plastic 150 cm^2 flasks in Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose and 10% fetal calf serum. Postconfluent cultures (seventh to twelfth passage) were routinely subcultured by trypsinization into four new flasks at 10-d intervals, or were harvested by trypsinization for preparation of particulate extracts, as described (8). S49 mouse lymphoma cells were cultured as described (9). Proteins were metabolically labeled with [^{35}S]methionine (1,000 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) as described (10).

Fibroblast cytoskeletons containing actin filaments and intermediate filaments were prepared by a modification of described methods (11). Confluent fibroblasts in 35-mm plastic dishes were washed with 0.1 M PIPES, pH 6.9, 0.5 mM $MgCl_2$, and 0.1 mM EDTA (stabilization buffer). Membrane and cytosol proteins were extracted by incubating with 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) in stabilization buffer for 1 min, and washing three times with stabilization buffer. All solutions were kept at 30°C. The cytoskeleton preparations were then incubated for 30 min at 30°C with 300 μl stabilization buffer containing 50 μM [^{32}P]NAD (10 Ci/mMol), 1 mM GTP, 0.4 mM ATP, and, when appropriate, 50 μg/ml DTT-treated cholera toxin (12). The preparation was then aspirated, washed with stabilization buffer, aspirated free of the buffer, and solubilized with 100 μl of 5% β-mercaptoethanol (vol/vol), 7.5% glycerol (wt/vol) and 1% SDS (wt/vol).

Microtubule protein was radiolabeled by incubating the purified protein at 1 mg/ml in 200 mM sodium phosphate, pH 6.8, 2 mM GTP, 0.4 mM ATP, 10 mM thymidine, 12 mM arginine-HCl, and 40 μM [^{32}P]NAD (10 Ci/mMol) and 50 μg/ml DTT-treated cholera toxin (12). The incubation was terminated by diluting 5 μl of the reaction mixture with 5 μl 2% SDS, 20% β-mercaptoethanol, and 15% glycerol.

Two-dimensional electrophoresis of these samples was done using a modification (8) of the O'Farrell method (13). A charge shift of one unit is arbitrarily defined as the distance a peptide moves due to phosphorylation in comparable pH ranges of two-dimensional gels of S49 cell proteins (10). No radiolabeled peptides were detected if cholera toxin was omitted from incubations.

Purified microtubule protein from chicken brain was a gift from Dr. Qui-Lin Choo and Dr. Marc W. Kirschner of the Department of Biochemistry, University of California, San Francisco, Calif., as described (14, 15). PIPES and NAD$^+$ase (N9629) were from Sigma Chemical Co. Other methods and the sources of other materials have been described (8, 16).

RESULTS

Cholera toxin catalyzed the transfer of radiolabel from [^{32}P]NAD$^+$ to several peptides in particulate extracts of human

[1] Abbreviations: N, guanine nucleotide-binding regulatory component of adenylate cyclase; PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; MAPs, microtubule-associated proteins; IFP, intermediate filament protein; Gpp(NH)p, β-γ-imidoguanosine 5'-triphosphate.

THE JOURNAL OF CELL BIOLOGY · VOLUME 91 NOVEMBER 1981 410–413

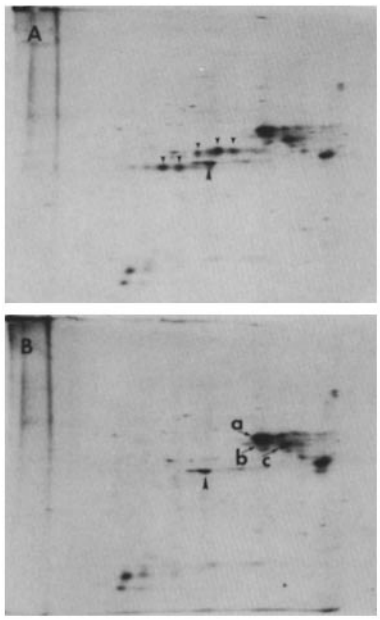

FIGURE 1 Autoradiograms of particulate cell extracts incubated with [^{32}P]NAD$^+$ and cholera toxin, and then subjected to equilibrium two-dimensional gel electrophoresis. Extracts were from human foreskin fibroblasts ($H_{27}F_{14}$) that were cultured either under control conditions (*A*) or with cholera toxin (1 μg/ml, 16 h) (*B*) before preparation of the extract. In each panel the first (isoelectric focusing) dimension runs from basic (left) to acidic (right); the second dimension shows migration (from top to bottom) in SDS-polyacrylamide gel electrophoresis. Extracts from cells metabolically labeled with [^{35}S]methionine were added to each sample before electrophoresis, to serve as markers. The upward-pointing arrow indicates ^{35}S-labeled actin (M_r = 45,000). Spots corresponding to the M_r, 42,000 and 52,000 peptide components of the regulatory subunit of adenylate cyclase (N) are indicated by small downward-pointing arrows. In panel B, the ^{32}P spot thought to be the satellite of IFP is indicated by a, and the two subunits of tubulin by b and c.

foreskin fibroblasts.[2] Of those peptides resolved by two-dimensional gel electrophoresis, two sets (indicated by small arrows in Figure 1*A*) appear to be subunits of the N component of adenylate cyclase. Their size (M_r = 42,000 and 52,000) and charge were similar to those of N peptides previously characterized in S49 mouse lymphoma cell membranes (8, 17). The cause of the charge heterogeneity of these peptides in human fibroblasts, like that in the mouse cells (8, 17), is unknown.

A second group of labeled peptides focused at substantially more acidic pH, with M_r ranging from 50,000 to 65,000 (Fig. 1*A* and *B*). These ^{32}P-labeled peptides were resolved about two acidic charge units away from a constellation of relatively abundant proteins, thought to include intermediate filament protein (IFP) and tubulin, that stained with Coomassie blue. An acidic charge shift of two units is consistent with ADP-ribosylation of a basic group on the peptide. Coomassie Blue stained a number of other abundant peptides (staining pattern not shown), including actin; these peptides were not radiolabeled, nor were they associated with labeled, acidic-shifted satellites.

The location of the ^{32}P-labeled peptides suggested that they included the cytoskeletal proteins IFP and tubulin. The following results support this hypothesis.

When purified microtubule protein was incubated with [^{32}P]NAD$^+$ and cholera toxin, radiolabeled spots (Fig. 2*B*) were resolved in positions that corresponded to those of several peptides labeled in particulate fibroblast extracts (Fig. 1). The ^{32}P-labeled proteins focused at a position two charge units to the acidic side of the unlabeled protein (Fig. 2). Several peptides in the purified microtubule protein preparation not easily detected in the Coomassie Blue-staining pattern (Fig. 2*A*), were also radiolabeled by incubation with toxin and [^{32}P]NAD$^+$ (Fig. 2*B*). Two of these spots were also seen in autoradiograms of particulate preparations (compare Figs. 1 and 2). The higher molecular weight protein migrated to a position characteristic of tau, a specific microtubular associated protein (MAP). The lower molecular weight peptide may be a degradation product of tubulin or a MAP.

Mild detergent extraction of a fibroblast monolayer can leave a cytoskeletal network of pure IFP and actin attached to the dish (11). When such cytoskeletons were incubated in situ with cholera toxin and [^{32}P]NAD$^+$, ^{32}P was detected in IFP, but not in actin (Fig. 3). As in the experiments with purified extracts and purified microtubule protein, the ^{32}P-labeled peptide was shifted approximately two acidic charge units away

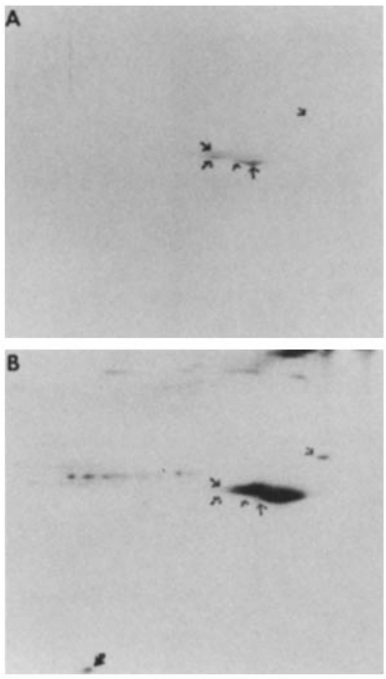

FIGURE 2 Coomassie Blue-staining pattern (*A*) and autoradiogram (*B*) of purified microtubule protein incubated with [^{32}P]NAD$^+$ and cholera toxin, and then subjected to equilibrium two-dimensional gel electrophoresis. For details, see Fig. 1. Arrows were drawn on the gel (*A*) with ^{14}C-ink pointing to stained peptides, and exposed the autoradiogram (*B*). These arrows designate, to increasing migration in SDS: tau, and the α_1, α_2, β_1, and β_2 subunits of tubulin. The curved arrow points to another labeled peptide: a similar peptide is seen in Fig. 1.

[2] To demonstrate that this transfer of ^{32}P was not the result of nonenzymatic addition of free ADP-ribose to protein, we conducted the following experiment: Cytoskeletons and microtubule proteins were incubated for 30 min at 30° with [^{32}P]NAD$^+$ (25 μM) and either (*a*) no addition (control), (*b*) NAD$^+$ase (140 mU/ml), (*c*) heat-treated NAD$^+$ase, or (*d*) cholera toxin (100 μg/ml). Aliquots were electrophoresed, and labeled proteins were detected only in tbe sample containing the toxin. Cholera toxin was then added to the first three fractions and the incubation continued for another 30 min. Only the sample containing NAD$^+$ase failed to incorporate label into proteins. The control and heat-treated NAD$^+$ase samples were nearly identical.

from the Coomassie Blue-stained IFP. A small amount of stained protein was resolved one acidic charge unit from the major Coomassie Blue-staining peptide of IFP. This protein was probably phosphorylated IFP (10). No label was detected in a position two acidic charge units from phosphorylated IFP (Fig. 3). Thus, phosphorylation of IFP may block ADP-ribosylation.

In experiments involving both types of purified proteins (microtubule and intermediate filaments), we consistently failed to detect any cholera toxin-induced shift of Coomassie-stained protein. Thus, the conditions used for ADP-ribosylation described in this report led to ADP-ribosylation of only a small portion of the incubated protein.

Does cholera toxin ADP-ribosylate cytoskeletal proteins in intact cells? We addressed this question with two types of experiments. First, we exposed fibroblasts to cholera toxin (1 μg/ml) for 16 h before harvesting and preparation of particulate extracts. As previously shown in S49 cells (3), this treatment prevented labeling of the M_r = 42,000 and 52,000 subunits of the fibroblast N protein during subsequent incubation of particulate extracts with [^{32}P]NAD$^+$ and cholera toxin (Fig. 1 *B*). Presumably cholera toxin filled the available ADP-ribosylation sites, using nonradioactive cellular NAD$^+$ as substrate, before particulate extracts were prepared. Exposure of intact fibroblasts to cholera toxin did not, however, affect subsequent toxin-catalyzed incorporation of radiolabel into tbe acidic group of M_r = 50,000 to 65,000 peptides (Fig. 1 *B*). Thus, exposure of cells to cholera toxin did not fill all ADP-ribosylation sites of these peptides.

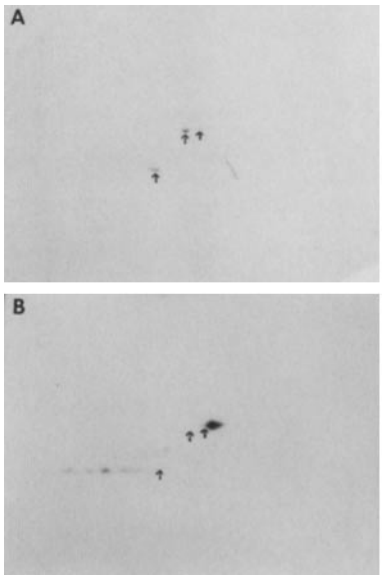

FIGURE 3 Coomassie Blue-staining pattern (*A*) and autoradiogram (*B*) of a cytoskeleton preparation from $H_{27}F_{14}$ fibroblasts incubated in situ with [^{32}P]NAD$^+$ and cholera toxin, and then subjected to equilibrium two-dimensional gel electrophoresis. For details, see Fig. 1. The arrows point to proteins detected by Coomassie Blue and were drawn on the gel (*A*) in ^{14}C-ink. The lower arrow points to actin. The upper two arrows point to IFP on the left and phosphorylated IFP on the right. The ^{14}C-ink exposed the autoradiogram, and serves to show the relationship of the ^{32}P-peptides to the Coomassie Blue-stained peptides. The ^{32}P-spots to the left of the arrow pointing to actin are probably due to ^{32}P-labeled adenylate cyclase. This type of contamination was seen only once in four experiments, but is shown because it serves to allow a better comparison to the autoradiograms shown in Fig. 1.

Nonetheless, the possibility remained that a small but potentially biologically relevant fraction of these peptides were ADP-ribosylated by cholera toxin in intact cells. In a second series of experiments (not shown), we looked for charge shifts in these peptides caused by cholera toxin in human fibroblasts and S49 kin$^-$ cells, using metabolic labeling with [^{35}S]methionine. Fibroblasts simultaneously exposed to [^{35}S]methionine and cholera toxin (1 μg/ml, 4 h) showed increased labeling of a spot one chart unit to the acidic side of IFP. This label probably represents phosphorylated IFP, as shown in S49 cells (10). No increase in ^{35}S was detected, however, in tbe positions corresponding to any of the ^{32}P-labeled satellites of the cytoskeletal proteins. To eliminate charge shifts caused by cAMP-dependent phosphorylation, we also studied a cell that lacks of cAMP-dependent protein kinase, the S49 kin$^-$ variant (18). The kin$^-$ variant accumulates cAMP in response to cholera toxin, but fails to show any of the charge shifts caused by cAMP activation of cAMP-dependent kinase in wild type S49 cells (10). Cholera toxin caused no detectable charge shifts in the [^{35}S]methionine autoradiograms of kin$^-$ cells. Thus we have found no evidence that cholera toxin ADP-ribosylates cytoskeletal proteins in intact cells.

DISCUSSION

Our results indicate that cholera toxin can catalyze the ADP-ribosylation of cytoskeletal proteins in broken but not in intact cells. Nonetheless, the existence of these sites suggests that endogenous enzymes may ADP-ribosylate cytoskeletal proteins and regulate their polymerization-depolymerization reactions.

What functional consequences result from ADP-ribosylation of cytoskeletal proteins? Knowledge of regulation of adenylate cyclase by cholera toxin suggests one possibility. GTP supports both polymerization of microtubule proteins (19) and activation of adenylate cyclase (20). Hydrolysis-resistant analogs of GTP, such as Gpp(NH)p, act as effective but not equivalent substitutes for GTP in both systems (19, 21). Cholera toxin activates adenylate cyclase by inhibiting an associated GTPase activity, thus allowing GTP and Gpp(NH)p to activate the enzyme in a similar fashion (22). Thus, ADP-ribosylation of a cytoskeletal protein may inhibit a GTPase, and make the effects of GTP more closely resemble those of Gpp(NH)p.

In addition, cholera toxin may exert effects on cells that are not mediated by cAMP. For example, recent data indicate that the toxin stimulates mitogenesis of 3T3 cells by a mechanism that does not involve cAMP (23). If so, then ADP-ribosylation of N by the toxin may have effects in addition to activation of adenylate cyclase, or ADP-ribosylation of other peptides may mediate the toxin's effect. Cytoskeletal proteins remain as potential substrates for the toxin in mediating its cAMP-independent action(s), even though we have failed to detect ADP-ribosylation of these proteins in intact cells.

We thank Dr. Marc Kirschner for useful discussions.

This work was supported by National Institutes of Health grants AM 27307, GM 28310, GM 27800, and GM 07546.

Received for publication 6 April 1981, and in revised form 17 August 1981.

REFERENCES

1. Gill, D. M., and R. Meren. 1978. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* 75:3050–3054.
2. Cassel, D., and T. Pfeuffer. 1978. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natl. Acad.*

Sci. U. S. A. 75:2669–2673.
3. Johnson, G. L., H. R. Kaslow, and H. R. Bourne. 1978. Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. *J. Biol. Chem.* 253:7120–7123.
4. Ross, E. M., A. C. Howlett, K. M. Ferguson, and A. G. Gilman. 1978. Reconstitution of hormone sensitive adenylate cyclase activity with resolved components of the enzyme. *J. Biol. Chem.* 243:6401–6412.
5. Pfeuffer, T. 1979. Guanine nucleotide-controlled interactions between components of adenylate cyclase. *Febs. Fed. Eur. Biochem. Soc. Lett.* 101:85–89.
6. Johnson, G. L., H. R. Kaslow, and H. R. Bourne. 1978. Reconstitution of cholera toxin-activated adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* 75:3113–3117.
7. Watkins, P. A., J. Moss and M. Vaughan. 1980. Effects of GTP on choleragen-catalyzed ADP-ribosylation of membrane and soluble proteins. *J. Biol. Chem.* 255:3959–3963.
8. Kaslow, H. R., D. Cox, V. E. Groppi, and H. R. Bourne. 1981. An M_r = 52,000 peptide can mediate the effects of cholera toxin in intact cells. *Mol. Pharmacol.* 19:406–410.
9. Bourne, H. R., P. Coffino, and G. M. Tomkins. 1975. Selection of a variant lymphoma cell deficient in adenylate cyclase. *Science (Wash. D. C.).* 187:950–952.
10. Steinberg, R. A., P. H. O'Farrell, V. Friedrich, and P. Coffino. 1977. Mutations causing charge alterations in regulatory subunits of the cAMP-dependent protein kinase of cultured S49 lymphoma cells. *Cell.* 10:381–391.
11. Heuser, J. E., and M. W. Kirschner. 1980. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.* 86:212–234.
12. Johnson, G. L., and H. R. Bourne. 1977. Influence of cholera toxin on the regulation of adenylate cyclase by GTP. *Biochem. Biophys. Res. Commun.* 78:792–798.
13. O'Farrell, P. H. 1975. High resolution two dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
14. Shelanski, M. L., F. Gaskin, and C. R. Cantor. 1973. Microtubule assembly in absence of added nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* 70:765–768.
15. Weingarten, M. D., A. H. Lockwood, S-Y Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. U. S. A.* 72:1858–1862.
16. Kaslow, H. R., G. L. Johnson, V. M. Brothers, and H. R. Bourne. 1980. A regulatory component of adenylate cyclase from human erythrocyte membranes. *J. Biol. Chem.* 255: 2726–2741.
17. Schleifer, L. S., H. C. Garrison, P. C. Sternweis, J. K. Northup, and A. G. Gilman. 1980. The regulatory component of adenylate cyclase from uncoupled S49 lymphoma cells differs in charge from the wild type protein. *J. Biol. Chem.* 255:2641–2644.
18. Steinberg, R. A., T. Van Daalen Wetters, and P. Coffino. 1978. Kinase-negative mutants of S49 mouse lymphoma cells carry a trans-dominant mutation affecting expression of cAMP-dependent protein kinase. *Cell.* 15:1351–1361.
19. Terry, B. J., and D. L. Purich. 1980. Assembly and disassembly properties of microtubules formed in the presence of GTP, 5′ guanylyl imidodiphosphate, and 5′-guanylyl methylenediphosphate. *J. Biol. Chem.* 255:10532–10536.
20. Rodbell, M., L. Birnbaumer, S. L. Pohl, and H. M. J. Kraus. 1971. The glucagon-sensitive adenylate cyclase system in plasma membranes of rat liver. *J. Biol. Chem.* 246:1877–1882.
21. Rodbell, M. 1980. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.).* 284:17–22.
22. Cassel, D., and Z. Selinger. 1977. Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. *Proc. Natl. Acad. Sci. U. S. A.* 74:3307–3311.
23. Pruss, R. M., and H. R. Herschman. 1979. Cholera toxin stimulates division of 3T3 cells. *J. Cell. Physiol.* 98:469–474.