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Effect of Substitution of Glycine for Arginine at Position 146 of the A1 Subunit on Biological Activity of *Escherichia coli* Heat-Labile Enterotoxin

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The ADP-ribosyltransferase activity of polypeptide A1 of cholera toxin and that of *Escherichia coli* heat-labile enterotoxin (LT) are primarily responsible for the toxic activities of these toxins. Since the amino acid sequences of the two A1 polypeptides are very similar, their functional mechanisms are considered to be the same. Arg-146 of polypeptide A1 is thought to be involved in the active site, because this amino acid of cholera toxin has been identified as the site of self-ADP-ribosylation. However, the exact role of Arg-146 and the significance of self-ADP-ribosylation in toxicity remain unclear. We substituted Arg-146 of polypeptide A1 of LT with Gly by oligonucleotide-directed mutagenesis and examined the biological property of the resultant mutant LT. The substitution changed the mobility of subunit A on sodium dodecyl sulfate-polyacrylamide gel but did not reduce the vascular permeability activity of LT. This result indicates that Arg-146 is not absolutely required for toxic activity and that LT can express its toxic activity without self-ADP-ribosylation at Arg-146.

Escherichia coli heat-labile enterotoxin (LT) and cholera enterotoxin (CT) resemble one another strongly in many aspects (2, 3, 8, 21). The effects of both toxins stem from their activation of adenylate cyclase and a resultant increase in intracellular cyclic AMP (9, 14). Both toxins are composed of two subunits, A and B. Subunit A consists of polypeptides A1 and A2 linked to one another by a single disulfide bond (7, 8, 24). Polypeptide A1 was found to be responsible for stimulation of adenylate cyclase in the presence of NAD. Subsequently, it was found that polypeptide A1 catalyzes ADP-ribosylation of the GTP-binding regulatory component of adenylate cyclase in the presence of NAD (14, 24). It is established that the ADP-ribosyltransferase activity of fragment A1 is primarily responsible for activation of adenylate cyclase in toxin-treated cells. However, the exact structure and amino acid sequences of polypeptide A1 required for exerting ADP-ribosyltransferase activity are not known. Lai et al. (15, 16) isolated the fragment of polypeptide A1 that contained ADP-ribosyltransferase activity and showed that the fragment contained the region from Gly-47 to Arg-148. Furthermore, they observed strong labeling of polypeptide A1 itself during attempts to label the membrane protein by ADP-ribose transfer by using polypeptide A1 of CT. They showed that Arg-146 of polypeptide A1 was the site of ADP-ribosylation in vitro and that the self-ADP-ribosylated A1 polypeptide could transfer over 70% of the ADP-ribose moiety to the acceptor in 25 min on incubation with polyarginine. Therefore, Arg-146 seemed to play an important role in expression of the biological activity of CT. However, direct evidence for the role of Arg-146 has not been obtained. The amino acid sequence and secondary structure in the proximity of Arg-146 of CT are highly conserved in the LTs produced by porcine and human strains of *E. coli* (LTp and LTh, respectively; 17, 26). Therefore, Arg-146 of LT is thought to play a role in toxic activity identical to that of CT. Recently, development of the oligonucleotide-directed mutagenesis method has permitted the construction of mutants of many proteins in which individual amino acid residues of potential interest have been modified (1). We replaced Arg-146 of LTp with Gly and examined the properties of the mutant LTp.

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MATERIALS AND METHODS

Bacterial strain and plasmid. *E. coli* HB101 was used as the host strain throughout these experiments and was cultured in Luria broth (20). Plasmid EWD299, which carries the *E. coli* LTp gene, was kindly provided by W. S. Dallas. The plasmid has two *Sma*I recognition sites, and one *Pvu*I recognition site as shown in Fig. 1. The *Pvu*I site is located in the ampicillin resistance gene. The DNA region of LTp in the plasmid is 1.2×10^6 daltons in mass (5).

Oligonucleotide-directed mutagenesis and DNA sequence. Oligonucleotide-directed mutagenesis was performed on plasmid EWD299 by using the plasmid method reported by Inouye and Inouye (12). The mutagenic oligonucleotide was synthesized on an Applied Biosystems model 380B DNA synthesizer by the phosphoramidite method. The sequence of the oligonucleotide was GTCTCC*ATATTCCC, which is complementary to the DNA sequence of the wild type (Fig. 2). The asterisk indicates the mismatch with the sequence of the wild type. This mismatch brings about the substitution of Arg-146 with Gly. Oligomer purification was performed by denaturing polyacrylamide gel electrophoresis, followed by DEAE-cellulose column chromatography as described by Vlasuk et al. (25). This oligonucleotide was phosphorylated by using T4 polynucleotide kinase.

The larger DNA fragment, which was generated by cutting with *Sma*I, the fragment generated by cutting with *Pvu*I, and the oligonucleotide were heated and reannealed by stepwise cooling. The new circular DNAs were incubated with the Klenow fragment of DNA polymerase I, T4 ligase, and the four deoxyribonucleotide triphosphates. *E. coli* HB101 was

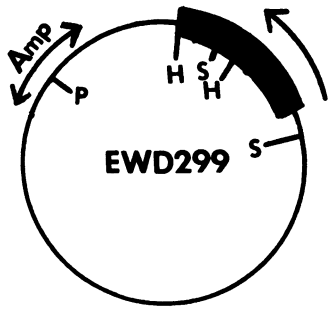

FIG. 1. Structure of plasmid EWD299. The thick bar indicates the *E. coli* LTp gene. Amp, Ampicillin resistance gene; H, *Hin*dIII; P, *Pvu*I; S, *Sma*I.

transformed with the treated DNA plasmid, and the transformants carrying the mutated plasmid were screened by colony hybridization with the ^{32}P-labeled oligonucleotide. Mutation was confirmed by DNA sequence determination by the procedure of Maxam and Gilbert (19). All enzymes used in these experiments were obtained from Takara Shuzo Co., Kyoto, Japan.

Purification of LTp. *E. coli* HB101 strains harboring the appropriate plasmids were cultured in Luria broth containing ampicillin (50 μg/ml) for 18 h at 37°C with shaking. The bacteria were collected by centrifugation and lysed by sonication. The crude lysate was clarified by centrifugation at 37,000 × *g* for 120 min at 4°C, and then the LT in the lysate was purified by successive column chromatographies on Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, Calif.) and Sephadex G-75 (Pharmacia, Uppsala, Sweden) (23). Subunit B of LTp (LTp-B) was purified as described previously (22).

Protein content was determined by the method of Lowry et al. (18), with bovine serum albumin as a standard.

Preparation of antisera. Antiserum against LTp-B (αLTp-B) was obtained by several subcutaneous injections of 20 μg of purified LTp-B into a rabbit.

Antiserum against subunit A of LTp (LTp-A) was prepared from antiserum to holotoxin of LTp by absorption with purified LTp-B. Purified holotoxin of LTp (25 μg) was emulsified with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), and the emulsion was inoculated intramuscularly into a rabbit. Several booster injections were given every 3 weeks. The antiserum was obtained 10 days after the last injection. The serum was applied to the purified LTp-B-coupled Sepharose 4B column, and the column was washed with 0.14 M NaCl solution (10). The eluted fraction was concentrated to the original volume by ultrafiltration on an Amicon PM-10 membrane. The antiserum thus obtained, which did not react with LTp-B in an immunodiffusion test, was used as the antiserum against LTp-A (αLTp-A).

Vascular permeability test. The purified toxins were treated with trypsin to attain full activity as described previously (23). The trypsin-treated toxins were diluted serially with borate-buffered saline (BG buffer) of the following composition: H_3BO_3, 0.05 M; NaCl, 0.12 M; gelatin, 0.1% (pH 7.5). The diluted samples were injected intradermally in quadruplicate into marked spots on the backs of rabbits which had been clipped. After 24 h, the rabbits were injected intravenously with 1.2 ml of a 5% solution of Diphenyl Brilliant Blue FF Supra (CIBA-GEIGY Corp., Greensboro, N.C.), in 0.45% NaCl per kg. At 1 h later, the intensity and diameter of the blue lesions in the skin were read. Blueing scores were calculated by multiplying mean blueing diameter by mean blueing intensity, as estimated on an arbitrary scale of 1 to 8 as described by Craig et al. (4). Four rabbits were used for determination of the vascular permeability activity of each sample.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS; 0.1%)-polyacrylamide gel slab (0.2 by 13.5 by

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                                        146
Wild-type sequence        -------Glu Tyr Arg Asp-----

          Template      5'----GG GAA TAT AGA GAC-----3'

Oligonucleotide           3' CC CTT ATA *CCT CTG 5'
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FIG. 2. Mutagenic oligonucleotide and partial sequences of fragment A1 of the LTp gene. The oligonucleotide is complementary to the DNA sequence of the wild type. The asterisk indicates a mismatch. The mismatch was designed to bring about the substitution of Arg-146 with Gly.

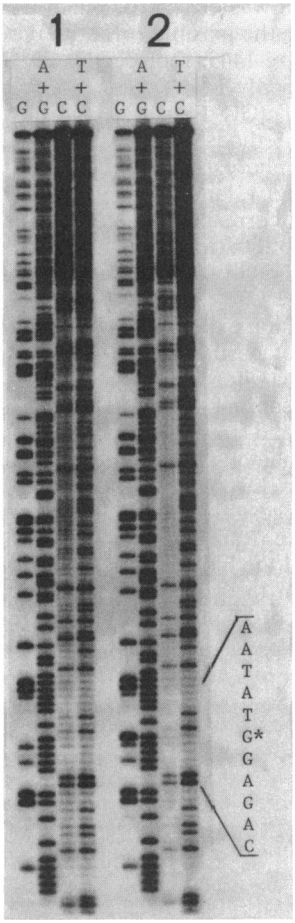

FIG. 3. Determination of mutant nucleotide sequence. Plasmids EWD299 and pKK501 were digested with *Hin*dIII and *Sma*I. The fragments carring LTp-A subunit genes were purified and labeled with [α-^{32}P]dATP by the Klenow enzyme. The labeled DNAs were cleaved with *Rsa*I. The 428-base-pair fragments carring the gene near position 146 of fragment A1 were purified and used for sequencing by the method of Maxam and Gilbert (19). The chemical cleavage products were applied to a denaturing 10% polyacrylamide gel and subjected to electrophoresis. Numbers 1 and 2 indicate the nucleotide sequences around the region of position 146 of fragment A1 of plasmids EWD299 and pKK501, respectively. The asterisk indicates the base that is different from the sequence of plasmid EWD299.

12.0 cm) electrophoresis was performed as described by Laemmli (13), with 15% acrylamide. Electrophoresis was performed at a constant current of 25 mA for 4.5 h. The gels were stained with Coomassie brilliant blue and then destained as described previously (6).

RESULTS

Oligonucleotide-directed mutagenesis was performed as described in Materials and Methods. The plasmid carrying the mutated LTp gene was identified by screening transformants with the ^{32}P-labeled mutagenic oligonucleotide. To determine the nucleotide sequences around the mutated LTp gene, the *Sma*I-*Hin*dIII fragments of the mutant plasmid and EWD299 were purified and labeled at the 3′ end of the *Hin*dIII site. The labeled DNA fragments were cleaved with *Rsa*I. The fragments carrying the object gene were purified and used for sequencing by the method of Maxam and Gilbert (19). Substitution of the nucleotide sequence in the predicted region was observed (Fig. 3). This means that the strain harboring the plasmid (pKK501) produces the mutant LTp in which Arg-146 is replaced by Gly. The mutant LTp is denoted as LTp(Gly-146).

LTp and LTp(Gly-146) were purified as described in Materials and Methods. The results of SDS-polyacrylamide gel slab electrophoresis of purified LTp and LTp(Gly-146) are shown in Fig. 4, lanes 1 and 2, respectively. Both toxins gave two bands corresponding to the A and B subunits. The B subunits of both toxins migrated to the same position. But subunit A of LTp(Gly-146) migrated slightly faster than that of LTp.

Immunodiffusion experiments to examine the immunological character of purified LTp(Gly-146) were performed in 1% Noble agar (Difco) in TEAN buffer (22) (Fig. 5). The antiserum to subunit A of LTp(αLTp-A) reacted with LTp(Gly-146) and formed a precipitin line that showed identity with that of LTp. Fusion formation was also observed between LTp-αLTp-B and LTp(Gly-146)–αTLp-B. These data suggest that LTp(Gly-146) is immunologically indistinguishable from LTp.

We examined the biological activities of purified LTp and LTp(Gly-146) in the vascular permeability test. The dose-response curve of LTp(Gly-146) in this test did not differ so much from that of LTp (Fig. 6). The concentrations of LTp(Gly-146) and LTp that gave a blueing score of 25 were 0.51 and 0.33 ng/ml, respectively. This result indicates that Arg-146 is not absolutely required for expression of the toxic activity of LTp.

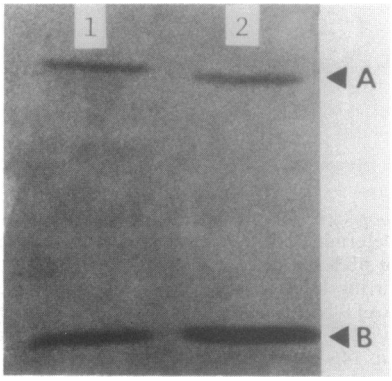

FIG. 4. Different mobilities of the A fragments of LTp and LTp(Gly-146) on SDS-polyacrylamide gel slab electrophoresis. About 2 μg of purified toxin was heated in the presence of SDS at 100°C for 2 min and applied to an SDS-polyacrylamide slab gel. The positions of subunits A and B are indicated. Lanes: 1, LTp; 2, LTp(Gly-146).

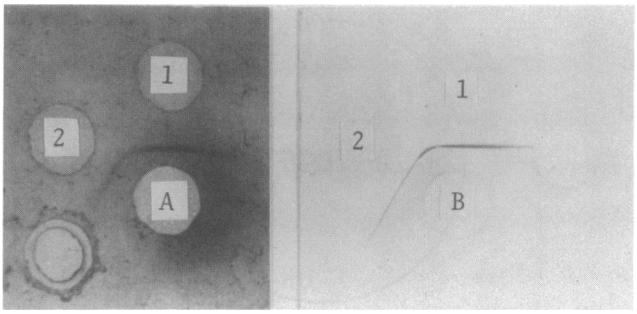

FIG. 5. Reactions of purified LTp and LTp(Gly-146) with antisera to subunit A or B of LTp. Antisera were prepared as described in Materials and Methods. Reactions: 1, LTp(Gly-146); 2, LTp; A, antiserum to subunit A; B, antiserum to subunit B.

DISCUSSION

The A subunits of CT and LT carry ADP-ribosyltransferase activity and catalyze the ADP-ribosylation of guanine nucleotide regulatory protein and, at least in vitro, of a variety of proteins, such as histone H1, protamine, and cytoskeletal proteins (24). The Arg-146 of polypeptide A1 has been proposed to be involved in the active site of ADP-ribosylation on the basis of the in vitro observation that the arginine is self-ADP-ribosylated (14, 16). However, it has not been demonstrated that formation of an intermediate, ADP-ribosylated A1 polypeptide is an indispensable step for the function of polypeptide A1. To understand the exact role of the amino acid residue, we substituted Arg-146 of LTp-A

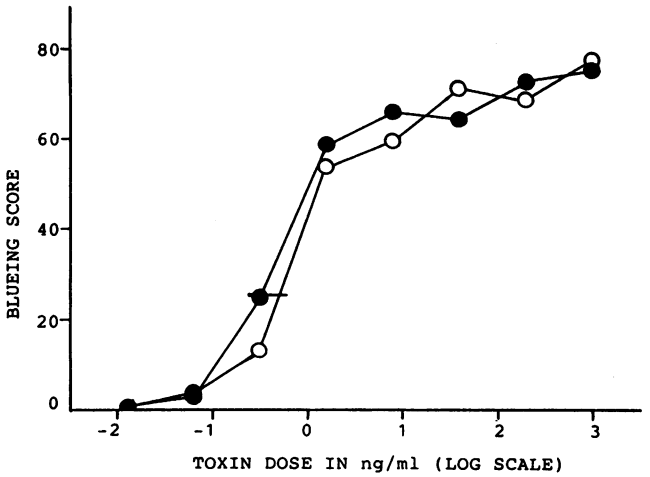

FIG. 6. Vascular permeability of purified LTp and LTp(Gly-146). Purified toxin was treated with trypsin and diluted serially with borate-buffered saline. The dilutions were injected intradermally in quadruplicate into each of four rabbits which had been clipped. After 24 h, the rabbit was injected with blue dye. The blueing scores for each sample were calculated by multiplying mean blueing diameter by mean blueing intensity as described in Materials and Methods. Each value represents the mean of blueing scores obtained from four rabbits. Symbols: ●, LTp; ○, LTp(Gly-146).

with Gly and examined the biological and immunological properties of the mutant LTp. The substitution brought about an alteration in subunit A as exhibited by a different mobility on SDS-polyacrylamide gel electrophoresis (Fig. 4). However, the biological activity of the mutant LTp, assessed by the vascular permeability test, was much the same as that of the parent LTp (Fig. 6). The result shows that self-ADP-ribosylation of Arg-146 is not absolutely required for expression of the activity of LTp.

Secondary-structure analysis of LT has revealed that position 146 is located in a region of predicted random coil structure of high hydrophilicity (27). It is known that hydrophilic regions are usually located in or immediately adjacent to a protein antigenic determinant (11), but the epitopes of subunit A of LTp have not been defined. It was assumed that the extreme antigenic alterations were not induced by substitution of Arg-146 on the basis of the results shown in Fig. 5. Further studies with mutant LTp and monoclonal antibodies will help our understanding of the epitopes of subunit A of LT.

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