

Endogenous ADP-ribosylation of eukaryotic elongation factor 2 and its 32 kDa tryptic fragment

KIVANÇ ERGEN*, MUHAMMET BEKTAŞ**, SINA GÖKÇE** AND RÜSTEM NURTEN**

* Kocaeli University Medical Faculty, Department of Biophysics, 41380 Umuttepe, İzmit-Kocaeli, TURKEY.

** Istanbul University Istanbul Faculty of Medicine, Department of Biophysics, 34390 Çapa-İstanbul, TURKEY

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ABSTRACT: Eukaryotic elongation factor 2 (eEF-2) can undergo ADP-ribosylation in the absence of diphtheria toxin. The binding of free ADP-ribose and endogenous transferase-dependent ADP-ribosylation were distinct reactions for eEF-2, as indicated by different findings. Incubation of eEF-2 tryptic fragment 32/33 kDa (32F) with NAD was ADP-ribosylated and gave rise to the covalent binding of ADP-ribose to eEF-2. 32F was revealed to be at the C-terminal by Edman degradation sequence analysis.

In our study, the elution of 32F from SDS-PAGE was ADP-ribosylated both in the presence and absence of diphtheria toxin. These results suggest that endogenous ADP-ribosylation of 32F might be related to protein synthesis. This modification appears to be important for the cell function.

Introduction

Eukaryotic Elongation factor 2 (eEF-2) is one of the three protein factors involved in eukaryotic polypeptide chain elongation and promotes translocation in this process (Bermek, 1978). eEF-2 provides a unique site of attack for diphtheria toxin and *Pseudomonas* exotoxin in eukaryotic cell (Collier, 1967; Honjo *et al.*, 1971; Iglewski and Kabat, 1975). These toxins inactivate eEF-2 by catalyzing ADP-ribosylation of its diphthamide residue (Van Ness *et al.*, 1980) thereby inhibiting protein synthesis (Pappenheimer, 1977).

It has been previously argued that the toxin effect

may simulate a normal cellular mechanism (Collier, 1975). Endogenous ADP-ribosyltransferase activity specific for eEF-2 has been reported in mammalian systems (Lee and Iglewski, 1984; Sitikov *et al.*, 1984; Sayhan *et al.*, 1986), and it appears to be an inherent property of eEF-2 (Sayhan *et al.*, 1986). This activity, which co-migrates with eEF-2 purification, has been implicated as an inherent property of the factor (Sayhan *et al.*, 1986).

Another line of research revealed that eEF-2 is also specifically phosphorylated by Ca/calmodulin-dependent kinase (protein kinase III or eEF-2 kinase) (Palfrey, 1983; Nairn *et al.*, 1985; Ryazanov *et al.*, 1988; Zamboni *et al.*, 1991). The phosphorylated form of eEF-2 is also inactive in protein synthesis. These data suggest that eEF-2 is an important site for cellular control mechanism through posttranslational modifications, as underlined by the presence of modified variants of eEF-2 in cell lysates (Celis *et al.*, 1990; Marzouki *et al.*, 1989). Increases in the relative proportions of phospho-

Address correspondence to: Dr. Kivanç Ergen. Kocaeli University Medical Faculty, Department of Biophysics, 41380 Umuttepe, İzmit-Kocaeli, TURKEY.
Phone: (+90 262) 303 84 97. E-mail: kergen@hotmail.com
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rylated variant of eEF-2 during mitosis (Celis *et al.*, 1990) and of the ADP-ribosylated variant in aged human cell cultures (Riis *et al.*, 1990) have been reported. Another eEF-2-mediated control mechanism for elongation appears to be the cellular proteolytic degradation of eEF-2, as indicated by the presence of varying amounts of ADP-ribosylatable fragments in different cell types and different stages of cell growth (Zamboni *et al.*, 1991; Giovane *et al.*, 1987). It has been suggested that the binding of free ADP-ribose, diphtheria toxin and endogenous transferase-catalysed ADP-ribosylations in the presence of NAD are distinct reactions (Bektaş *et al.*, 2006). Bektaş *et al.* (2006) showed that both types of endogenous ADP-ribosylation give rise to inhibition of polyphenylalanine synthesis.

In this report, we prove that incubation of eEF-2 tryptic fragment 32/33 kDa (32F) with NAD was ADP-ribosylated. Endogenous ADP-ribosylation of 32F was revealed to be at the C-terminal by Edman degradation. This site appears to be important for the functional integrity of the factor.

Material and Methods

eEF-2 purification

All reagent grade chemicals were obtained from the Sigma Chemical Co. (St. Louis, USA). The diphtheria toxin (DT) was a gift from the Refik Saydam Institute in Ankara. eEF-2 was purified from the rat livers as previously described (Nurten and Bermek, 1980). Briefly, finely minced rat livers were homogenized in 50 mM Tris-HCl, at pH 7.4, 25 mM KCl, 5 mM MgCl₂, 7 mM mercaptoethanol and 250 mM sucrose, which is also used as dialysis buffer (DB), with a Potter homogenizer. The homogenate was subjected to differential centrifugation; following the initial centrifugation for 20 min at 30,000 xg, the postmitochondrial supernatant was centrifuged for 2 h at 130,000 xg. eEF-2 was obtained from the rat liver postribosomal supernatant by adsorption to hydroxyapatite and subsequent elution. eEF-2 was eluted between 50 mM and 150 mM potassium phosphate pH 7.0. eEF-2 was further purified by chromatography on DEAE-cellulose (DE52, Whatman) and eluted between 15–65 mM KCl in 50 mM Tris-HCl, pH 7.4, 7 mM mercaptoethanol and 0.1 mM EDTA. The eEF-2 proteins were then fractionated on phosphocellulose (P11, Whatman) and eluted between 50 mM and 250 mM KCl/phosphate buffer, pH 6.8 and 7 mM 2-mercaptoethanol.

ADP-ribosylation

Molar amounts of EF-2 were determined by ADP-ribosylation for 10 min at 20°C in the presence of 50 mM Tris HCl, pH 7.4, 7 mM mercaptoethanol and 5 µM [¹⁴C] NAD labelled in adenine moiety (specific activity 252 mCi/mmol, Amersham Pharmacia Biotech UK), and 120 µg/ml diphtheria toxin (Nurten and Bermek, 1980). Endogenous ADP-ribosylation was carried out for 1 h at 20°C in the presence of the same components except the diphtheria toxin. Aliquots from the reaction mixtures were applied to GF/A (Whatman) filters, which were washed successively in cold 5% TCA, ether-ethanol and ether. After being dried, the filters were transferred to vials containing 5 ml 0.4% 2,5-diphenyloxazol in toluene, and TCA-precipitated radioactivity was determined in a liquid scintillation spectrometer (Packard Tri-Carb 1000TR) (Nurten and Bermek, 1980).

Trypsin digestion and identification of radiolabeled fragment

For partial digestion, eEF-2 was incubated in 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.1 mM EDTA, 5 mM mercaptoethanol and 10% glycerol with trypsin (at a molar trypsin/EF-2 ratio of 1/500) for 30 min at 37°C. The reaction was stopped by adding a stoichiometric amount of soya bean inhibitor and subsequent denaturation of the sample as described (Bilgin *et al.*, 1990). Alternatively, toxin-mediated and endogenously ADP-ribosylated eEF-2 was incubated under the same condition for 30 min. Tryptic peptides from endogenously ADP-ribosylated eEF-2 separated by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) were electrophoretically transferred to PVDF membrane and, thereafter, subjected to sequence analysis by Edman degradation (carried out by Eurosequence Netherland using an automatic sequenator Model 494 Procise or Model 477A, Applied Biosystems).

Elution of eEF-2 and 32F and zymographic in situ ADP-ribosylation

eEF-2 and 32F eluted from SDS-PAGE were renatured as described (Sayhan *et al.*, 1986). Briefly, the slices were homogenized in 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA and 0.1 mg/ml BSA. The homogenates were left at room temperature overnight, followed by centrifugation through siliconized glasswool for 15 min at 2000 xg. The centrifugates were

precipitated with 4 volumes of cold (-20°C) acetone, dissolved in 100 µl 6 M guanidine hydrochloride and renatured by dialysis against 50 mM Tris HCl, pH 7.4, 150 mM KCl, 1 mM DTE, 0.1 mM EDTA, 0.1 mg/ml BSA and 2% glycerol. From each sample 10 µl aliquots were assayed for ADP-ribosylation in the presence or absence of diphtheria toxin. Zymographic in situ ADP-ribosylation following SDS-PAGE was performed according to (Hager and Burgess, 1980; Nilsson and Nygard, 1985). Then eEF-2 was renatured in 20 volumes of 2.5% Triton X-100 for two cycles of 30 min and in H₂O for an additional 30 min, and the gel was incubated for 1 h at room temperature in 1.5 ml of zymographic reaction mixture containing 100 mM phosphate, pH 7.5, 1 mM DTT, 400 mM NaCl, 2 mg/ml histone, 50 µM ADP-ribose and 2 µM [¹⁴C] NAD labelled in the adenine moiety. The reaction was stopped in 50% TCA, 0.1% Coomassie Brilliant Blue.

Electrophoretic and autoradiographic analysis

SDS-PAGE analysis was performed as described (Laemmli, 1970). β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and soya bean inhibitor were used as molecular weight standards. The proteins were stained with Coomassie Brilliant Blue, destained in 7% acetic acid and 30% methanol and exposed to Kodak X-Omat K films.

Results

Electrophoretic and autoradiographic analysis of ADP-ribosylated eEF-2 and its tryptic fragments

Mild trypsin digestion of eEF-2 (and of ADPR-EF-2) resulted in SDS-PAGE with the appearance of major polypeptides of about 66, 47, 32/33 and 23 kDa, plus a number of minor products (Fig. 1). Autoradiographic analysis and radioactive counting revealed that, in addition to ADPR-EF-2 corresponding to the 97 kDa, the 32/33 kDa polypeptide (32F) carried the radioactive label (Fig. 1 and Fig. 2). Both native eEF-2 and ADP-ribosylated eEF-2 (whether mediated by diphtheria toxin or by endogenous ADP-ribosylation) gave the same profile with trypsin digestion (Fig. 2, lane 1 and 3). The eluates corresponding to intact eEF-2 and to 32F were also ADP-ribosylated both in the presence and absence of diphtheria toxin, and both ADP-ribosylations were resistant to cold TCA-treatment and the radioactive label was retained after SDS-PAGE, which shows the co-

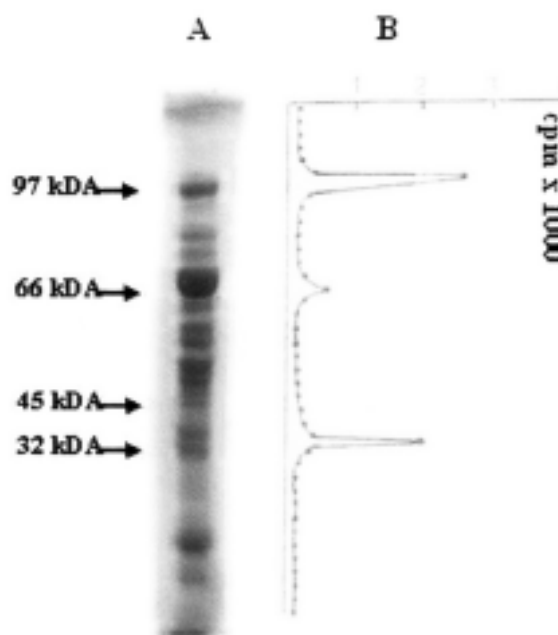


FIGURE 1. Mild trypsin digestion of diphtheria toxin ADP-ribosylated eEF-2. 60 µg of ADP-ribosylated eEF-2 was partially digested at 37°C for 30 min. After separation by SDS-PAGE, 1 mm slices were incubated at 50°C for 24h in 3% H₂O₂. The radioactivity of 1 mm slices was counted. (A) ADP-ribosylated eEF-2 after trypsin treatment. (B) Radioactivity of slices.

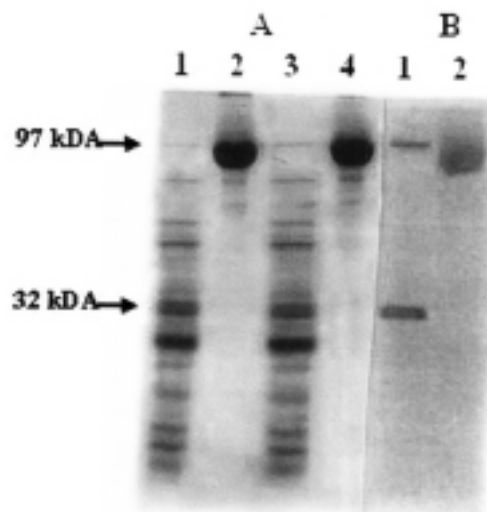


FIGURE 2. Mild trypsin digestion of endogenous ADP-ribosylated eEF-2. 60 µg eEF-2 and 60 µg ADP-ribosylated eEF-2 were partially digested at 37°C for 30 min. After separation by SDS-PAGE, autoradiography was applied. (A) Lane 1- eEF-2 (native) + trypsin; Lane 2- eEF-2 (native); Lane 3- ADP-ribosylated eEF-2 (by endogenous ADP-ribosylation) + trypsin; Lane 4- ADP-ribosylated eEF-2. (B) Lanes 1 and 2 show the autoradiography corresponding to lanes 3 and 4 of panel A, respectively.

valent binding character. (Fig. 1 and Fig. 2A, lane 3).

Zymographic in situ ADP-ribosylation

Endogenous ADP-ribosylation of the 32F was also shown by zymographic in situ reaction, which gave rise to the radioactive labeling of the same band(s) (Fig. 3).

Edman degradation sequence analysis

N-terminal sequence analysis of 32/33F revealed the presence of two fragments with the N-termini corresponding to L526 and S529 on eEF-2, respectively. Therefore, in this study, we proved that endogenous ADP-ribosylation of eEF-2 with NAD takes place at the C-terminal part of eEF-2, namely at 32F.

Discussion

eEF-2 is known to undergo ADP-ribosylation in the absence of diphtheria toxin (Lee and Iglewski, 1984; Sitikov *et al.*, 1984; Sayhan *et al.*, 1986). An endogenous ADP-ribosyltransferase has been implicated to account for this reaction (Lee and Iglewski, 1984;

Bermek, 1976; Iglewski and Dewhurst, 1991). However, ADP-ribosylation has also been observed with purified eEF-2 fractions and under conditions excluding the presence of such presumptive cellular activity (Iglewski, 1994). ADP-ribosylation of eEF-2 appears to be a common cellular event. Indeed, eEF-2 fractions reveal varying degrees of endogenous ADP-ribosylation, which appears to increase with aging (Marzouki *et al.*, 1989). Recently, it was shown that ADP-ribosylation of eEF-2 takes place under physiological conditions. However, H₂O₂-promoted oxidative stress gave rise to an increase that is two times greater than the *in vivo* labeling eEF-2 in a K562 cell culture and was accompanied by a loss of eEF-2 activity in polypeptide chain elongation (Bektaş *et al.*, 2005). It has been suggested that the binding of free ADP-ribose, diphtheria toxin and endogenous transferase-catalysed ADP-ribosylations in the presence of NAD are distinct reactions (Bektaş *et al.*, 2006). Bektaş *et al.* (2006) showed that both types of endogenous ADP-ribosylation give rise to inhibition of polyphenylalanine synthesis.

Mild trypsin digestion of eEF-2 (and of ADP-ribosylated eEF-2) resulted in SDS-PAGE patterns similar to those previously reported (Nilsson and Nygard, 1985) (Fig. 1). 32/33 kDa polypeptide (32F) carried the radioactive label, a finding seemingly consistent with a previous report (Fig. 2B) (Nilsson and Nygard, 1985). These findings extended the scope of the former data showing that the fragments arising from the proteolytic cleavage of eEF-2 can be ADP-ribosylated in the presence of diphtheria toxin (Giovane *et al.*, 1987). As indicated by additional data (not shown), purified fractions of eEF-2 underwent a specific breakdown with the appearance of minor polypeptides with a molecular range of 20-36 kDa. A report on the presence of an eEF-2-specific proteolytic activity (Servillo *et al.*, 1988) is consistent with these findings.

In our study, ADP-ribosylation of 32F appeared to result in the formation of covalent linkage on the basis of its product to cold TCA-treatment as well as retention of bound radioactive label after SDS-PAGE, as is the case with intact eEF-2, as has been previously reported (Bektaş *et al.*, 2005). In that report, binding of free ADP-ribose, and binding of ADP-ribose which took place in the presence of NAD due to endogenous ADP-ribosyltransferase action, also appeared to result in the formation of covalent linkage.

In the present study, it was also shown that the C-terminal part of eEF-2, which is 32kDa, carried the ADP-ribosylated fragment. This finding is consistent with a previous report that the sites involved in this

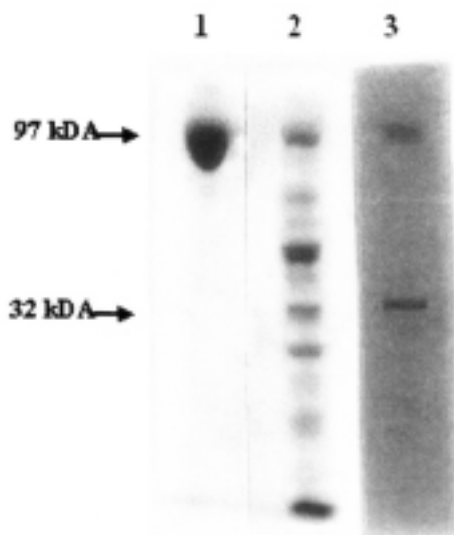


FIGURE 3. Zymographic and autoradiographic analysis of mild trypsin digestion of eEF-2. 60 µg eEF-2 was partially digested for 30 min at 20°C. After separating by SDS-PAGE, Zymography (in-situ hybridization) was applied. Lane 1- Control eEF-2; Lane 2- Zymography of eEF-2 after trypsin treatment; Lane 3- Autoradiography of 2.

modification by the toxin and endogenous transferase have been found to reside on the same tryptic peptide from the carboxyl terminal region of the factor (Fendrick *et al.*, 1992). This may suggest an alternative function of 32F apart from the intact eEF-2. GTP interaction was shown to be at 158-161 amino acid residues of eEF-2 (McKeehan, 1972); therefore, it can be excluded the likely interaction of NAD at the GTP binding site, since 32F has N-termini corresponding to L526 and S529. Moreover, as previously shown, by cleaving with the tryptophan-specific reagent N-chlorosuccinimide, the site of ADP-ribosylation by diphtheria toxin, could be located at a distance of 40-60 kDa from the GTP-binding site and about 4-11 kDa from the nearest terminus (Nilsson and Nygard, 1985) and diphtheria toxin-catalyzed ADP-ribosylation takes place at histidine residue of codon 715 that is modified post-translationally to diphthamide (Omura *et al.*, 1989; Kohno and Uchida, 1987). It was shown that purified eEF2 recovered from SDS-PAGE can be radiolabeled by using [¹⁴C]NAD (Sayhan *et al.*, 1986). In agreement with that report, it was suggested that eEF2 can contain an ADP-ribosyltransferase site, which can be activated *in vitro* and give rise to auto-ADP-ribosylation (Iglewski and Dewhurst, 1991). The finding of this study that the elution of 32F from SDS-PAGE was ADP-ribosylated both in the presence and absence of diphtheria toxin extended this view.

ADP-ribose generated from NAD and/or poly ADP-ribose under glycohydrolase action can bind to, following an Amadori rearrangement, a protein amide group under formation of a Schiff base (Fendrick and Iglewski, 1989; Parrado *et al.*, 1999; Vary *et al.*, 1994; Ayala *et al.*, 1996; Jacobsen *et al.*, 1994). This ketoamine adduct can, in turn, give rise under oxidation to protein glycooxidation products, i.e. advanced glycosylation end products (AGE) (Khalifah *et al.*, 1996; Cervantes-Laurean *et al.*, 1993). Protein glycation by pentoses and hexoses appears to play a role in the physiopathology of a number of diseases such as diabetes as well as in oxidative stress and aging (Jacobson *et al.*, 1997). Our work does not explain whether 32F was ADP-ribosylated by binding free ADP-ribose or binding ADP-ribose under the endogenous transferase action in the presence of NAD.

The modification of eEF-2 within the context of such a condition, the formation of ADP-ribosylated 32F, may well be related to protein synthesis as in the case of ADP-ribosylated eEF-2. Increased NAD/ADP-ribose turnover following DNA damage might depress protein synthesis in the cell by ADP-ribosylation. This also

shows the unique binding reaction of ADP-ribose to eEF-2.

In conclusion, the results presented in this report attest to the presence of ADP-ribosylation site on 32F at the C-terminal half of eEF-2. Work is in progress on the elucidation of the molecular mechanism of endogenous ADP-ribosylation and characterization of the sites involved.

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