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Production of an antiserum specific to the ADP-ribosylated form of elongation factor 2 from archaebacteria and eukaryotes

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An antiserum to ADP-ribosylated elongation factor 2 (ADPR-EF-2) from *S. acidocaldarius* was raised in rabbits using stained, homogenized, ADPR-EF-2-containing slices from SDS-gels as a source of antigen. Elongation factor 2 (EF-2) from *S. acidocaldarius* was cloned in *E. coli* and the expressed gene product was used in order to adsorb all anti-EF-2 antibodies which do not contain the ADP-ribosyl group within their epitopes, as *E. coli* is unable to synthesize the ADP-ribosyl acceptor diphthamide. The remaining antibodies were specific to ADP-ribosylated EF-2 from *Thermoplasma acidophilum*, *S. acidocaldarius* and *Desulfurococcus mucosus*. ADP-ribosylated EF-2 from eukaryotic sources also reacted with the adsorbed antiserum as shown for EF-2 isolated from the killi-fish *Cynolebias whitei*, the mouse species BALB/c and Han/Wistar rats. The adsorbed antiserum did not cross-react with ADP-ribosylated actin or rho protein or with FAD-containing D-amino acid oxidase.

Antiserum; Elongation factor 2; ADP-ribosylation; *Sulfolobus acidocaldarius*

1. INTRODUCTION

Elongation factor 2 from eukaryotes and archaebacteria is specifically ADP-ribosylated by diphtheria toxin and *Pseudomonas* toxin A [1,2]. In both kingdoms diphthamide, a modified histidine, is the specific acceptor of the ADP-ribosyl group, but only in eukaryotic cells ADP-ribosyl transferases have been found [3,4] which transfer the ADP-ribose moiety from NAD to EF-2. This reaction always results in a block of protein synthesis [3,5]. Since only part of the EF-2 is ADP-ribosylated in vivo and the ADP-ribosyl-EF-2 (ADPR-EF-2) had a short half-life [3] it is possible that the endogeneous transferase regulates protein synthesis at the level of elongation. But the physiological conditions that induce or repress ADP-ribosylation are not well understood, although it has been shown that the amount of ADP-ribosylatable EF-2 varies in cultured cells during the cell cycle [6], during aging [7] and at low serum concentration [3].

A rabbit anti-(baby hamster kidney ADPR-EF-2) antiserum has been used to immunoprecipitate [^{32}P]ADPR-EF-2 from various cell types [4] but it crossreacted with a lot of other proteins making the interpretation of the results difficult. We now have succeeded to raise an antiserum to ADPR-EF-2 from *S. acidocaldarius* in rabbits and to render it specific by adsorbtion with recombinant *S. acidocaldarius* EF-2 expressed in *E. coli* [8,9]. This EF-2 is free of diphthamide which cannot be built in *E. coli*; therefore, it is not ADP-ribosylatable by diphtheria toxin.

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Abbreviations: EF-2, elongation factor 2; ADPR-EF-2, ADP-ribosylated elongation factor 2; PBS, phosphate buffered saline; rEF-2, recombinant elongation factor 2.

2. MATERIALS AND METHODS

2.1. *Preparation of ADP-ribosylated EF-2 and immunization of rabbits*

ADP-ribosylated isoform 1 (p*I* = 6.2) of EF-2 from *S. acidocaldarius* was prepared by ammonium sulfate precipitation, gel filtration on Sephadex G-200 and preparative isoelectric focusing before and after ADP-ribosylation with diphtheria toxin (Siegmund and Klink, unpublished). Minor impurities of the ADPR-EF-2 preparation were removed by preparative SDS-PAGE in slab gels (3 × 200 × 200 mm). The Coomassie-stained EF-2 band was cut out and chilled into small pieces which were washed three times with 0.9% NaCl, by stirring for 10 min. Finally, the gel pieces were minced by a Potter homogenizer to become small enough to be passed through a 22-gauge needle. The gel homogenate was washed with 0.9% NaCl until the pH of the supernatant was raised above 4.0. The gel was stored at −70°C in portions containing 100 μg of ADPR-EF-2. Immediately before immunization, one portion was washed three times with phosphate buffered saline (PBS) and suspended in PBS. 0.5 ml suspension were injected into the hind legs of the rabbits nearby the popliteale lymph nodes. The rest was injected intradermally in multiple sites of the backs. Six weeks later, the animals were boostered accordingly and bled after ten days in two-day intervals for serum collection, until slaughtering after another 10 days.

2.2. *Preparation of recombinant EF-2 and adsorbtion of antiserum*

Plasmid pEX9-encoded S. acidocaldarius EF-2 (rEF-2) was prepared from *E. coli* JM 109 cell extracts as described [9]. The purified rEF-2 was bound to CNBr-activated Sepharose (Pharmacia-LKB) accordingly to the instructions of the manufacturer. The antiserum was diluted 5-fold with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20. 10 ml of the diluted antiserum were adsorbed by 160 μg of rEF-2-Sepharose by slowly rotating the suspension for 2 h.

2.3. *Preparation and isoelectric focusing of EF-2 from rat liver*

EF-2 from rat liver was partially purified by ultracentrifugation and DEAE-cellulose chromatography as described [10]. 10 ml of eluate from DEAE-cellulose, containing about 300 μg of EF-2, were dialyzed against 20 mM Tris-HCl, pH 7.5, 5 mM mercaptoethanol, 1 mM EDTA, 0.1 mM PMSF. The dialysate was mixed with 40 g wet IEF-Sephadex (Pharmacia), 3.5 ml ampholyte pH 4–9, 30 mg DTT and double-distilled water to get a smoothable slurry. Flat bed isoelectric focusing was performed using ampholyte diluted 1:15 as electrode solutions until 8,000 V·h were achieved.

2.4. *Preparation of other elongation factors and source of enzymes*

EF-2 from *Thermoplasma acidophilum* and *Desulfurococcus mucosus* were partially purified as described for *T. acidophilum* [10]. EF-2 from mouse liver was isolated as follows. 3.5 g of mouse liver (BALB/c) were homogenized in 16.5 ml of 100 mM Tris, 50 mM DTT, 1 mM EDTA, 25 μg/ml DNase I (Boehringer), 28 μg/ml RNase (Boehringer) and 0.1 mM PMSF by two strokes with an Elvehjem homogenizer at 300 rpm. Cell debris was removed by centrifugation for 15 min at 30,000 × *g*. The supernatant protein was precipitated with ammonium sulfate at 70% saturation. After centrifugation, the pellet was washed with 55% ammonium sulfate in 50 mM Tris-HCl, pH 8.5, 5 mM DTT, 1 mM EDTA, 0.1 mM PMSF and dissolved in the same buffer. ADP-ribosylation of the different EF-2 preparations was performed by incubation for 10 min with 100 μg/ml of diphtheria toxin and 10 μM NAD. Fish EF-2 from *Cynoleblas whitei* was prepared according to Ludwig et al. (unpublished).

D-Amino acid oxidase EC 1.4.3.3 from porcine kidney was obtained from Sigma (Deisenhofen) and xanthine oxidase EC 1.1.3.22 from cow milk was a product from Boehringer (Mannheim). ADP-ribosylated actin and rho protein were a generous gift of K. Aktories (Homburg/Saar).

2.4. *Enzyme-linked immunosorbent assay and Western blot*

ELISA was performed in polystyrol microplates, Western blots on nitrocellulose. For blocking and washing steps, a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20 was used. Anti-(rabbit IgG) peroxidase conjugate (Sigma, Deisenhofen) was used as the second antibody. Colour was developed from *o*-phenylenediamine in ELISA or 4-chloro-1-naphthol in blot applications.

3. RESULTS AND DISCUSSION

Direct immunization of rabbits with Coomassie-stained, ADPR-EF-2 containing gel slices was chosen

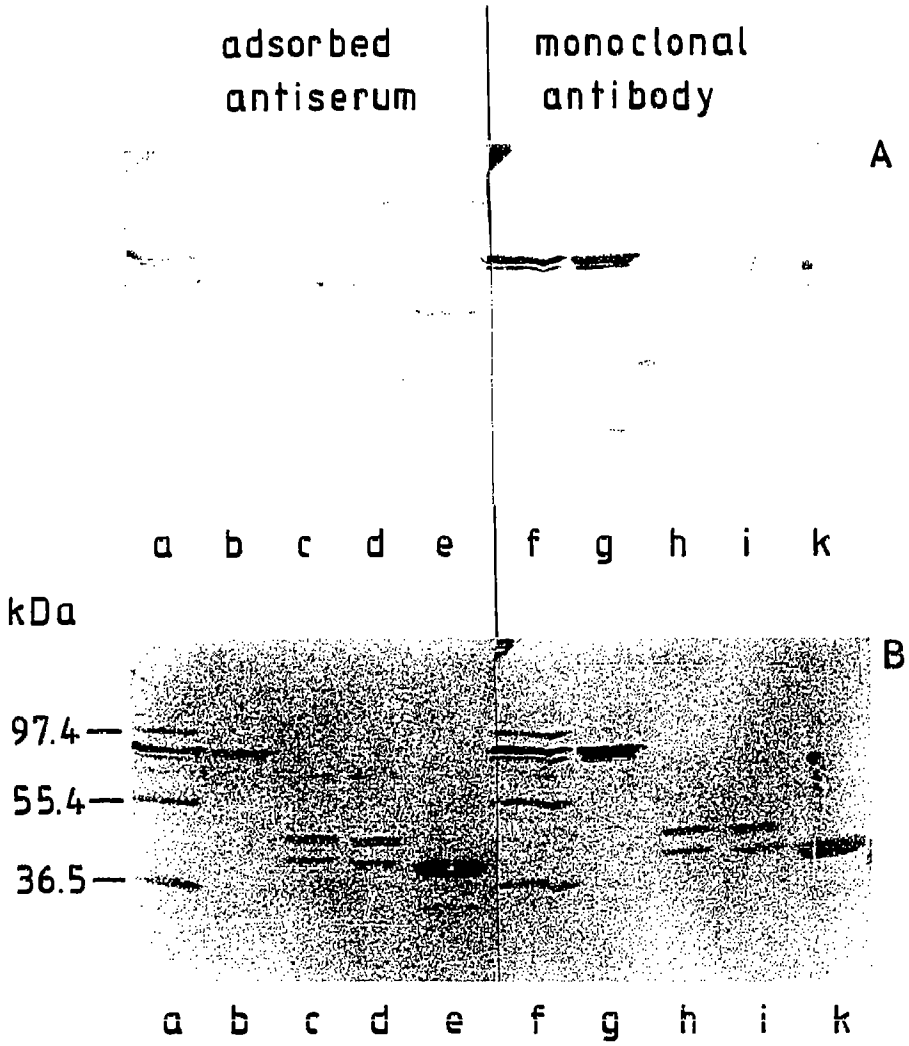

Fig. 1. Test on cross-reactivity of adsorbed anti-(ADPR-EF-2) antiserum in comparison with a monoclonal anti-(*S. acidocaldarius* EF-2) antibody. (A) Immunostained blot after SDS-PAGE. (B) Additionally staining of protein by Ponceau S. Lanes: (a,f) ADPR-EF-2 from *S. acidocaldarius*, (b,g) EF-2 from *S. acidocaldarius*, (c,h) actin from rabbit skeletal muscle, (d,i) ADP-ribosylated actin, (e,k) xanthine oxidase.

Table I

Titer of anti-(ADPR-EF-2) antiserum before and after adsorption with recombinant *S. acidocaldarius* EF-2 from *E. coli*

Antigen in ELISA	Titer before adsorption	Titer after adsorption
Recombinant EF-2	440	2×10^{-9}
EF-2 from *S. acidocaldarius*	730	3.8
ADPR-EF-2	320	20

*Titer is defined as the dilution producing OD = 1.0 in ELISA after incubation for 30 min at 300 rpm on a Titertek microplate shaker (Flow Laboratories).

because of the considerably lower amount of antigen needed for immune stimulation [11]. Addition of Freund's adjuvant was unnecessary, as the antigen was diffusing slowly out of the gel pieces which resulted in a depot effect without inducing an inflammation like it is caused by the complete adjuvant. Therefore, this procedure reduced the pain which the animals could suffer from immunization.

The adsorbtion procedure of the antiserum with recombinant *S. acidocaldarius* EF-2 (rEF-2) resulted in an about 10^{12}-fold reduction of the antiserum titer for rEF-2 compared to an only 16-fold reduction of the titer for ADPR-EF-2 and a 190-fold reduction for EF-2 isolated from *S. acidocaldarius* (Table I). The considerable lower reduction of the titer for native EF-2 might be due to trace amounts of in vivo ADP-ribosylated EF-2 in the preparation or to the fact that native EF-2 contains diphthamide which is lacking in rEF-2. However, Fig. 1, lanes a and b, show that the adsorbed antiserum was specific to the ADP-ribosylated form of *S. acidocaldarius* elongation factor 2 while a monoclonal antibody to EF-2 from *S. acidocaldarius* (which was kindly provided by K. Lemkemeier) detected both forms of the factor (lanes f and g). No cross-reaction was observed with rabbit skeletal muscle actin ADP-ribosylated by *Clostridium perfringens* iota toxin (Fig. 1, lanes d and i) or with the flavoprotein xanthine oxidase (lanes e and k) in either case, using the antiserum specific to ADPR-EF-2 (lanes a–e) or the monoclonal antibody to EF-2 from *S. acidocaldarius* (lanes f–k). Similarly, rho protein ADP-ribosylated by *Chlostridium botulinum* ADP-ribosyl transferase C3 [12] did not react with the antiserum (data not shown). On the other hand, Fig. 2 shows that the adsorbed antiserum specifically detected ADPR-EF-2 on Western blots of partially purified EF-2 from various archaebacterial as well as eukaryotic sources. With the exception of the fish extract, a slight reaction of the antiserum with the EF-2 band in the samples which were not treated by diphtheria toxin and NAD, may indicate that the factors were in vivo ADP-ribosylated to some extent. Besides the EF-2 band at 85 kDa, a smaller ADP-ribosylatable EF-2 fragment of 80 kDa was detected in the extracts from the archaebacteria. In contrast to these observations, for eukaryotes only the extract from mouse liver showed an ADP-ribosylated fragment of EF-2 or another small ADP-ribosylated protein with a molecular weight of about 40 kDa (Fig. 2, lanes l and m).

Meyer and Hilz [13] have produced an anti-(ADP-ribose) antiserum which did react with ADP-ribosylated actin or rho protein (K. Aktories, personal communica-

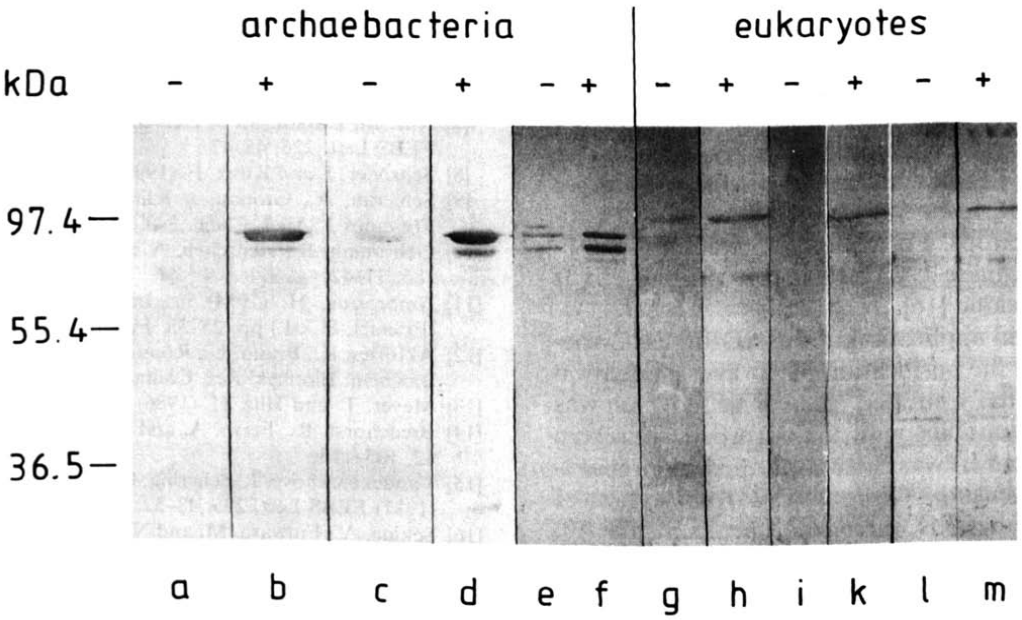

Fig. 2. Specificity of adsorbed anti-(ADPR-EF-2) antiserum to partially purified elongation factor 2 from different sources. Immunostained blot after SDS-PAGE of in vitro ADP-ribosylated (+) and not ADP-ribosylated (−) EF-2: (a,b) *T. acidophilum* (180 ng each), (c,d) *S. acidocaldarius* (180 ng), (e,f) *D. mucosus* (90 ng), (g,h) Han/Wistar rat liver (110 ng), (i,k) *Cynolebias whitei* (10 ng), (l,m) mouse liver (BALB/c) (4 ng).

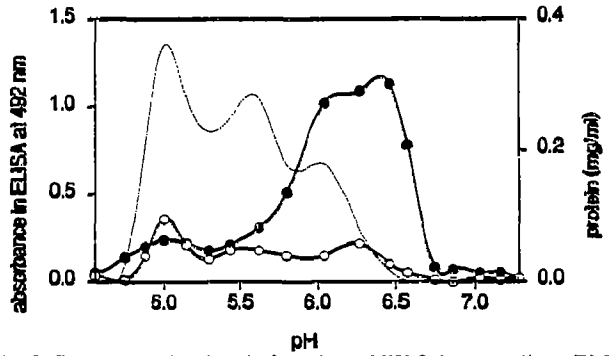

Fig. 3. Preparative isoelectric focusing of EF-2 from rat liver. ELISA with anti-(ADPR-EF-2) antiserum before (○) and after (●) in vitro ADP-ribosylation of the EF-2 contained in each fraction, (----) protein.

tion) as well as with FAD, ADPR-EF-2 and mono-ADP-ribosylated nuclear proteins [13]. These authors also reported on difficulties in immunization of rabbits with ADP-ribosylated proteins due to phosphodiesterase which hydrolyzed the diphosphate group of the hapten [14]. They have circumvented this problem by synthesis of an ADP[CH_2]-ribose serum albumin conjugate which was used to raise an antiserum in rabbits. In our experiments, hydrolysis was possibly prevented by the use of SDS-gel pieces as the antigen depot. On diffusion of the protein to the gel surface, the ADPR groups might have been presented to the immune cells and have stimulated antibody production, before they have been recognized by the hydrolytic enzymes. However, in contrast to the anti-(ADP-ribose) antiserum produced by Meyer and Hilz [13] which showed a rather broad specifity to all kinds of ADP-ribosylated proteins, our adsorbed antiserum, although raised with ADPR-EF-2 from *S. acidocaldarius*, was specific to ADPR-EF-2 from various archaebacterial and eukaryotic sources. This indicates that some of the highly conserved amino acids within the ADP-ribosylatable domain of EF-2 [8], but at least the ADPR acceptor diphthamide itself, must have been a part of the antibody inducing epitops. For comparison, actin is ADP-ribosylated at arginine-177 [15] and the rho protein at an asparagine residue [16].

Fig. 3 depicts an application of the ADPR-EF-2-specific antiserum in the purification of rat liver elongation factor 2. The partially purified rat liver EF-2 which was analyzed by immunoblot with the adsorbed antiserum (Fig. 2, lanes g and h) was further purified by preparative isoelectric focusing. Without ADP-ribosylation of the EF-2 in the fractions, three small peaks at pH 5.0, 5.5 and 6.25 was detected by ELISA. But only the peak at pH 6.25 was raised about 5-fold by ADP-ribosylation resulting in a peak at pH 6.4 with a broad shoulder at pH 6.0 while the peak at pH 5.0 rather was reduced. This indicates that the peak at pH 6.25 contained at least two ADP-ribosylatable isoforms of EF-2 while the peaks at pH 5.0 and 5.5 may have contained trace amounts of in vivo ADP-ribosylated EF-2.

In summary, we have produced an antiserum specific to ADP-ribosylated EF-2 from archaebacteria as well as eukaryotes. The existence of some antibodies to unsubstituted diphthamide in the serum as mentioned above seems rather unlikely as the EF-2 used for raising the antiserum was almost completely ADP-ribosylated and the ribose-phosphate bound to diphthamide cannot be removed by phosphodiesterase. Thus, we have obtained a valuable tool for elucidation of the role which this posttranslational modification might play in regulation of metabolism. Studies are currently underway to identify the physiological conditions of in vivo ADP-ribosylation of EF-2 from S. acidocaldarius.

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