ADP-ribosyltransferase from beef liver which ADP-ribosylates elongation factor-2

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Fragment A of diphtheria toxin and *Pseudomonas* toxin A intoxicate cells by ADP-ribosylating the diphthamide residue of elongation factor-2 (EF-2) resulting in an inhibition of protein synthesis [1–3]. A cellular enzyme from polyoma virus transformed baby hamster kidney (pyBHK) cells ADP-ribosylates EF-2 in an identical manner [4]. Here we describe a similar cellular enzyme from beef liver which transfers [*adenosine*-^{14}C]ADP-ribose from NAD to EF-2. The ^{14}C-label can be removed from the EF-2 by snake venom phosphodiesterase as a soluble product which comigrates with AMP on TLC plates, indicating the ^{14}C-label is present on EF-2 as monomeric units of ADP-ribose. Furthermore, the forward transferase reaction catalyzed by the beef liver ADP-ribosyltransferase is reversible by excess diphtheria toxin fragment A, with the formation of ^{14}C-labeled NAD, indicating that both transferases ADP-ribosylate the same site on the diphthamide residue of EF-2. Thus, beef liver and pyBHK mono(ADP-ribosyl)transferases both modify the diphthamide residue of EF-2, in a manner identical to diphtheria toxin fragment A and *Pseudomonas* toxin A. These results suggest the cellular enzyme is probably ubiquitous among eukaryotic cells.

ADP-ribosyltransferase *Elongation factor-2* *Bacteria toxin* *Posttranslational modification* *Protein synthesis* *Liver*

1. INTRODUCTION

We have recently described the first cellular mono(ADP-ribosyl)transferase from polyoma virus transformed baby hamster kidney (pyBHK) cells which ADP-ribosylates eukaryotic EF-2 in a manner identical to fragment A of diphtheria toxin and *Pseudomonas* toxin A [4]. We suggested that the cellular enzyme was part of an endogenous enzyme system of eukaryotic cells which is taken over by these two microbial toxins during the process of intoxication. Since these bacterial toxins affect a number of different types of cells [5–8], the endogenous cellular ADP-ribosyltransferase system should also have a broad distribution. The initial isolation of the cellular ADP-ribosyltransferase was from pyBHK cells which are both virus infected and transformed and consequently might contain a unique enzyme not found in normal tissue. In this report, however, we describe an ADP-ribosyltransferase which has now been isolated from normal beef liver. The finding of this enzyme in two different tissues from two different species of animals suggests it is probably ubiquitous among eukaryotic cells.

2. MATERIALS AND METHODS

2.1. *Partial purification of beef liver elongation factor-2*

A fresh 21 lb beef liver was obtained from a local slaughterhouse, divided into 100 g portions and frozen at −70°C. Elongation factor-2 (EF-2) from beef liver was partially purified according to a procedure for purification of EF-2 from hamster tumors [9]. Briefly, rapidly thawed liver was homogenized in a sucrose containing buffer at 5°C

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in a Waring blender. A postmitochondrial fraction was selected by centrifugation and precipitated with ammonium sulfate added to 80% saturation. The precipitate was dissolved in buffer A [4] and sequentially chromatographed on DEAE-cellulose, Sephadex DEAE A50, Sephacryl S-200 and phosphocellulose. Chromatographic fractions rich in EF-2 were assayed by the specific transfer of [*adenosine*-^{14}C]ADP-ribose from NAD (534 mCi/mM) to EF-2 by diphtheria toxin fragment A as in [10]. The preparations of partially purified EF-2 were analyzed by SDS–polyacrylamide gel electrophoresis [9].

2.2. *ADP-ribosylation of preparations of beef liver EF-2*

The partially purified liver EF-2 preparations also served as the source of the cellular ADP-ribosyltransferase. Diphtheria toxin fragment A was purified as in [11]. 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 as in [4,9]. The reaction mixture contained 25 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol, 6.33 μM [*adenosine*(U)-^{14}C]NAD (534 mCi/mM) (New England Nuclear Co.), and EF-2 at 50 μg/ml with or without added fragment A at 0.5 μg/ml. After intervals of incubation at 22°C, 10 μl samples were trichloroacetic acid precipitated and the radioactivity was measured [4,9].

2.3. *Snake venom phosphodiesterase digestion of ADP-ribosylated EF-2*

Digestion with snake venom phosphodiesterase (Worthington Biochemical) was used to distinguish between mono- or poly-ADP-ribosylation of EF-2 by the endogenous transferase as described [4]. EF-2 was ADP-ribosylated by the cellular ADP-ribosyltransferase in the presence of ^{14}C-labeled NAD and then dialyzed to remove NAD. Then 25 μl of snake venom phosphodiesterase was added and incubated 15 min at 37°C. The digestion products were chromatographed on thin-layer polyethyleneimine cellulose plates (J.T. Baker) with 0.3 M lithium chloride, dried and exposed to X-ray film (Kodak XPP-5) to locate the ^{14}C-labeled products relative to the UV light adsorbing AMP, adenosine, ADP-ribose and NAD markers (Sigma).

2.4. *Reversal of the ADP-ribosylation reaction*

Reversal reactions were done by incubating the product of the forward reaction ([^{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 previously described [4]. The radioactive products were analyzed by thin-layer polyethyleneimine cellulose chromatography as described above.

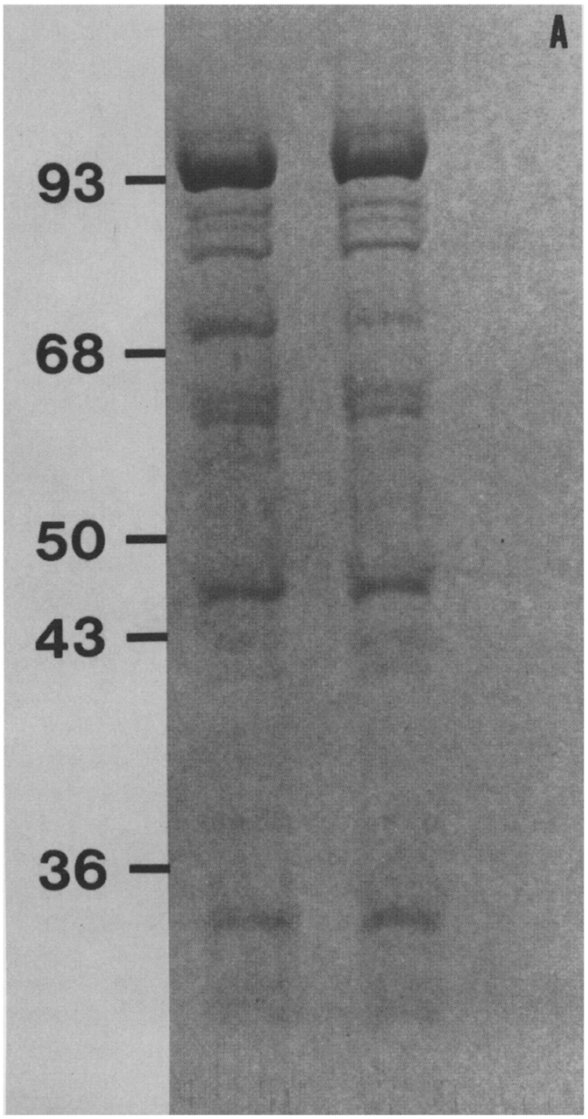
3. RESULTS AND DISCUSSION

3.1. *Transfer of [^{14}C]adenosine from NAD to EF-2 by the endogenous transferase*

Beef liver EF-2 was partially purified by sequential column chromatography on DEAE-cellulose, Sephadex DEAE A50, Sephacryl S-200 and phosphocellulose as in [9]. The partially purified beef liver EF-2 preparation consisted of a major band of 93-kDa protein and trace bands of lower molecular mass proteins when reduced and analyzed by SDS–polyacrylamide gel electrophoresis (fig.1A). The major band represented >90% of the stainable protein on the gels. Incubation of the partially purified EF-2 preparation which contained the endogenous transferase with [*adenosine*-^{14}C]NAD resulted in the transfer of ^{14}C-label to an acid precipitable form. EF-2 could also be labeled by addition of diphtheria toxin fragment A. Since the fragment A catalyzed reaction reaches saturation in <5 min and the endogenous transferase catalyzed reaction requires >160 min of incubation, the ^{14}C-labeled acceptor could be preferentially labeled by either enzyme [4]. Moreover, the transfer of label to the acceptor by sequential addition of the two enzymes is not additive (unpublished data). SDS–polyacrylamide gel analysis of the products labeled by the cellular transferase or fragment A indicated that the label was transferred to a 93-kDa protein which comigrates with EF-2 [4,9], as shown in fig.1B.

3.2. *Snake venom phosphodiesterase digestion of ^{14}C-labeled EF-2*

To determine if the EF-2 modified by the beef liver ADP-ribosyltransferase contains monomers or polymers of ADP-ribose, we synthesized the labeled product formed in the presence of the endogenous cellular transferase, [*adenosine*-^{14}C]NAD and EF-2 and then treated the labeled product with snake venom phosphodiester-



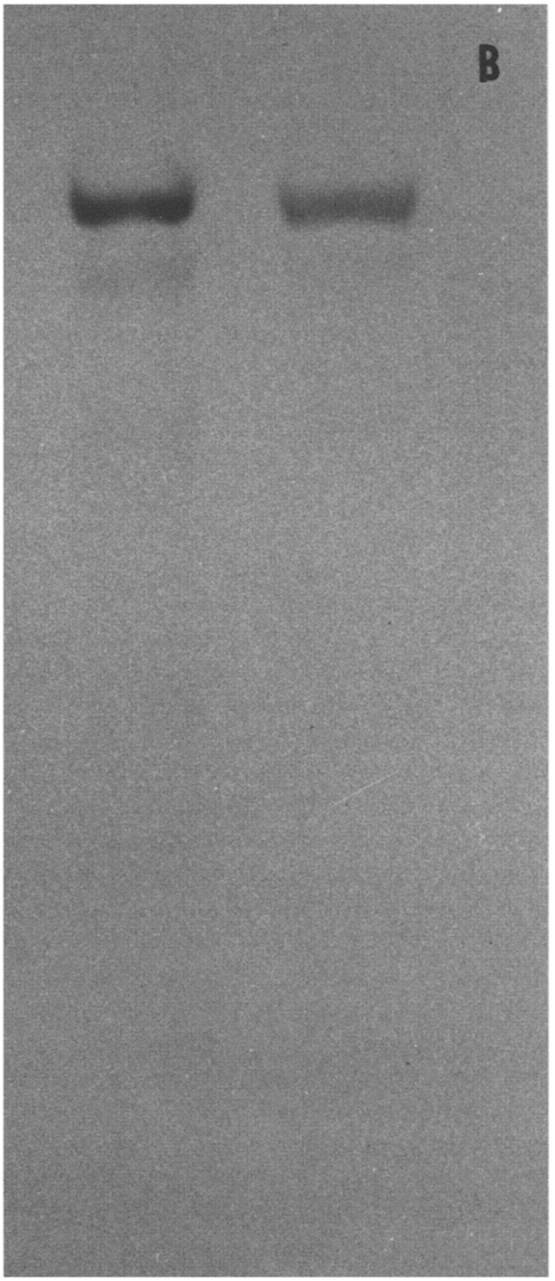


Fig.1. SDS–polyacrylamide gel analysis of partially purified beef liver EF-2 and of the EF-2 preparation labeled with [^{14}C]adenosine by diphtheria toxin fragment A and the endogenous transferase. EF-2 was partially purified from homogenates of beef liver as described in section 2. Gels containing 8.5% acrylamide and 0.1% sodium dodecyl sulfate (SDS) were prepared as in [9]. Following electrophoresis the protein bands were stained with Coomassie brilliant blue stain as shown in (A). Lanes from right to left: beef liver EF-2 preparation incubated with [*adenosine*-^{14}C]NAD for 160 min; and beef liver EF-2 incubated with [*adenosine*-^{14}C]NAD and 0.05 μg of diphtheria toxin fragment A for 160 min. The numbers represent $M_r \times 10^{-3}$ of the protein standards. Following electrophoresis, the dried gel was exposed to X-ray film to locate proteins labeled with [^{14}C]adenosine by the two transferases as shown in (B). Lanes from right to left: proteins labeled with [^{14}C]adenosine by the endogenous transferase in beef liver EF-2 preparations and proteins labeled with [^{14}C]adenosine by fragment A. The major stained and ^{14}C-labeled protein comigrates with the 93-kDa phosphorylase *b* molecular mass marker protein.

ase (SVP). EF-2 modified by fragment A was used in an accompanying reaction mixture as a positive control. In both reactions the soluble products resulting from digestion with snake venom phosphodiesterase cochromatographed with each other and with the UV light absorbing AMP marker (fig.2). Some tailing of ^{14}C-labeled AMP in the chromatogram was observed due to the salt effect of the $MgCl_2$ component in the SVP catalyzed reaction. The same tailing effect of the AMP standard was observed when the standards were treated and chromatographed under the same conditions as the unknowns. The effect was identical in all reactions and does not obscure the region of the chromatogram where the ADP-ribose standard migrates. Since AMP is the product of snake venom phosphodiesterase action on mono ADP-ribosylated proteins, we conclude that ADP-ribose is present on the acceptor in EF-2 as monomeric units.

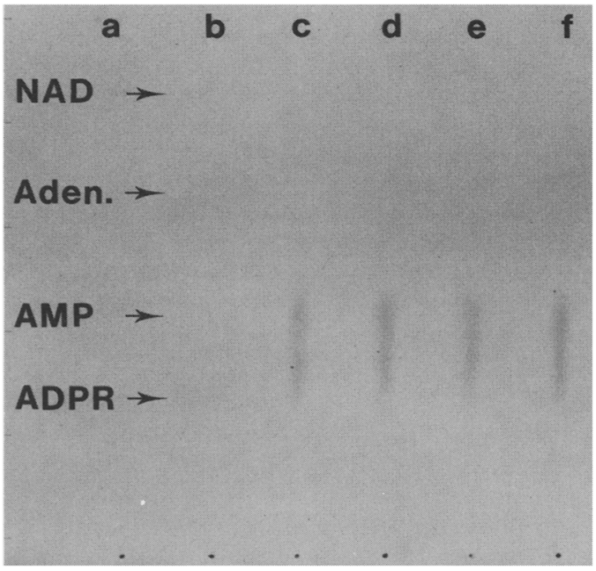


Fig.2. Thin-layer chromatography of the snake venom phosphodiesterase digestion products from beef liver EF-2 labeled by fragment A or the beef liver endogenous transferase with [*adenosine*-^{14}C]NAD. The beef liver EF-2 was labeled by fragment A or by the endogenous transferase in our standard reaction mixture, the products were digested with snake venom phosphodiesterase (SVP) and chromatographed on thin-layer plates as described in section 2. The radioactive products are visualized by autoradiography. The lanes from left to right represent the soluble products from EF-2 labeled in the presence of [*adenosine*-^{14}C]NAD by: (a) fragment A; (b) endogenous transferase; (c) fragment A followed by 5 min digestion with SVP; (d) fragment A followed by 15 min digestion with SVP; (e) endogenous transferase followed by 5 min digestion with SVP; and (f) endogenous transferase followed by 15 min digestion with SVP. ADPR, AMP, Aden. and NAD refer to the chromatographic positions of the UV-light absorbing standards of ADP-ribose, AMP, adenosine and NAD which have the R_f values of 0.65, 0.53, 0.40, and 0.25, respectively.

3.3. *Reversal of the ADP-ribosylation reaction*

The ADP-ribosylation of EF-2 by mono(ADP-ribosyl)transferases is reversible [4,12,13]. Since these ADP-ribosyltransferases synthesize identical products, it is possible to drive the forward reaction with one type of ADP-ribosyltransferase and then catalyze the reversal reaction with a second type of transferase [4,12,13]. A preparation of [^{14}C]adenosine labeled beef liver EF-2 containing a small amount of beef liver ADP-ribosyltransferase (used in the forward reaction) was incubated in the absence or presence of excess fragment A and nicotinamide to reverse the reaction. The ^{14}C-labeled product generated in this reversal reaction was analyzed by thin-layer chromatography. The ^{14}C-labeled soluble product found in the reverse reaction mixtures cochromatographed with the marker NAD (fig.3), indicating that the beef liver enzyme ADP-ribosylates the diphthamide residue of EF-2 in a manner identical to that of fragment A of diphtheria toxin, *Pseudomonas* toxin A and pyBHK cellular mono(ADP-ribosyl)transferase.

The mono(ADP-ribosyl)transferases from beef liver and from pyBHK cells are found in EF-2 preparations when the EF-2 is partially purified as in [4,9]. Although the enzyme is found in our EF-2 preparations the activity can be separated from pyBHK EF-2 using an immunoadsorbant column containing polyvalent antibody prepared by immunizing Balb/c mice with an EF-2 preparation having the cellular transferase activity (unpublished data). The cellular ADP-ribosyltransferase binds to the immunoadsorbant column and can be eluted in an enzymatically active form, while pyBHK EF-2 does not bind to the resin. Thus, the transferase activity is distinct from EF-2 and either co-purifies with EF-2 or is a minor contaminant in our EF-2 preparations.

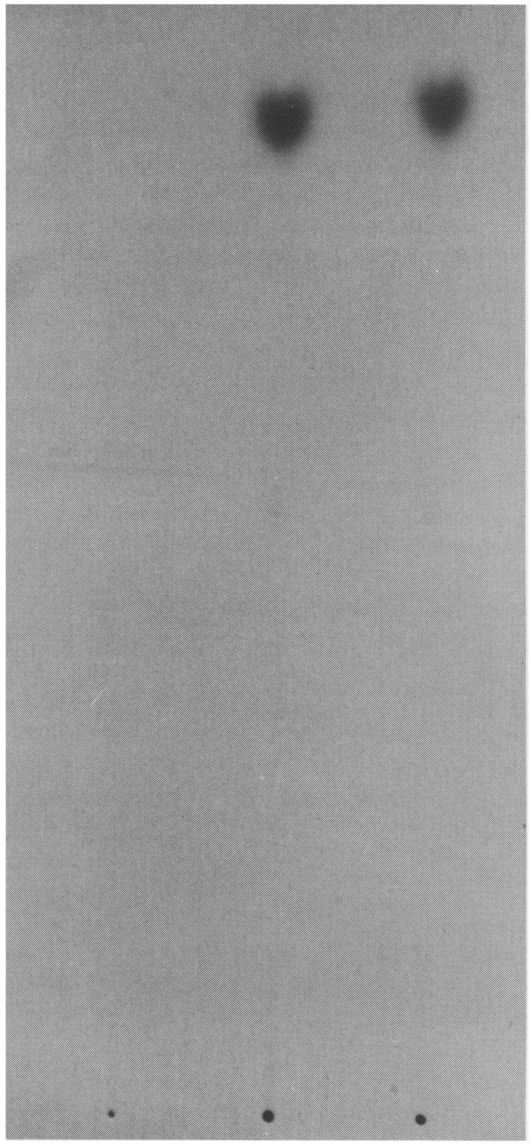


Fig.3. Thin-layer chromatography of the products formed by the reversal reaction. Beef liver EF-2 was radioactively labeled by diphtheria toxin fragment A (0.5 μg/ml) or the endogenous ADP-ribosyltransferase from the beef liver EF-2 preparation as shown in fig.1B. After exhaustive dialysis to remove NAD, the labeled proteins were incubated in Tris–HCl buffer (pH 6.6). For the reverse reaction the ^{14}C-labeled EF-2 preparations were incubated with an excess of fragment A (10 μg/ml) and 2 mM nicotinamide. The products of the reversal were analyzed by thin-layer chromatography using the same chromatographic system shown in fig.2. Untreated ADP-ribosylated EF-2 was spotted at the origin of one lane as a control. Radioactive compounds were visualized by autoradiography. Lanes from left to right: untreated ADP-ribosylated EF-2; reversal product from EF-2 labeled in the presence of [*adenosine*-^{14}C]-NAD by fragment A; and reversal product of EF-2 labeled by the beef liver endogenous transferase. The ^{14}C-labeled soluble product of the reversal cochromatographed with the NAD standard.

Beef liver serves as a convenient source for the cellular ADP-ribosyltransferase but we have found that the yield of beef liver EF-2 and its ADP-ribosyltransferase to be much lower (approximately 1/20) than the yield of pyBHK EF-2 and its copurifying ADP-ribosyltransferase enzyme. We have also found that the activity of the beef liver ADP-ribosyltranferase is much less stable than the enzyme isolated from pyBHK cells. Enzyme activity is rapidly lost when frozen at −70°C, suggesting the beef liver ADP-ribosyltransferase may be inactivated by the process of freezing. We normally store the beef liver EF-2 preparations in ice and use them as rapidly as possible. In contrast, the ADP-ribosyltransferase from pyBHK cells is stable when stored at −70°C and is more suitable for long term studies.

Similar mono(ADP-ribosyl)transferases have now been isolated from two different tissues of different species of animals. Since the unique diphthamide residue of EF-2 has been stringently conserved in eukaryotic cells from yeast to man [5,6,14] it seems likely that the mono(ADP-ribosyl)transferase which ADP-ribosylates this acceptor site will also be common to these eukaryotic cells. The stringent conservation of the diphthamide residue and, apparently, the cellular mono(ADP-ribosyl)transferase suggests an essential physiological function, perhaps 'fine tuning' of protein synthesis at the level of functional EF-2. A model for the potential relationship of microbial toxins and cellular ADP-ribosyltransferases has recently been presented together with a potential explanation for the selective toxicity of the microbial ADP-ribosyltransferase over its cellular counterpart [15]. It will now be important to demonstrate the active form of this enzyme in vivo and to determine under what conditions that proposed 'fine tuning' mechanism is operational.

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