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.

RESULTS

Cholera toxin catalyzed the transfer of radiolabel from $[^{32}P]NAD^+$ to several peptides in particulate extracts 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.
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 (H27F,4) that were cultured either under control conditions (A) or with cholera toxin (1 \(\mu\)g/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 \(^{31}\)S\_labeled actin (Mr = 45,000). Spots corresponding to the Mr, 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 1A) appear to be subunits of the N component of adenylate cyclase. Their size (Mr = 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 Mr, ranging from 50,000 to 65,000 (Fig. 1A 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. 2B) 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. 2A), were also radiolabeled by incubation with toxin and \([^{32}\)P]NAD\(^{+}\) (Fig. 2B). 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 from IFP, and could be shown to be due to ADP-ribosylation (Fig. 3B). 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 Mr = 42,000 and 52,000 subunits of the fibroblast N protein during subsequent incubation of particulate extracts with [32P]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 the acidic group of Mr = 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.

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 [35S]methionine. Fibroblasts simultaneously exposed to [32P]methionine and cholera toxin (1 µg/ml, 4 h) showed increased labeling of a spot one charge unit to the acidic side of IFP. This label probably represents phosphorylated IFP, as shown in S49 cells (10). No increase in [35S] was detected, however, in the positions corresponding to any of the 32P-labeled satellites of the cytoskeletal proteins. To eliminate charge shifts caused by cAMP-dependent phosphorylation, we also studied a cell that lacks 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 [32P]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.

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