Inactivation of the Elongation Factor Tu by Mosquitocidal Toxin-Catalyzed Mono-ADP-Ribosylation

Jörg Schirmer,1 Hans-Joachim Wieden,2 Marina V. Rodnina,2 and Klaus Aktories1*

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität
Freiburg, D-79104 Freiburg,1 and Institute of Physical Biochemistry, University of Witten/Herdecke,
D-58448 Witten,2 Germany

Received 29 March 2002/Accepted 1 July 2002

The mosquitocidal toxin (MTX) produced by Bacillus sphaericus strain SSII-1 is an ~97-kDa single-chain toxin which contains a 27-kDa enzyme domain harboring ADP-ribosyltransferase activity and a 70-kDa putative binding domain. Due to cytotoxicity toward bacterial cells, the 27-kDa enzyme fragment cannot be produced in Escherichia coli expression systems. However, a nontoxic 32-kDa N-terminal truncation of MTX can be expressed in E. coli and subsequently cleaved to an active 27-kDa enzyme fragment. In vitro the 27-kDa enzyme fragment of MTX ADP-ribosylated numerous proteins in E. coli lysates, with dominant labeling of an ~45-kDa protein. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry combined with peptide mapping identified this protein as the E. coli elongation factor Tu (EF-Tu). ADP ribosylation of purified EF-Tu prevented the formation of the stable ternary EF-Tu–aminoacyl-tRNA·GTP complex, whereas the binding of GTP to EF-Tu was not altered. The inactivation of EF-Tu by MTX-mediated ADP-ribosylation and the resulting inhibition of bacterial protein synthesis are likely to play important roles in the cytotoxicity of the 27-kDa enzyme fragment of MTX toward E. coli.

The mosquitocidal toxin (MTX) from Bacillus sphaericus strain SSII-1 is a member of the family of bacterial ADP-ribose transferases (8, 22). These toxins transfer the ADP-ribose moiety of NAD to a protein target in eu-karyotic cells, affecting essential cell functions (8). Diphtheria toxin, Pseudomonas exotoxin A, cholora toxin, pertussis toxin, C2 and iota toxins, the family of C3-like transferases, and Pseudomonas exoenzyme S are well-described members of this family of bacterial toxins. Diphtheria toxin and Pseudomonas exotoxin A block protein synthesis by ADP-ribosylation of the eukaryotic elongation factor 2 at diphthamide, a modified histidine residue (26). Cholora toxin and pertussis toxin ADP-ribosylate G proteins at specific arginine and cysteine residues, respectively, to modify signal transduction by G-protein-coupled receptors (4, 14, 25, 29). Members of the subfamily of binary ADP-ribosylating toxins, such as Clostridium botulinum C2 toxin and Clostridium perfringens iota toxin, ADP-ribosylate G actin at Arg177 (23, 24). Several bacterial exoenzymes ADP-ribosylate small GTP-binding proteins, including C. botulinum C3 exoenzyme and related C3-like transferases, which ADP-ribosylate Rho GTPases at Asn41 (2, 19, 21, 30), and Pseudomonas aeruginosa exotoxin S, which preferentially modifies Ras proteins at several arginine residues (10).

The mature MTX (without the putative signal sequence of 29 amino acid residues) is a 97-kDa protein (MTX30–870), MTX30–870 is processed into a 27-kDa N-terminal enzyme component (MTX30–264) and a 70-kDa C-terminal putative binding component (MTX265–870) by crude mosquito larval gut extracts or chymotrypsin. MTX30–870 enzyme activity is strictly dependent on proteolytic activation. Following proteolytic cleavage, the putative MTX binding component remains non-covalently bound to the enzyme component, thereby reducing enzyme activity. However, no repression is observed when the 27-kDa fragment is generated from a 32-kDa N-terminal fragment of MTX (MTX30–308), resulting in enzyme activity 150-fold higher than that of processed MTX30–870. The production of recombinant MTX30–264 in Escherichia coli is not possible due to its severe toxicity toward the bacterial cell. This toxicity is dependent on the transferase activity of the enzyme, as catalytically inactive MTX30–264 constructs can be produced in E. coli (20).

In this study, we investigated the possible reasons for the toxicity of MTX toward E. coli. We identified the bacterial elongation factor Tu (EF-Tu) as one of the target proteins of MTX in E. coli cell lysates and showed the inactivation of EF-Tu by mono-ADP-ribosylation.

MATERIALS AND METHODS

Materials. MTX30–308 and MTX30–308 with an E197Q mutation (MTX30–308 E197Q) were purified and proteolytically processed as described previously (20). Cellular EF-Tu from E. coli MRE 600 and [14C]Phe-tRNA were purified by published procedures (11, 18). [14C]phenylalanine was purchased from ICN (Braunschweig, Germany), and adenylyl-[32P]NAD (30 Ci/mmol) was obtained from NEN (Vilvoorde, Belgium). 3′-[25P]GTP (mant-GTP) was obtained from Molecular Probes (Leiden, The Netherlands). All other reagents were obtained from Sigma (Deisenhofen, Germany) unless otherwise indicated. Buffer A contained 10 mM MgCl2, 50 mM NH4Cl, and 25 mM Tris-HCl (pH 7.4).

Cloning and purification of EF-Tu. DNA was amplified from plasmid pEECAHis (coding for EF-Tu) by PCR with the following primers: EF-Tu sense, 5′-CGCGGATCCTCTAAAGAAAAGTTTGAACGTAC-3′, and EF-Tu antisense, 5′-CCGGAATTCTGCTAGAAGAAGGTGTGAACGTAC-3′. The EF-Tu fragment was cut with BamHI and EcoRI, purified, and ligated to the digested pGEX-2TGL vector. This vector was previously designed in our laboratory (Freiburg) and is a modification of the pGEX-2T vector from Amersham Pharmacia Biotech, Freiburg, Germany. The vector contains an additional oli-
ADP-RIBOSYLATION OF EF-Tu

VOL. 68, 2002

ADP-RIBOSYLATION OF EF-Tu

4895

The porcine brain EF Tu plasmids were transformed into E. coli TG1. Cells were grown in Luria-Bertani medium to stationary phase at 37°C, harvested, resuspended in fresh medium containing 1 mM isopropyl-1-thio-β-D-galactopyranoside, and incubated for 2 h at 30°C. Then, cells were harvested, lysed by sonication in 20 mM Tris·HCl (pH 7.4)–10 mM NaCl–1 mM MgCl2–1% Triton X-100–1 mM phenylmethylsulfonyl fluoride–5 mM diithiothreitol (lysis buffer), and purified by affinity chromatography with glutathione-Sepharose beads (Amersham Pharmacia Biotech). Loaded beads were washed twice with lysis buffer and twice with thrombin cleavage buffer (50 mM Tris·HCl [pH 7.4]; 150 mM NaCl; 5 mM MgCl2). EF-Tu was eluted with thrombin directly from the beads in thrombin cleavage buffer. Thrombin was removed with benzamidine-Sepharose beads (Amersham Pharmacia Biotech).

ADP-ribosyltransferase assay. ADP-ribosylation was performed as follows: 2.5 μM cellular or recombinant EF-Tu·GDP or 12 μg of total E. coli cell lysate protein was incubated with 100 μM [32P]NAD and 50 nM MTX30–264 for various time periods at room temperature in the presence of 1 mM diithiothreitol–2 mM MgCl2–50 mM Tris·HCl (pH 7.4) in a total volume of 20 μL. Toxin-free control experiments were performed in the presence of the storage buffer for MTX proteins (thrombin cleavage buffer). For the time course experiment, 300 nM recombinant EF-Tu and 100 nM MTX30–264 were used to achieve complete ADP ribosylation of EF-Tu. The reaction was stopped by the addition of Laemmli buffer and heating for 5 min at 95°C, and the samples were subsequently subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) by the methods of Laemmli (13). [32P]ADP-ribosylated proteins were detected with a PhosphorImager from Molecular Dynamics. Quantification of PhosphorImager data was performed with ImageQuant software.

Two-dimensional gel electrophoresis. A [32P]ADP-ribosylation assay with E. coli cell lysate (150 μg of total protein) and 1 μM MTX30–264 was performed in a total volume of 200 μL, followed by isoelectric focusing (IEF) with Immobiline DryStrips (Amersham Pharmacia Biotech), pHs 3 to 10, for first-dimension separation. IEF was carried out with a Multiphor II apparatus as described in the Amersham Pharmacia Biotech manual. In the second dimension, proteins were separated by SDS-PAGE and stained with Coomassie blue. Radioabeled proteins were detected by autoradiography.

Mass spectrometry. The radioactively labeled proteins were excised from the gel and destained for 1 h at 50°C in 40% acetonitrile–60% ammonium carbonate (50 mM, pH 7.8) to remove stain, gel buffer, SDS, and salts. The gel plugs were subsequently dried by vacuum centrifugation. Thereafter, 20 μL of ammonium hydrogen carbonate solution containing 0.2 μL of trypsin was added, and digestion was carried out for 12 h at 37°C. A saturated matrix solution of 4-hydroxy-α-cyanocinnamic acid in a 1:1 solution of acetonitrile–aqueous 0.1% trifluoroacetic acid was prepared and mixed with 2 μL of the proteolytic peptide mixture in equal parts. For internal calibration, 5 pmol of a human adrenocorticotropic hormone (18 to 39 amino acids) (mass, 2645.20 Da; Sigma) and 5 pmol of human angiotensin II (mass, 1046.54 Da; Sigma) were added to the matrix solution. Using the dried-droplet method of matrix crystallization, 1 μL of the sample matrix solution was placed on the mass spectrometer target and dried at room temperature, resulting in a fine granular matrix layer.

Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry (MS) was performed with a Bruker Biflex mass spectrometer equipped with a nitrogen laser (λ = 337 nm) to desorb and ionize the samples. Mass spectra were recorded in the reflector-negative mode in combination with delayed extraction. The computer program ProFound (Rockefeller University, New York, N.Y.) was used for protein identification. This program matches measured peptide masses to virtually trypsin-digested proteins in a database (peptide mapping).

Characterization of the ternary complex aminoacyl-tRNA·EF-Tu·GTP. Cellular EF Tu·GDP (3 μM) was ADP-ribosylated by MTX30–264 (500 nM) in the presence of 100 μM NAD primarily as described above. ADP-ribosylated EF Tu·GTP (final concentration, 1 μM) was prepared by incubation with 4 μM GTP, 5 mM phosphoenolpyruvate, and 250 μg of pyruvate kinase/ml in 40 μL of buffer A for 30 min at 37°C. Purified [14C]tRNAψψψ (0.5 μM; 942 dpm/μmol) in 20 μL of buffer A was added, and the reaction mixture was placed on ice for 10 min. The reaction was carried out at 37°C, and at various time points, 10 μL of the reaction solution was transferred to trichloroacetic acid-soaked Whatman paper. After extensive washing with 5% trichloroacetic acid, the filter paper was dried, 2.5 mL of scintillation mixture was added, and nonhydrolyzed [14C]tRNAψψψ was measured with a scintillation counter (Packard RT2500). Control assays were performed with wild-type EF Tu but without ADP-ribosylation, with EF Tu treated with catalytically defective MTX30–264E197Q (20), and in the absence of EF Tu.

Binding of mann-GTP to ADP-ribosylated EF Tu. EF Tu·GDP (4 μM) was incubated with 100 μM NAD and 500 nM MTX30–264. Next, ADP-ribosylated EF Tu was incubated with 1 mM phosphoenolpyruvate and 100 μg of pyruvate kinase/ml in buffer A for 30 min at 37°C to form the ADP-ribosylated EF Tu·GTP complex. The exchange of GTP with mann-GTP was accomplished by rapidly mixing 60 μL of the EF Tu·GTP solution (1 μM) with 60 μL of a mann-GTP solution (10 μM) by using the stopped-flow technique (SX-18MV spectrometer; Applied Photophysics). The fluorescence of mann-GTP was excited via fluorescence resonance energy transfer from tryptophan 184 in EF Tu excited at 280 nm and measured after passage through 4K08 filters (Schott). Experiments were performed at 20°C, and the change in fluorescence was recorded over 10 s. Time courses (see Fig. 4) were obtained by averaging six or seven individual transients. The fluorescence at zero time was set to 1, and data were evaluated by fitting to a two-exponential function with characteristic parent time constants (kapp) and amplitudes (A) according to the equation F = A1 × exp(−kapp1 × t) + A2 × exp(−kapp2 × t), where F is the fluorescence at time t. Calculations were performed by using SigmaPlot (Jandel Scientific).

Determination of the acceptor amino acid of MTX. Platelet cytosol (150 μg) was incubated with Clotrimazole lincomycin C3 toxin (250 nM) and Clotrimazole botulinum C2 toxin (250 nM) in the presence of 100 μM [32P]NAD–2 mM MgCl2–1 mM diithiothreitol–5 mM thymidine for 30 min at 37°C in a total volume of 200 μL. Recombinant EF Tu (1.5 μM) was incubated with 250 nM MTX30–264 in the presence of 100 μM [32P]NAD–2 mM MgCl2–1 mM diithiothreitol for 30 min at room temperature in a total volume of 200 μL. The reactions were stopped by adding SDS to a final concentration of 2%. The reaction mixtures were then divided into equal parts and incubated with either 0.5 M NaCl for 4 h or 0.5 M hydroxyamine (pH 7.5) for 2 or 4 h at 37°C. After precipitation of proteins with chloroform-methanol, the samples were subjected to SDS-PAGE and analyzed by phosphorimaging.

RESULTS

ADP-ribosylation of multiple proteins in E. coli cell lysates. To understand why ADP-ribosylation by MTX30–264 is toxic for E. coli (20), possible protein targets of MTX30–264 were identified in E. coli cell lysates. ADP-ribosylation of total E. coli cell lysates with MTX30–264 in the presence of [32P]NAD labeled various proteins, as detected by SDS-PAGE and subsequent phosphorimaging (Fig. 1A). The labeling was dependent on MTX30–264 enzyme activity. Labeling was not detected in the absence of MTX30–264 or in the presence of catalytically inactive MTX30–264E197Q. Two-dimensional gel electrophoresis (Fig. 1B) of the [32P]-labeled proteins showed that while several proteins were ADP-ribosylated by MTX30–264, two substrates of ~45 kDa were predominantly radiolabeled (Fig. 1C).

Identification of EF Tu as a target protein of MTX. The ADP-ribosylated ~45-kDa proteins from E. coli lysates were excised from the SDS gel and digested with trypsin. The resulting peptides were analyzed by MALDI-TOF MS. By use of the computer program ProFound, measured peptides of both proteins were assigned to E. coli EF Tu (SWISS-PROT accession number P02990) with a sequence coverage of 25% (Table 1). It was not possible to elucidate the difference between the two EF Tu protein spots by MALDI-TOF MS, as the measured peptide masses were the same. However, the differences might have arisen from altered mobility during IEF when EF Tu was bound to different ligands.

Mono-ADP-ribosylation of purified EF Tu. To verify that EF Tu is a substrate for MTX30–264-catalyzed ADP-ribosylation, cellular EF Tu·GDP and recombinant EF Tu·GDP were tested in an ADP-ribosylation assay with MTX30–264. Both cellular EF Tu·GDP and recombinant EF Tu·GDP were tar-
gets for ADP-ribosylation by MTX30–264 (Fig. 2A), whereas no labeling was detected in the absence of MTX30–264 or after treatment of EF-Tu with catalytically inactive MTX30–264E197Q (Fig. 2A). A difference in the efficiency of ADP-ribosylation was not observed when EF-Tu–GTP was used instead of EF-Tu–GDP (data not shown).

EF-Tu modification by MTX30–264 was time dependent, with about 0.75 mol of ADP-ribose incorporated per mol of EF-Tu upon completion of the assay (Fig. 2B). Adding more MTX30–264 to the ADP-ribosylation mixture after 45 min of incubation did not increase the incorporation of ADP-ribose, indicating saturation of the reaction.

**Functional effects of EF-Tu ADP-ribosylation.** EF-Tu in its GTP-bound state forms a stable complex with aminoacyl-tRNA, protecting the 3'end of aminoacyl-tRNA from hydrolysis (28). ADP-ribosylation of EF-Tu prevented the formation of a stable ternary complex with GTP and aminoacyl-tRNA, as indicated by the rapid hydrolysis of [14C]Phe-tRNAPhe (Fig. 3). Similar time courses of [14C]Phe-tRNA hydrolysis were observed in the absence of EF-Tu or in the presence of ADP-ribosylated EF-Tu, indicating that ADP-ribosylated EF-Tu is inactive in aminoacyl-tRNA binding. EF-Tu treated with catalytically inactive MTX30–264E197Q was as active as native EF-Tu in protecting [14C]Phe-tRNAPhe from hydrolysis (Fig. 3).

**Binding of mant-GTP to ADP-ribosylated EF-Tu is not altered.** The nucleotide binding of ADP-ribosylated EF-Tu to mant-GTP, a fluorescent GTP derivative, was studied by using fluorescence resonance energy transfer from Trp184 in EF-Tu (27). Binding of the nucleotide to native or ADP-ribosylated EF-Tu increased mant-GTP fluorescence. To verify that EF-Tu used in this assay was fully ADP-ribosylated, aliquots were tested in the hydrolysis protection assay described above. The time courses of nucleotide exchange were identical for native EF-Tu and ADP-ribosylated EF-Tu (Fig. 4). Calculation of the

<table>
<thead>
<tr>
<th>Peptide mass (Da)</th>
<th>Position in sequence</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>836.56</td>
<td>117–123</td>
<td>EHIILGR</td>
</tr>
<tr>
<td>1,026.54</td>
<td>270–279</td>
<td>AGENVGVLLR</td>
</tr>
<tr>
<td>1,128.60</td>
<td>280–288</td>
<td>GIKEEIER</td>
</tr>
<tr>
<td>1,217.54</td>
<td>177–187</td>
<td>ALLEGDAEWEAK</td>
</tr>
<tr>
<td>1,375.65</td>
<td>45–56</td>
<td>AFDOIDNAPEEK</td>
</tr>
<tr>
<td>1,656.94</td>
<td>238–252</td>
<td>VGEEVEIVGIKETOK</td>
</tr>
<tr>
<td>1,802.89</td>
<td>59–74</td>
<td>GITINSTHVEYDTPTR</td>
</tr>
<tr>
<td>1,963.96</td>
<td>155–171</td>
<td>ELLSOYDFPDDTPIVR</td>
</tr>
</tbody>
</table>

*Protein spot 1 (Fig. 1) was identified as E. coli EF-Tu by MALDI-TOF MS and subsequent peptide mapping (ProFound). Monoisotopic masses are shown. Mass differences between measured and calculated peaks were below 0.1 Da. The sequence coverage of EF-Tu was 25%. Analysis of protein spot 2 yielded identical peptide masses, so that it represented EF-Tu as well.
apparent time constants ($k_{app}$) from the time courses indicated that there was no significant difference in the velocity of nucleotide exchange between native EF-Tu and ADP-ribosylated EF-Tu (Fig. 4). Therefore, we concluded that ADP-ribosylation does not alter the GTP-binding properties of EF-Tu.

An arginine residue in EF-Tu is modified by MTX. ADP-ribose-amine acid bonds can be characterized by their stability toward neutral 0.5 M hydroxylamine (1, 3). Whereas the ADP-ribose bond to an aspartate or cysteine residue is stable toward hydroxylamine, the ADP-ribose bond to an arginine residue has a half-life of ~2 h (1, 12). The ADP-ribosylation of the small GTPase Rho (Asn41) by C3 exoenzyme (21) and that of actin (Arg177) by C2 toxin (24) were used for comparison. As shown in Fig. 5A, a time-dependent decrease in the signal of ADP-ribosylated actin was observed after 2 and 4 h of treatment with hydroxylamine, whereas the signal for ADP-ribosylated Rho remained largely unchanged. The signal intensity of ADP-ribosylated EF-Tu decreased after incubation with hydroxylamine, suggesting that an arginine residue in EF-Tu was modified (Fig. 5B).
DISCUSSION

In this study, we report on a target protein of MTX whose inactivation by ADP-ribosylation may play an important role in cytotoxicity toward *E. coli*. Separation of [32P]ADP-ribosylated *E. coli* proteins by two-dimensional gel electrophoresis and detection of modified proteins by autoradiography revealed two dominantly labeled proteins with apparent masses of ~45 kDa. Both protein spots were identified as *E. coli* EF-Tu. Cellular EF-Tu and recombinant EF-Tu were ADP-ribosylated by MTX30–264, supporting the MS findings. ADP-ribosylation of EF-Tu by MTX30–264 led to the incorporation of about one ADP-ribose residue per molecule of EF-Tu.

EF-Tu is a bacterial translation elongation factor that delivers aminoacyl-tRNA to the A site of the ribosome during the elongation cycle of protein synthesis. ADP-ribosylation of EF-Tu prevents the formation of the stable ternary complex with GTP and aminoacyl-tRNA due to a deficiency in aminoacyl-tRNA binding. This functional inactivation of EF-Tu leads to the inhibition of protein synthesis and is likely to result in the retardation of cell growth and lethality. However, MTX30–264 ADP-ribosylated several substrates in *E. coli* cell lysates, an action which may have contributed to the cytotoxic effect.

All biglutamic acid ADP-ribosyltransferases so far studied attach ADP-ribose to arginine residues of their protein substrates (15). The instability of the ADP-ribose-amino acid bond in EF-Tu toward neutral hydroxylamine suggests the modification of arginine residues by MTX30–264-mediated ADP-ribosylation as well. ADP-ribose-asparagine bonds or ADP-ribose-cysteine bonds are stable toward neutral hydroxylamine, and so these amino acids can therefore be ruled out as acceptor amino acids (1, 5, 16). The exact arginine residue that is targeted by MTX30–264 in EF-Tu is not known yet, but possible candidates are currently under investigation.

Most ADP-ribosyltransferases are specific; i.e., they have only one specific eukaryotic target protein and exhibit no toxicity toward their host organisms. In contrast, MTX30–264 ADP-ribosylates several proteins in *E. coli* lysates. Also, in HeLa cell lysates (20) as well as in other eukaryotic cell lysates (unpublished observations), MTX30–264 modifies numerous proteins. Furthermore, Thanabalu et al. (22) reported that a nonactivated N-terminal MTX truncation (amino acids 30 to 439) modifies two proteins with apparent masses of 42 and 38 kDa in lysates of a *Culex quinquefasciatus* cell line. In contrast, the active MTX30–264 fragment used in our experiments led to the labeling of a large array of proteins in lysates of the same *C. quinquefasciatus* cell line, with predominant labeling of a 55-kDa protein (unpublished data).

So far, none of the labeled proteins in cell lysates other than those from *E. coli* could be assigned to an elongation factor. As
reported previously (20), MTX 30–264 leads to the rounding up of HeLa cells and the formation of actin-containing protrusions after 12 h. This effect is unlikely to be mediated by an alteration of the function of an elongation factor. Therefore, at least in HeLa cells, different protein targets of MTX seem to contribute to cytotoxicity. However, we cannot exclude the possibility that elongation factors may serve as additional substrates in eukaryotic cells as well. With respect to the sequence similarity between E. coli EF-Tu and the functionally equivalent human elongation factor 1 alpha (45% at the amino acid level), it seems possible that the latter serves as a substrate for elongation factor. Therefore, at 12 h. This effect is unlikely to be mediated by an ADP-ribosyltransferase after 12 h. This effect is unlikely to be mediated by a eukaryotic host factor. Venkataraman, D., P. T. Lo

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (Sonnerforschungsbereich 388) and by the Fonds der Chemische Industrie. We thank Kirill Gromadski for help with the stopped-flow experiments.

REFERENCES


