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Cellular ADP-ribosyltransferase with the same mechanism of action as diphtheria toxin and *Pseudomonas* toxin A

(bacterial toxins/protein synthesis)

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ABSTRACT **An ADP-ribosyltransferase was found in elongation factor 2 (EF-2) preparations from polyoma virus-transformed baby hamster kidney (pyBHK) cells. Like fragment A of diphtheria toxin and *Pseudomonas* toxin A, this eukaryotic cellular enzyme transfers [^{14}C]adenosine from NAD$^+$ to EF-2. However, the cellular transferase is immunologically distinct from fragment A. The transferase also can be distinguished from fragment A and *Pseudomonas* toxin A by the inhibition of the activity of the former by cytoplasmic extracts and by histamine. Snake venom phosphodiesterase digestion of the [^{14}C]adenosine-labeled EF-2 product of the cellular transferase reaction yielded [^{14}C]AMP, indicating that the cellular enzyme is a mono(ADP-ribosyl)transferase. The forward ADP-ribosylation reaction catalyzed by the cellular enzyme is reversed by fragment A, yielding [^{14}C]NAD$^+$. The results strongly suggest that the cellular transferase is a mono(ADP-ribosyl)transferase, which ADP-ribosylates the same diphthamide residue of EF-2 as does fragment A and *Pseudomonas* toxin A.**

Mono(ADP-ribosyl)ated proteins have been found in a variety of eukaryotic tissues (1-3). These ADP-ribosylated proteins are present in practically every major compartment of the cell (4), suggesting a diversity of biological functions. However, little is known about the identity of these mono(ADP-ribosyl)ated acceptor proteins and their physiological functions. Moss and Vaughn (5) have described a cytosolic ADP-ribosyltransferase from turkey erythrocytes that catalyzes the mono(ADP-ribosyl)ation of several endogenous proteins and the activation of brain adenylate cyclase. They were the first to suggest that the ADP-ribosyltransferase mechanisms of bacterial toxins, such as cholera toxin and heat-labile enterotoxin of *Escherichia coli*, are not entirely foreign to vertebrate cells.

The fragment A portion of diphtheria toxin and toxin A of *Pseudomonas aeruginosa* transfer ADP-ribose from NAD$^+$ to the diphthamide acceptor site of elongation factor 2 (EF-2) (6–10). ADP-ribosylated EF-2 no longer functions in protein synthesis, resulting in intoxication of the cells. In this paper we describe an endogenous mono(ADP-ribosyl)transferase from polyoma virus-transformed baby hamster kidney (pyBHK) cells that also transfers ADP-ribose from NAD$^+$ to the diphthamide residue of EF-2.

MATERIALS AND METHODS

Enzymatic Activity. EF-2 was purified from pyBHK cells as described (11). These EF-2 preparations also served as the source of the pyBHK transferase activity described in this report. Fragment A of diphtheria toxin was purified as described (12). ADP-ribosyltransferase activity was measured by the incorporation of radioactivity from [*adenosine*-U-^{14}C]NAD$^+$ into trichloroacetic acid-precipitable material in the presence of EF-2 by a modification of a previously described procedure (11, 13). The modification was the absence of histamine and the use of purified EF-2 in the reaction. Unless otherwise noted, the reaction was performed at 22°C in 0.1 ml of 25 mM Tris·HCl, pH 8.0/1 mM dithiothreitol/6.33 μM [*adenosine*-U-^{14}C]NAD$^+$ (534 mCi/mmol, New England Nuclear; 1 Ci = 37 GBq) containing EF-2 at 50 μg/ml and with or without various amounts of *Pseudomonas* toxin A, fragment A, or a mixture of fragment A and anti-fragment A antiserum. After various intervals of incubation, 10-μl samples were withdrawn and coprecipitated with 0.1 ml of bovine serum albumin at 0.1 mg/ml in 1 ml of 10% trichloroacetic acid. Precipitates were collected and washed, and the radioactivity was measured as described (11).

Analysis of Reaction Product. Radiolabeled products formed in the ADP-ribosyltransferase assays were electrophoresed in NaDodSO$_4$/polyacrylamide gels as described (11). Gels were dried and developed by autoradiography (11).

To determine if ADP-ribose was present in the acceptor protein as monomeric units or as poly(ADP-ribose), EF-2 was labeled by incubation for 160 min with [*adenosine*-U-^{14}C]NAD$^+$ and the endogenous pyBHK transferase or fragment A as described for our standard reaction mixture. The reaction mixture was dialyzed extensively to remove NAD$^+$, evaporated to dryness under nitrogen, and resuspended in 25 μl of 20 mM ammonium bicarbonate buffer (pH 9.0). Then 25 μl of snake venom phosphodiesterase (Worthington) at 1 mg/ml in 40 mM $MgCl_2$ was added (10), and the mixture was incubated at 37°C for 15 min, 30 min, and 6 hr. The digested products were then chromatographed on thin-layer polyethyleneimine (PEI)-cellulose plates (J. T. Baker) with 0.3 M lithium chloride as the solvent (14). AMP, adenosine, ADP-ribose, and NAD$^+$ (Sigma) were cochromatographed as markers. The chromatogram was exposed to x-ray film (Kodak XRP-5) to locate the ^{14}C-labeled products relative to the UV light-adsorbing markers. Radioactive material was scraped into vials and analyzed for radioactivity in a liquid scintillation spectrophotometer.

Reversal of the ADP-Ribosylation Reaction. Reverse reactions were done by incubating [U-^{14}C]adenosine-labeled EF-2 at pH 6.6 with an excess of fragment A (10 μg/ml) and 2 mM nicotinamide at 22°C as described (7). The radioactive products were analyzed by polyethyleneimine-cellulose thin-layer chromatography as described by Randerath and Randerath (15). The ^{14}C-labeled samples were cochromatographed with standards of NAD$^+$, AMP, adenosine, and ADP-ribose in 0.3 M LiCl. Radioactive compounds were located by autoradiography on x-ray film. Radioactive materi-

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Abbreviations: pyBHK cells, polyoma virus-transformed baby hamster kidney cells; EF-2, elongation factor 2.

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al was then scraped into vials and analyzed for radioactivity in a liquid scintillation spectrophotometer.

RESULTS

Transfer of [^{14}C]Adenosine from NAD$^+$ to EF-2. Fragment A of diphtheria toxin rapidly ADP-ribosylated EF-2 in the presence of NAD$^+$ (Fig. 1). It was also possible to transfer [^{14}C]adenosine from NAD$^+$ to an acid-precipitable form in the absence of fragment A. Maximum transfer of label in the absence of fragment A occurred between 80 and 160 min, depending on the purified EF-2 preparation used, and always approached the maximum level of ADP-ribosylation catalyzed by fragment A. The transfer of [^{14}C]adenosine from NAD$^+$ to an acid-precipitable form suggested that our EF-2 preparations contained an unusual enzyme activity, which seemed to mimic the activity of fragment A. An autoradiogram of the [^{14}C]adenosine-labeled protein analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis indicated that both fragment A and the endogenous enzyme in our EF-2 preparations transferred most of the label to a protein with an apparent M_r of 93,000 (Fig. 2). We previously had characterized this protein in our preparations as EF-2 (11). The preparations of EF-2 also contained traces of products from partial proteolysis of the EF-2 incurred during final steps in EF-2 purification and not during the ADP-ribosylation reaction. These products of partial proteolysis were readily detected in autoradiograms and were labeled identically with radioactivity by fragment A or the endogenous transferase in the EF-2 preparations. Similar labeling of ADP-ribosylated EF-2 degradation products by fragment A was observed by Van Ness *et al.* (16).

A comparison of tryptic peptides of EF-2 labeled in the presence of [*adenosine*-^{14}C]NAD$^+$ and fragment A or the endogenous transferase and then analyzed by thin-layer chromatography produced identical autoradiograms (data not shown). These data indicate that a similar peptide of the EF-2 molecule is labeled by both fragment A and the pyBHK endogenous transferase.

Properties of the Endogenous Transferase. Since large amounts of purified fragment A were used routinely in our laboratory, it was essential to eliminate the possibility that our EF-2 preparations were accidentally contaminated with traces of fragment A. Fragment A antibody greatly inhibited

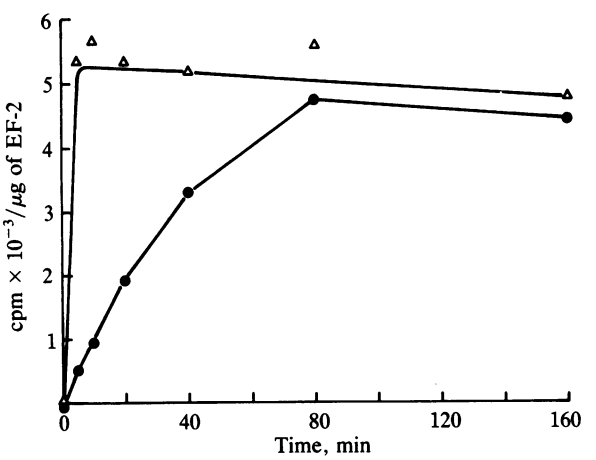

FIG. 1. Incorporation of label from NAD$^+$ into EF-2 catalyzed by fragment A or the pyBHK endogenous transferase found in EF-2 preparations. Five micrograms of an EF-2 preparation containing endogenous transferase plus 0.05 μg of fragment A (△) or 5 μg of an EF-2 preparation containing the endogenous transferase (●) were added to 0.1 ml of reaction buffer containing 6.33 μM [*adenosine*-^{14}C]NAD$^+$ and incubated at 22°C. At intervals, aliquots (10 μl) were taken, and trichloroacetic acid-precipitable material was assayed for radioactivity.

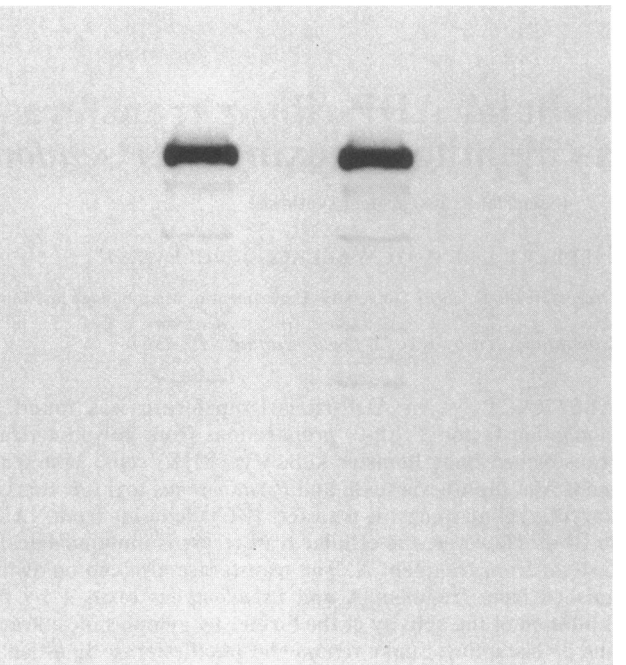

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of the proteins in the EF-2 preparations labeled with [^{14}C]adenosine from NAD$^+$ by fragment A or by the pyBHK endogenous transferase in the EF-2 preparations. EF-2 preparations were labeled as described in Fig. 1, and the radioactive proteins were analyzed by electrophoresis on 12% NaDodSO$_4$/polyacrylamide gels along with marker proteins. Lanes: right, fragment A-labeled EF-2; left, endogenous transferase-labeled EF-2. The radioactive proteins were visualized by autoradiography. The major band coelectrophoresed with the phosphorylase *b* marker with an M_r of 93,000, as does the major band of stained protein (11).

the transfer of label from NAD$^+$ to EF-2 in a fragment A-catalyzed reaction (Fig. 3*A*). In fact, increasing the antibody concentration 5-fold provided similar results (data not shown). The residual activity seen in the presence of anti-fragment A antiserum is presumably due to the endogenous transferase activity present in the EF-2 preparation. In contrast, the fragment A antibody had essentially no effect on the endogenous transferase of the EF-2 preparations (Fig. 3*B*), indicating that this ADP-ribosyltransferase associated with EF-2 is immunologically distinct from fragment A.

Two other properties clearly distinguish the pyBHK endogenous transferase from the known ADP-ribosyltransferases that specifically modify EF-2. First, addition of a charcoal-adsorbed cytoplasmic extract from pyBHK cells to purified EF-2 inhibited the endogenous transferase activity from the EF-2 preparations by 90% but had no effect on the ADP-ribosyltransferase activities of fragment A (Table 1). However, boiling the cytoplasmic extract prior to use did destroy the inhibitory effect on the endogenous transferase activity from the EF-2 preparation. Second, 0.25 M histamine almost totally inhibited the activity of the endogenous transferase but had no appreciable effect on the activity of either of the bacterial toxins (Table 1). Similar results were obtained at a histamine concentration of 0.15 M.

Endogenous Transferase Is a Mono(ADP-Ribosyl)Transferase That Modifies Diphthamide of EF-2. Previously described bacterial ADP-ribosyltransferases have been shown to transfer mono(ADP-ribose) from NAD$^+$ directly to proteins (17). On the other hand, many eukaryotic ADP-ribosyltransferases yield poly(ADP-ribosyl)ated proteins (3). To determine if the EF-2 modified by the endogenous transferase contained monomers or polymers of ADP-ribose, we synthe-

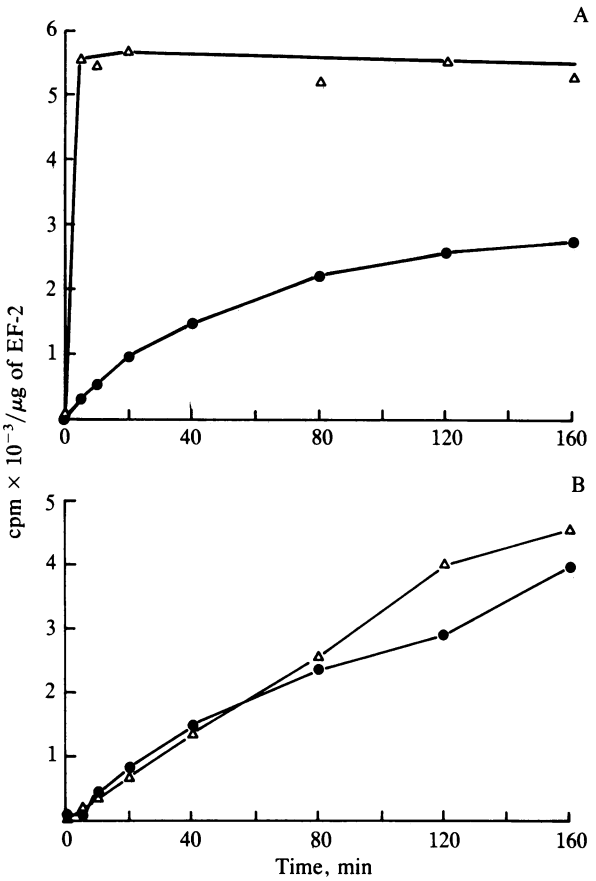

FIG. 3. Effect of fragment A antibody on incorporation of [^{14}C]adenosine from NAD$^+$ into EF-2 catalyzed by fragment A or the pyBHK endogenous transferase. A 1:5 dilution of rabbit anti-fragment A antiserum or control serum was incubated with 0.05 μg of fragment A or an EF-2 preparation containing the pyBHK endogenous transferase activity for 5 min at 37°C. The enzyme and serum mixtures were then added to our standard transferase assay as described in Fig. 1 and *Materials and Methods*. The transfer of [^{14}C]-adenosine from NAD$^+$ to EF-2 is shown after various times of incubation. (*A*) Fragment A (△)- or fragment A/anti-fragment A antiserum (●)-catalyzed reaction. (*B*) Endogenous transferase (△)- or endogenous transferase/anti-fragment A antiserum (●)-catalyzed reaction.

sized the product formed in the presence of the endogenous transferase, [*adenosine*-^{14}C]NAD$^+$, and EF-2 and then treated the labeled product with snake venom phosphodiesterase. EF-2 modified by fragment A was used in an accompanying reaction mixture as a positive control. The digestion products were chromatographed and exposed to film. In both reactions the low molecular weight ^{14}C-labeled material chromatographed with AMP (Fig. 4). No radioactivity migrated with the marker ADP-ribose [which comigrates in the system with iso-ADP-ribose, the product of venom phosphodiesterase action on poly(ADP-ribose) (17)]. Therefore, the ADP-ribose is present on the acceptor in EF-2 as monomeric units, rather than as poly(ADP-ribose).

In addition, an extremely prolonged time of digestion (6 hr) resulted in the apparent conversion of [^{14}C]AMP to a ^{14}C-labeled material that comigrated with the adenosine marker (Fig. 4). This apparent conversion of AMP to adenosine represents a trace of contaminant enzyme activity in the snake venom phosphodiesterase preparation.

The ADP-ribosylation of EF-2 catalyzed by fragment A or *Pseudomonas* toxin A is reversible (7, 18, 19). This reverse reaction requires excess toxin (i.e., fragment A) and nicotinamide and has a lower pH optimum than that of the forward

Table 1. The effect of cytoplasmic extracts from pyBHK cells and histamine on transferase activity in EF-2 preparations

	[^{14}C]Adenosine incorporated per μg of EF-2, cpm				
	Cytoplasmic extract (5 mg of protein per ml)			Histamine at 0.25 M	
Transferase	Absent	Present	Boiled and centrifuged	Absent	Present
Fragment A	4700	4600	4600	4200	3900
Pseudomonas toxin A	NT	NT	NT	4500	4000
Endogenous transferase	4500	550	4000	4300	100

The standard reaction mixture described in *Materials and Methods* and Fig. 1 was supplemented by the addition of 10 μl of a cytoplasmic extract from pyBHK cells having a protein concentration of 5 mg/ml or by adding histamine to a final concentration of 0.25 M. In some experiments cytoplasmic extract was boiled for 5 min and centrifuged to remove denatured protein before use. Cytoplasmic extracts were incubated with EF-2 for 5 min at 37°C before addition to the reaction mixture. The reaction mixtures were incubated until there was no further transfer of label to EF-2. The inhibitory effect of the cytoplasmic extract on the pyBHK endogenous transferase occurred at 5 min of incubation and was maximal by 10 min. The inhibitory effect of histamine on the endogenous transferase was maximal by 5 min of incubation, which was the first sampling time. Similar results were obtained when histamine was used at a concentration of 0.15 M. NT, not tested.

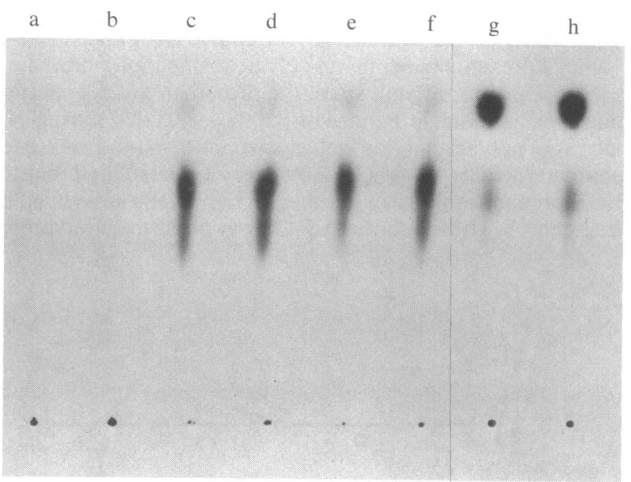

FIG. 4. Thin-layer chromatography of the snake venom (SV) phosphodiesterase digestion products from EF-2 labeled by fragment A or the pyBHK endogenous transferase with [^{14}C]adenosine from NAD$^+$. The radioactive products are visualized by autoradiography. EF-2 was labeled by fragment A or by the endogenous transferase in our standard reaction mixture, and the products were digested with SV phosphodiesterase and chromatographed on thin-layer plates as described. Lanes show the soluble products from EF-2 labeled in the presence of [adenosine-^{14}C]NAD$^+$ by: fragment A (lane a), endogenous transferase (lane b), fragment A followed by a 15-min digestion with SV phosphodiesterase (lane c), endogenous transferase followed by a 15-min digestion with SV phosphodiesterase (lane d), fragment A followed by a 30-min digestion with SV phosphodiesterase (lane e), endogenous transferase followed by a 30-min digestion with SV phosphodiesterase (lane f), fragment A followed by a 6-hr digestion with SV phosphodiesterase (lane g), and endogenous transferase followed by a 6-hr digestion with SV phosphodiesterase (lane h). The major products of the 15- and 30-min digestions comigrate with AMP. Increasing the time of digestion to 6 hr results in the further conversion of AMP to the major product, which comigrates with adenosine. No soluble product cochromatographed with ADP-ribose. The R_f values of NAD$^+$, adenosine, AMP, and ADP-ribose are 0.65, 0.53, 0.40, and 0.25, respectively.

reaction. The reversibility of the forward reactions catalyzed by fragment A or the pyBHK endogenous mono(ADP-ribosyl)transferase are shown in Fig. 5. A preparation of [^{14}C]adenosine-labeled EF-2 containing a small amount of endogenous transferase (used in the forward reaction) was incubated in the absence or presence of excess fragment A and nicotinamide. Although little or no radioactivity was released from EF-2 in the absence of excess fragment A, over 60% of the radioactivity was released when both fragment A and nicotinamide were present in large excess. Similarly, radioactivity was released from EF-2 labeled in the forward reaction with fragment A and then reversed in the presence of excess fragment A and nicotinamide. The radioactive products of the reverse reactions were analyzed by thin-layer chromatography. The low molecular weight soluble products found in the reverse reaction mixtures had the chromatographic behavior of NAD^+ (Fig. 6). In addition, the reaction mixtures contained some labeled EF-2, which remained at the origin.

DISCUSSION

The mono(ADP-ribosyl)transferase from pyBHK cells can be distinguished from fragment A and *Pseudomonas* toxin A by the sensitivity of its activity to cytoplasmic extracts and to histamine. These properties may explain why similar cellular ADP-ribosyltransferases were not previously found by standard techniques. Crude preparations of EF-2 are commonly used to assay for fragment A and *Pseudomonas* toxin A, and histamine is commonly used to inhibit poly(ADP-ribosyl)transferase activity in reactions containing preparations of EF-2 (13). We observed that either condition inhibits the activity of the pyBHK ADP-ribosyltransferase.

Fig. 1 presents the cpm of [^{14}C]adenosine incorporated per μg of EF-2. More than 95% of the protein in this preparation migrates as a single band with an M_r of 93,000 during Na-DodSO$_4$/polyacrylamide gel electrophoresis and has been characterized as EF-2 (11). However, we calculated that the EF-2 preparation shown in Fig. 1 is 51% ADP-ribosylated by fragment A. The amount of EF-2 in six of our purified preparations able to accept the transfer of ADP-ribose catalyzed by fragment A ranges from 43% to 90% (11), with a typical preparation having about 50% ADP-ribosylatable EF-2. This variation in ADP-ribosylatable EF-2 may represent an inactivation of the acceptor activity during purification. We have shown that ADP-ribose acceptor activity of EF-2 is inactivated by both freezing and thawing samples and by prolonged storage at −20 or −70°C (unpublished observations). Alternative explanations for the reduced acceptor activity of our EF-2 preparations are the presence of ADP-ribosylated EF-2 in EF-2 preparations extracted from cells or protein contaminants in our EF-2 preparations. The former explanation assumes that EF-2 is normally ADP-ribosylated in pyBHK cells and copurifies with EF-2.

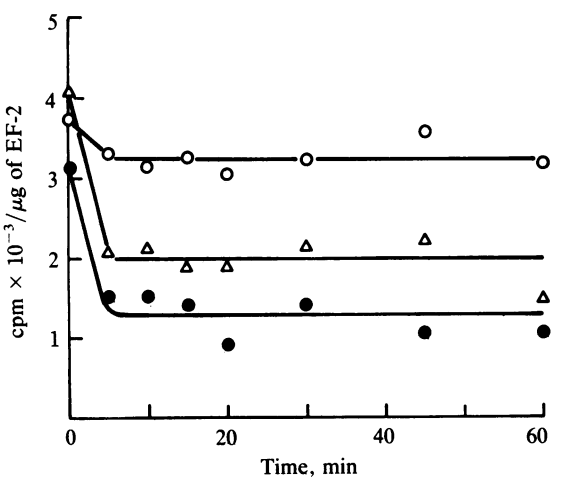

FIG. 5. Reversal of ADP-ribosylation of EF-2 catalyzed by fragment A or the endogenous ADP-ribosyltransferase from pyBHK cells. EF-2 preparations were labeled by incubation with [*adenosine*-^{14}C]NAD^+ and a low concentration of fragment A (0.5 μg/ml) or the endogenous transferase in our standard reaction mixture. After exhaustive dialysis, which removes all unreacted NAD^+, the labeled proteins were incubated in Tris·HCl buffer (pH 6.6). For the reverse reaction, the EF-2 labeled by the endogenous transferase was incubated with fragment A (10 μg/ml) and 2 mM nicotinamide (●) or with 2 mM nicotinamide in the absence of fragment A (○). The fragment A-catalyzed forward reaction was also reversed with fragment A (10 μg/ml) and 2 mM nicotinamide (△).

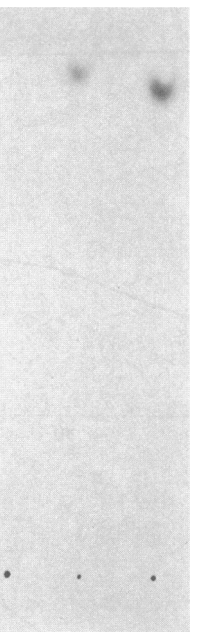

FIG. 6. Thin-layer chromatography of the products formed by the reversal reaction. Radioactive compounds were visualized by autoradiography. EF-2 was radioactively labeled by fragment A or the endogenous ADP-ribosyltransferase from pyBHK cells as described in Fig. 1. After extensive dialysis, the reaction was reversed as described in Fig. 5. The products of the reversal were analyzed by chromatography on CEL-300 thin-layer plates with 0.3 M LiCl as eluent. Lanes: right, reversal products from EF-2 labeled in the presence of [*adenosine*-^{14}C]NAD^+ by fragment A; left, reversal products of EF-2 labeled by the endogenous transferase. Only the major radioactive product of the reversal had the same chromatographic mobility as NAD^+. Radioactive EF-2 remains at the origin. No soluble radioactive labeled products were observed when labeled EF-2 from only the forward reaction was chromatographed. The R_f values of NAD^+, AMP, and ADP-ribose are 0.79, 0.74, and 0.84, respectively.

Fragment A and *Pseudomonas* toxin A transfer ADP-ribose from NAD^+ specifically to the diphthamide acceptor site of EF-2 (6, 9, 10). Diphthamide is a unique, modified histidine residue only found in EF-2 (9, 10). This residue apparently has been conserved throughout eukaryotic evolution (18, 20, 21), suggesting that it is involved in an important function. It seems unreasonable the diphthamide has been conserved for the convenience of microbial intoxication of cells. A more likely event is that diphthamide serves as the acceptor for an ADP-ribosylation reaction catalyzed by a cellular enzyme such as the one we have found in pyBHK cells. The microbial toxins simply take advantage of this existing system.

At the intracellular NAD^+ concentration in HeLa cells (about 0.5 mM), a steady-state concentration of a single molecule of fragment A is sufficient to inactivate all of the EF-2

within 24 hr (22). Yet the cellular enzyme activity described here has the same mechanism of action as that of fragment A. Such an endogenous enzyme must be under stringent control or it would cause cell death. This control may be exerted by a component (or components) in cytoplasmic extracts of pyBHK cells that we found to inhibit the activity of the pyBHK ADP-ribosyltransferase. We propose that the interaction of the cellular ADP-ribosyltransferase and its inhibitor is a mechanism of controlling protein synthesis at the level of functional EF-2. Since cells with mutant EF-2, resistant to ADP-ribosylation by fragment A and presumably by the pyBHK transferase, synthesize protein and grow as well as wild-type cells do (11, 23), the proposed mechanism for control of protein synthesis is probably not necessary for survival of all types of cells. It may only be operative under certain conditions in differentiated tissues. Nevertheless, the proposed control mechanism should result in cell death when out of balance and might explain pathological conditions characterized by cell death from unknown causes, such as cell senescence or diseases characterized by cell degeneration.

Using identical methods to those reported here, we have isolated a cellular ADP-ribosyltransferase from beef liver, which is also a mono(ADP-ribosyl)transferase that transfers ADP-ribose from NAD^+ to diphthamide of liver EF-2 (unpublished data). Thus, this enzyme activity can also be found in normal tissues from a different species and, therefore, may be ubiquitous.

To fully understand the reaction catalyzed by the pyBHK ADP-ribosyltransferase, a defined system containing purified enzyme, substrate, and inhibitor must be established. In addition, this enzyme system should be shown to be operative *in vivo* in several cell types.

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