

Vaccination against Gonadotropin-releasing Hormone (GnRH) Using Toxin Receptor-binding Domain-conjugated GnRH Repeats¹

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Abstract

A method for the preparation of an immunogen containing multiple copies of a self-peptide in linear alignment was designed in order to overcome the difficulty of inducing an immune response to poorly immunogenic peptide antigens. DNA fragments encoding multiple repeats of the self-peptide were generated by a new technique, termed template-repeated polymerase chain reaction (TR-PCR), which could be subcloned into an expression vector for production of peptide repeats as an immunogen. This approach was tested by constructing fusion proteins containing the receptor-binding domain of *Pseudomonas* exotoxin A and multiple copies of the 10-residue sequence of the peptide hormone gonadotropin-releasing hormone (GnRH). Immunization of female rabbits with the immunogen that contained the exotoxin receptor-binding domain and 12 copies of GnRH (PEIa-GnRH₁₂) resulted in the generation of high-titer antibodies specific for GnRH. Although at equal molar basis of the GnRH moiety, the immunogen that contained single copy of GnRH (PEIa-GnRH₁) induced low-titer anti-GnRH antibodies. These observations suggest that the presence of multiple peptide repeats is a key factor in eliciting an immune response. In addition, anti-GnRH antibodies effectively neutralized GnRH activity *in vivo*, as demonstrated by the degeneration of the ovaries in the injected rabbits. Because anti-GnRH antibody could be functionally analogous to GnRH antagonist, which has been used to treat patients with ovarian cancer, vaccination of PEIa-GnRH₁₂ presents a potential therapeutic application for the treatment of GnRH-sensitive ovarian cancer.

Introduction

The induction of an immune response against specific self-peptides is potentially beneficial for the treatment of certain diseases. The results of such peptide-based immunization in animals, however, have not been satisfactory because of the low immunogenicity of self-peptides, a low efficiency of chemical conjugation, and the heterogeneous nature of antigen preparations. To address these problems, we have investigated the use of peptide repeats conjugated to the receptor-binding domain of *Pseudomonas* exotoxin A to induce antibodies that might suppress the function of overproduced self-proteins. Application of cell-binding toxin subunit as an adjuvant and antigen vector has been reported (1). In this study, the receptor binding domain of *Pseudomonas* exotoxin A was chosen because of its ability to deliver antigens into antigen-presenting cells through receptor-mediated endocytosis, which is more efficient than phagocytosis. As a target antigen for the development of a therapeutic vaccine, we chose GnRH.³

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³ The abbreviations used are: GnRH, gonadotropin-releasing hormone; TR-PCR, template-repeated PCR; Pfu, *Pyrococcus furiosus*; GST, glutathione S-transferase.

GnRH is an endogenous hormone that contains 10 amino acid residues (2). If an antigen construct containing linear repeats of GnRH is able to induce an autoimmune response, it might be expected that any linear self-peptide repeat would also prove immunogenic. Furthermore, GnRH functions as a key hormone in the regulation of the pituitary-gonadal axis and also likely affects various other tissues in addition to the pituitary (2–4). The expression of GnRH or its receptors is increased in several human malignancies, including cancer of the breast, ovary, endometrium, and prostate (4–6). The induction of an immune response to GnRH might thus prove beneficial in the treatment of such GnRH-associated diseases.

Materials and Methods

Materials. Heat stable *Pfu* DNA polymerase, GnRH, angiotensin I and pGEM-T Easy vector system were purchased from Promega. Ex. *Taq* polymerase was obtained from Takara Co. His-Bind Resin was from Novagen. Bacteria strains, *E. coli* XL10-Glod and BL21(DE3)lysS, used for DNA cloning and protein production were from Stratagene. Female New Zealand White rabbits were acquired from the animal center at National Taiwan University. [³H]GnRH was purchased from NEN. Reagents for ELISA and immunoblotting were from GIBCOBRL. Most other chemicals, unless specified, were from Sigma Chemical Co.

Construction of DNA Fragments Encoding GnRH Repeats and Immunogen-containing Toxin Receptor-binding Domain-conjugated GnRH Repeats. Ten pM of GnRH coded single strain DNA sequences Oligonucleotide A and Oligonucleotide B (as a half complement to Oligonucleotide A) were used as both primers and templates in the TR-PCR. The thermal cycle is 30 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 30 s, and polymerization at 72°C for 30 s, followed by a final polymerization step at 72°C for 10 min. The products of TR-PCR at 100-fold dilution were subjected to adapter-PCR with the adapter primers as shown in Fig. 1c. The amplification protocol was identical to that for TR-PCR, with the exception that the denaturation step was performed for 1 min. The products of adapter-PCR, which contained a *SacII* site at the 5' end, and *EcoRI* site at the 3' end, and a stop codon at the end of the coding region, were then subcloned into a T Easy vector. Various repeats of GnRH clone were subcloned into plasmid pPEDI at the 3' end of the coding region for domain Ia, the receptor-binding domain, of *Pseudomonas* exotoxin A. The resulting plasmid, pPEDI_{Gn} (n for 1, 9, 12) was introduced into *E. coli* BL21(DE3) lysS, and the expressed fusion protein (PEIa-GnRH_n) was purified with affinity chromatography on a nickel-agarose column as described in Fig. 2b.

Animal Experiments. Sixteen-week-old female New Zealand White rabbits were injected subcutaneously with 0.1 to 1.0 mg of various antigens three times at biweekly intervals. Sera were collected weekly for immunoassay. Animals injected for three consecutive weeks were sacrificed at 26 weeks after the last injection. The ovaries were individually weighed for statistical analysis.

Immunocompetitive Assay. A trace amount of [³H]-GnRH (1 μCi, specific activity 54 Ci/mmol), 50 μl of protein A Sepharose-CL4B (0.05 mg protein A/ml), 7 μl antisera and various amounts of competitor (GnRH or angiotensin I) in PBS in a final volume of 0.5 ml were incubated at 37°C for 2 h, followed by centrifugation to remove unbound [³H]-GnRH. The pellets were washed three times with PBS prior to scintillation counting. [³H]-GnRH

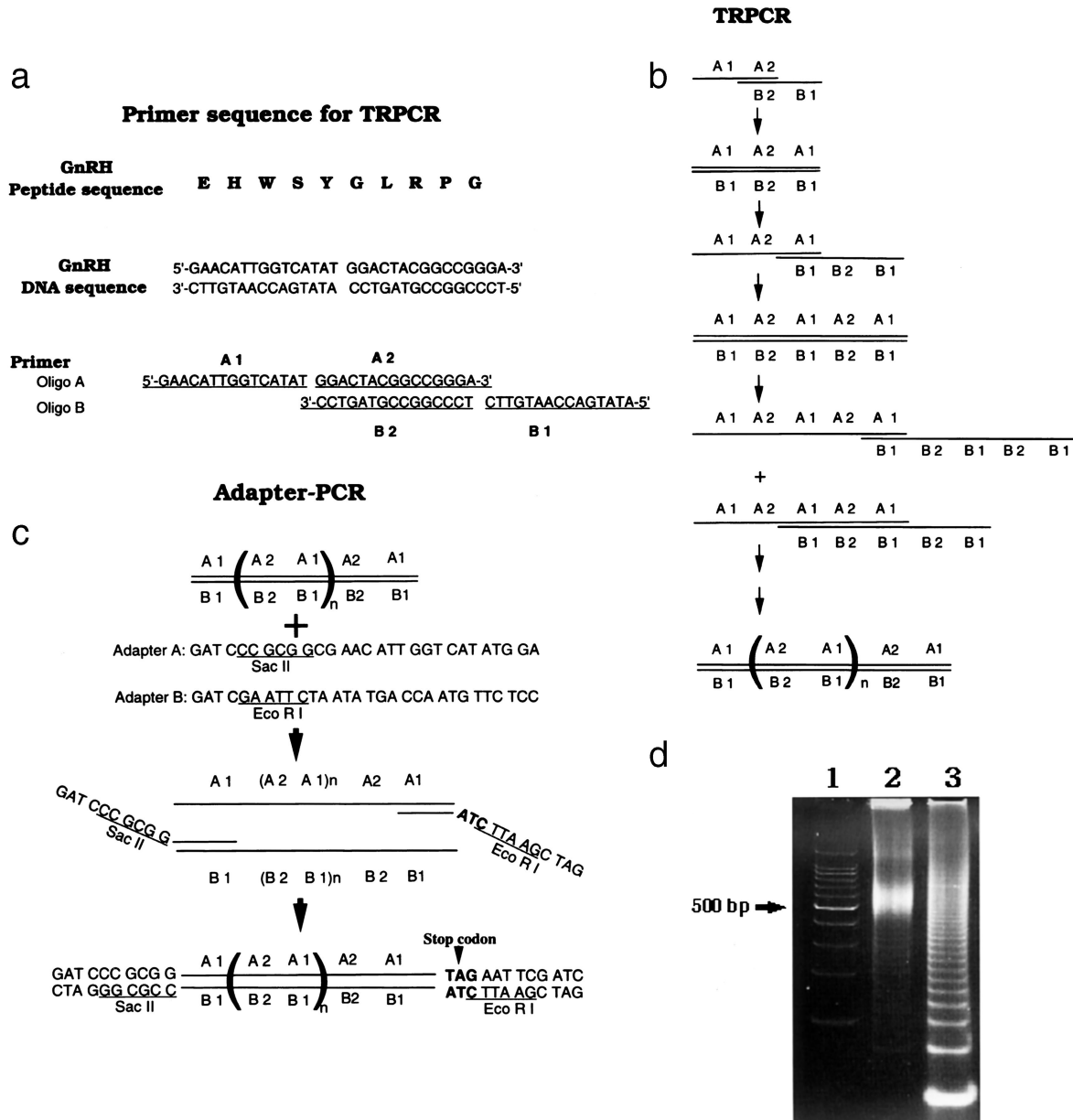


Fig. 1. The strategy for TR-PCR. *a*, oligonucleotide primers for TR-PCR. Oligo A encodes the target antigen (GnRH) and oligo B is complementary to oligo A in the manner indicated. Thus, the 5' half (A1) of oligo A is complementary to the 5' half (B1) of oligo B, and the 3' half of oligo A (A2) is complementary to the 3' half (B2) of oligo B. *b*, TR-PCR. Oligo A and oligo B were used as both primers and templates for TR-PCR, the protocol for which comprised 30 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 30 s, and polymerization at 72°C for 30 s, followed by a final polymerization step at 72°C for 10 min. *c*, adapter-PCR. The products of TR-PCR (1:100 dilution) were subjected to adapter-PCR with the adapter primers A and B. The amplification protocol was identical to that for TR-PCR, with the exception that the denaturation step was performed for 1 min. The products of adapter-PCR, which contained a *Sac*II site at the 5' end, an *Eco*RI site at the 3' end, and a stop codon at the end of the coding region, were then subcloned into a T vector. *d*, analysis of the products of TR-PCR and adapter-PCR. The products of TR-PCR (Lane 2) and adapter-PCR (Lane 3) were analyzed by PAGE on an 8% gel and ethidium bromide staining. Lane 1, DNA size markers.

bound to antisera in the absence of competitor was used as a control, representing 100% of the [³H]-GnRH binding activity.

Results

To synthesize toxin-conjugated GnRH repeats, we developed a new technique termed TR-PCR (Fig. 1). We designed two oligonucleotides, oligo A and B. Oligo A encodes the target antigen (rabbit GnRH, which is identical in sequence to the human hormone), and oligo B exhibits overlapping complementarity to oligo A as shown in Fig. 1*a*. Because the TR-PCR products also serve as DNA templates for the generation of linear GnRH repeat sequences (Fig. 1*b*), we chose *Pfu* DNA polymerase instead of *Taq* polymerase to catalyze the

reaction in order to prevent insertion or misincorporation of bases during amplification (7). To incorporate restriction sites for subcloning at both ends of the TR-PCR products (*Sac*II at the 5' end and *Eco*RI at the 3' end) as well as a stop codon at the 3' end of the coding region, we performed a second PCR (adapter-PCR) with two adapter primers (Fig. 1*c*). The products of TR-PCR and adapter-PCR were examined by PAGE. Most of the TR-PCR products were between 500 and 700 bp in size, whereas the size distribution of the products of adapter-PCR showed a ladder-like pattern (Fig. 1*d*). The prominent lower band in this ladder corresponds to the GnRH DNA dimer, with the higher bands corresponding to the trimer, tetramer, and so forth; as the size of the products increased, the intensity decreased gradually.

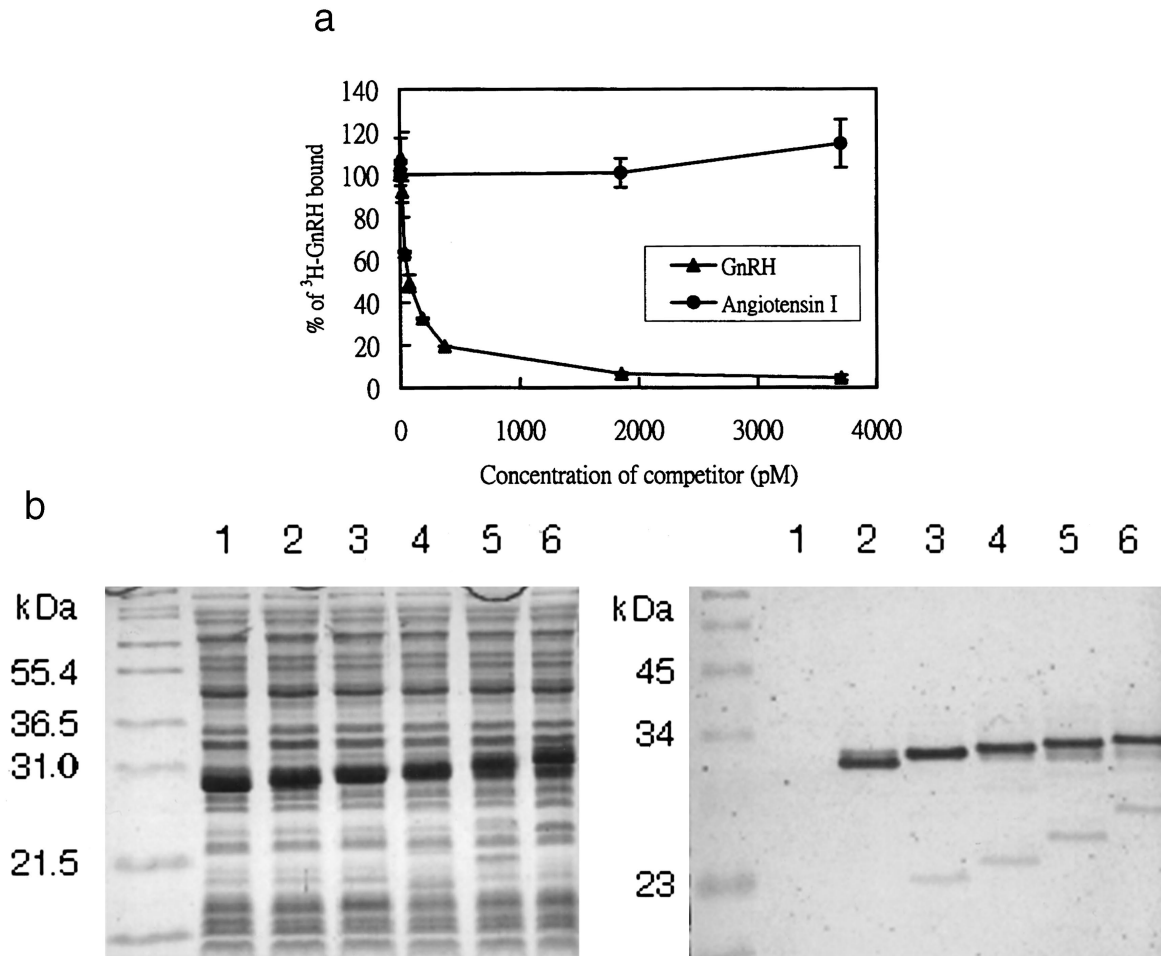


Fig. 2. Characterization of the antisera obtained from rabbits immunized with PEIa-GnRH₁₂. *a*, specificity analysis of the antisera. To examine whether the antibodies induced by PEIa-GnRH₁₂ reacted with native GnRH, an immunocompetitive assay was performed. A trace amount of [³H]GnRH (1 μ Ci; specific activity, 54 Ci/mmol), 50 μ l of protein A Sepharose-CL4B (0.05 mg of protein A/ml), 7 μ l of antisera, and various amounts of competitor (GnRH or angiotensin I) in PBS in a final volume of 0.5 ml were incubated at 37°C for 2 h, followed by centrifugation to remove unbound [³H]GnRH. The pellets were washed three times with PBS before scintillation counting. [³H]GnRH bound to antisera in the absence of competitor was used as a control, representing 100% of the [³H]GnRH binding activity. *b*, Epitope analysis of anti-GnRH antibodies. *Left*, test antigens for epitope analyses were prepared by subcloning DNA fragments encoding one to five copies of GnRH into plasmid pGEXKXG (16), and introducing the resulting constructs into *E. coli* BL21(DE3)lysS. The transformants were cultured at 37°C in Luria Bertoni medium containing ampicillin (50 μ g/ml), chloramphenicol (25 μ g/ml), and tetracycline (10 μ g/ml). When the absorbance at 600 nm of the culture reached 0.2, isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the cells were cultured for an additional 90 min. Cells (20×10^6 per lane) were dissolved in Laemmli sample buffer, heated at 90°C for 5 min, and then analyzed by SDS-PAGE on a 15% gel and Coomassie Blue staining. *Lane 1*, GST; *Lane 2*, GST-GnRH; *Lane 3*, GST-GnRH2; *Lane 4*, GST-GnRH3; *Lane 5*, GST-GnRH4; and *Lane 6*, GST-GnRH5. The left-most lane contains molecular size standards. (*kDa*, molecular weight in thousands.) *Right*, serum collected from rabbits immunized with PEIa-GnRH₁₂ was analyzed for the epitopes of anti-GnRH antibodies. Bacterial cells (1×10^6 per lane) expressing the various test antigens were fractionated by SDS-PAGE, and the separated proteins were transferred to a polyvinylidene difluoride filter (Millipore) and subjected to immunoblot analysis with the rabbit antibodies, followed by incubation with antirabbit antibody conjugated with alkaline phosphatases, and then stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Lanes are as in the left panel.

The adapter-PCR products were then subcloned into a T vector. We screened 24 positive clones and found that they contained from 3 to 12 GnRH DNA repeats. One clone containing 12 such repeats was chosen for further study.

The DNA fragment encoding the 12 repeats of GnRH was subcloned into plasmid pPEDI at the 3' end of the coding region for domain Ia, the receptor-binding domain, of *Pseudomonas* exotoxin A (8, 9). The resulting plasmid, pPEDIG₁₂, was introduced into *Escherichia coli* BL21(DE3)lysS, and the expressed fusion protein (PEIa-GnRH₁₂) was purified by taking advantage of the six-histidine (His6) tag at its NH₂ terminus (10). Affinity chromatography on a nickel-agarose column yielded a preparation of PEIa-GnRH₁₂ that was about 95% homogeneous (data not shown).

Sixteen-week-old female New Zealand White rabbits were injected three times with purified PEIa-GnRH₁₂, after which serum was individually collected for analysis. To demonstrate that the antibodies induced by PEIa-GnRH₁₂ actually reacted with the native peptide,

GnRH, reactivity of the antibody to native GnRH was measured by immunocompetitive assay using native GnRH as the competitor. As shown in Fig. 2*a*, GnRH could effectively block [³H]GnRH binding to anti-GnRH antibodies. GnRH, at equal molar concentration and 350-fold of [³H]GnRH, inhibited [³H]GnRH binding by 20 and 80%, respectively, whereas angiotensin I failed to compete for [³H]GnRH binding to anti-GnRH antibodies even at the concentration 3,700-fold to [³H]GnRH. To investigate whether the epitopes recognized by induced antibodies were localized within the GnRH sequence or spanned adjacent GnRH repeats, we examined the reactivity of the antibodies with GST fusion proteins that contained various numbers of GnRH repeats. Immunoblot analysis revealed that, although the antibodies did not recognize GST alone, they reacted (at a dilution of 1:5000) with a similar affinity to GST fusion proteins containing one to five copies of the GnRH sequence (Fig. 2*b*). In this study, we also observed that some extra bands appeared in low-molecular range. We believe that these extra bands represent the degraded products of the

fusion protein of GST and GnRH repeats. This result, thus, suggests that most of the epitopes recognized by the antibodies reside within the GnRH sequence rather than between adjacent GnRH repeats. Enzyme-linked immunosorbent assays also revealed that, at a dilution of 1:10,000, the antibodies reacted with a similar affinity to GST fusion proteins containing one or five copies of the GnRH sequence (data not shown). Thus, these observations suggest that antigens comprising linear peptide repeats can be used to generate monoclonal-like antibodies, and that these antibodies can be used for biochemical analysis of the target peptide.

To address the issue of whether multicopies or single-copy of GnRH is sufficient to induce anti-GnRH immunoresponses, we have performed animal immunization with immunogens containing various copies of GnRH. Our results showed that GnRH alone or PEIa-GnRH₁ at the dosage of 0.1 mg failed to induce an anti-GnRH immune response. However, PEIa-GnRH₉ and PEIa-GnRH₁₂ at the dosage of 0.1 mg were capable of raising high-titer anti-GnRH antibodies. PEIa-GnRH₁ at the dosage of 1 mg induced a weak anti-GnRH immune response (Fig. 3). The ELISA results of the PEIa-GnRH₁₂ group were not shown in Fig. 3, because the ELISA reading of 1,000-fold diluted anti-PEIa-GnRH₁₂ antiserum was out of scale. If the ELISA reading of the PEIa-GnRH₁₂ group were to be adjusted to the range between 1 and 2, the anti-PEIa-GnRH₁₂ antiserum needed to be diluted 10,000-fold. Under this situation, the ELISA reading of all of the other groups went down to basal levels and showed no difference from preimmune serum. This observation suggests that the copy number of GnRH in the immunogen play an important role in inducing a robust anti-GnRH immune response. In addition, the observation that PEIa-GnRH₁ at the dosage of 1.0 mg is able to induce weak immune response against GnRH, whereas GnRH at the dosage of 0.1 mg, which is in much higher molar ratio of GnRH moiety compared with 1.0 mg of PEIa-GnRH₁, fails to induce anti-GnRH immune response, clearly indicates that PEIa makes GnRH in PEIa-GnRH₁ more immunogenic. A complete study on the role of PEIa making the fusion peptides highly immunogenic is under investigation now.

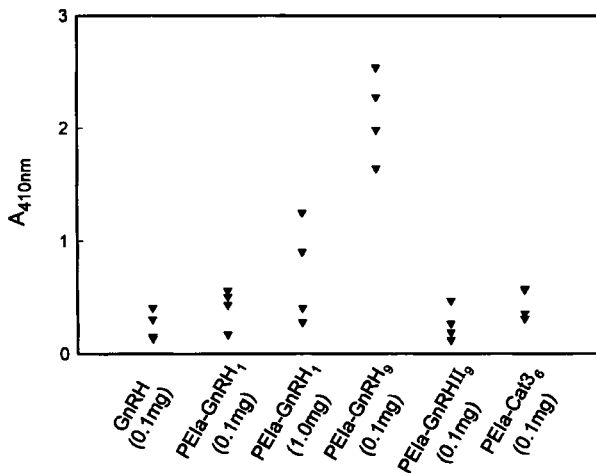


Fig. 3. Enhancement of peptide antigenicity by TR-PCR. To examine whether multicopies of peptide in linear repeat are needed to elicit peptide antigenicity, we have performed animal immunization with immunogen containing various copies of GnRH. Seven groups, four rabbits per group, of 5-month old female New Zealand White rabbits 5-month-old were injected s.c. with various dosages of immunogens three times at biweekly intervals. Serum collected from immunized rabbits (2 weeks after last immunization) was analyzed for anti-GnRH antibodies. The ELISA results were presented with 1000-fold diluted serum. The ELISA results of the PEIa-GnRH₁₂ group are not shown in this figure because the ELISA reading of 1000-fold-diluted anti-PEIa-GnRH₁₂ antiserum was out of scale. PEIa-GnRH₁₂ stands for the receptor-binding domain of *Pseudomonas* exotoxin A conjugated with nine copies of GnRH; the amino acid sequence is Gln-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly. PEIa-Cat36 stands for the receptor binding domain of *Pseudomonas* exotoxin A conjugated with six copies of the peptide, for which the amino acid sequence is Leu-Gly-Ile-Ser-Tyr-Gly-Arg-Lys-Lys-Arg.

a



b

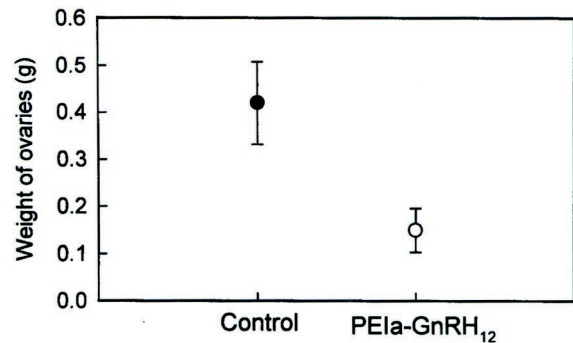


Fig. 4. Degeneration of the ovaries of rabbits immunized with PEIa-GnRH₁₂. Sixteen-week-old female New Zealand White rabbits were injected s.c. with purified PEIa-GnRH₁₂ for 3 consecutive weeks at weekly intervals. Aluminum phosphate (pH 7.0) was used as adjuvant in the injection. The injection volume was 0.5 ml and each injection contained 100 μ g PEIa-GnRH₁₂ and 0.125 mg of aluminum. a, set of control and degenerated ovaries. The ovaries of rabbits that had received three injections of either PEIa-GnRH₁₂ (left) or the control antigen PEIa-TopN₈ (right) were examined 26 weeks after the last immunization. Each experimental group contained four rabbits and all of the PEIa-GnRH₁₂ immunized rabbits showed degeneration of ovaries. The ovaries were individually weighed, statistical analysis of the control and degenerated ovaries is summarized in b.

We next investigated whether the induction of an autoimmune response to GnRH affected the ovaries of immunized rabbits. Whereas the ovaries of the four rabbits injected with a control antigen, PEIa-TopN₈ (the receptor-binding domain of *Pseudomonas* exotoxin A conjugated to eight repeats of the NH₂-terminal 10 amino acids of human DNA topoisomerase I), appeared normal, those of the four rabbits that were injected with PEIa-GnRH₁₂ exhibited substantial degeneration. One represented set of control and degenerated ovaries was shown in Fig. 4a. The ovaries were individually weighed and statistical analysis of the control and degenerated ovaries was summarized in Fig. 4b. These results indicate that the induced high-titer antibodies to GnRH appear to impair the function of this important hormone in the immunized rabbits. It has been shown that some patients with ovarian cancer have been treated with GnRH antagonist (11).

Because anti-GnRH antibody could be functionally analogous to GnRH antagonist, vaccination of PEIa-GnRH₁₂ presents a potential therapeutic application for the treatment of GnRH-sensitive ovarian cancer.

Discussion

To overcome the difficulty of inducing an immune response against poorly immunogenic self-peptides, we have designed a new and efficient delivery system for antigen presentation. In addition, we have developed a new method for increasing the copy number of the self-peptide in the antigen conjugate. We have tested our approach with GnRH as a model self-peptide. The immunogen comprised the receptor binding-domain of *Pseudomonas* exotoxin A and 12 copies of GnRH in linear alignment. This construct induced autoantibodies to GnRH that caused degeneration of the ovaries in immunized female rabbits.

In previous studies, antigens that were used to generate antibodies against small peptides have been prepared by chemical conjugation of the peptide to a carrier protein. A trial to induce antibody response to GnRH using the conjugation of GnRH to T-cell epitope has been reported (12). However, because the efficiency of such chemical conjugation varies, the antigen preparations were heterogeneous and differed from batch to batch. The immune responses to such antigen preparations were, therefore, inconsistent. With the use of our TR-PCR approach to generate a DNA template for the expression of linear peptide repeats, it is possible to synthesize large amounts of homogeneous immunogen with a high content of antigenic peptide, without the need for chemical conjugation and at lower cost. In addition to PEIa-GnRH₁₂, we have prepared several other autoantigens with the TR-PCR technique and shown that they all effectively induce an immune response (data not shown).

Our new technique may have potential applications in the development of therapeutic vaccines. Various antibodies have been shown to be effective in the treatment of cancer (13, 14). The most promising example is Herceptin, a humanized monoclonal antibody to HER2 that is now in clinical use in combination with chemotherapy (15). Vaccination with growth factor or cell surface autoantigens using our approach may offer a low-cost alternative for cancer therapy.

Acknowledgments

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Structure and Function Relationship of *Pseudomonas* Exotoxin A

AN IMMUNOCHEMICAL STUDY*

(Received for publication, June 2, 1988)

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We have raised antisera against *Pseudomonas* exotoxin A (PE) and domains Ia and III to study the structure-function relationships of PE. Anti-PE antibody (Ab^{PE}) was shown to abolish the ADP-ribosylation activity of PE. However, neither antidomain Ia antibody nor antidomain III antibody inhibited the ADP-ribosylation activity of PE. This suggests that the inhibition of ADP-ribosylation by Ab^{PE} results from the binding of Ab^{PE} to the region between domains Ia and III. Since the binding of Ab^{PE} to PE did not inhibit NAD hydrolysis in the absence of elongation factor 2, the inhibitory effect of Ab^{PE} on ADP-ribosylation may be due to steric hindrance rather than a direct action on the catalytic function. Thus, the interface between domain Ia and III may be the site of entry of elongation factor 2 during ADP-ribosylation. The antibodies were also used to study both the inhibitory effects of PE on protein synthesis and its cytotoxic activity. Either Ab^{PE} or antidomain Ia antibody, but not antidomain III antibody, was able to reverse the inhibition of protein synthesis by PE and to block its cytotoxicity. In addition, rabbits immunized with domain Ia acquired tolerance against 100 µg of PE injected subcutaneously. These results suggest that domain Ia is the cell-binding domain of PE and may be used for vaccination against PE-mediated diseases.

Pseudomonas exotoxin A (PE)¹ is the most toxic component of the cellular and extracellular fractions of *Pseudomonas aeruginosa* and has been identified to play a major role in the pathogenicity of *P. aeruginosa* infections (1-3). The toxic effect of PE results from the inhibition of protein synthesis in eukaryotic cells by ADP-ribosylation of EF-2 (4, 5). Recently, structural and functional studies of PE have yielded new insights into its mechanism of action. The three-dimensional structure of PE has been determined by x-ray crystallography (6). *Pseudomonas* exotoxin A contains three structurally distinct domains: domain I (domain Ia, residues 1-254 and domain Ib, residues 365-404); domain II (residues 253-364); and domain III (residues 405-613). Based on the three-dimensional structure and the use of recombinant DNA methods, domain Ia, and domain III with a portion of domain Ib have been identified as the cell binding and ADP-ribosylation domains, respectively (7). In this study, we have used immu-

nunochemical methods for further examine the ADP-ribosylation domain, with special focus on the interaction of PE and EF-2, and the possibility of using domain Ia for vaccination against PE-mediated disease. The rationale for this approach is that antibodies to PE would block the binding of its substrate (EF-2) or receptor. By this approach, we have found that the cell-binding domain of PE is located in domain Ia and that this domain can be used for vaccination against PE-mediated cytotoxicity. Furthermore, our results suggest that EF-2 interacts with the active site of PE through the interface between domain Ia and domain III during the ADP-ribosylation reaction.

EXPERIMENTAL PROCEDURES

Materials—*Pseudomonas* exotoxin A was obtained from ICN Biochemicals. Nicotinamide-[U-¹⁴C]adenine dinucleotide, ammonium salt ([U-¹⁴C]NAD) and [carbonyl-¹⁴C]nicotinamide adenine dinucleotide, ammonium salt ([carbonyl-¹⁴C]NAD) were purchased from Amersham Corp. The wheat germ extract enriched for elongation factor 2 was prepared as described by Legocki and Marcus (8) and Seal *et al.* (9). Reagents for gel electrophoresis were from Bio-Rad. Freund's adjuvant and medium for tissue culture were from GIBCO. All other chemicals were analytical reagent-grade.

Construction of Domains Ia and III Expression Plasmids—The domains Ia and III expression plasmids were derived from plasmid pJH4, which encodes a PE molecule with an additional methionine at the amino terminus (7). The construction of domain Ia expression plasmid (pJH14) is shown in Fig. 1A. First, pJH4 was partially cut with *Ava*I, and the linearized DNA was then completely cut with *Eco*RI. The 4.9-kilobase DNA, which is deleted for domains II, Ib, and III, was incubated with S1 to remove cohesive ends followed by ligation with T4 ligase. In this construction a stop codon, TGA, can be found right after the codon, CCT, of the 251st residue of PE. The plasmid pJH14 was then examined for its ability to express domain Ia of PE.

The construction of domain III expression plasmid (pJH7) is shown in Fig. 1B. Plasmid pJH4 was partially cut with *Aat*II. The linearized DNA was then completely cut with *Hind*III to remove DNA encoding for domains I and II. The right-sized DNA fragment, 4.7 kilobases, was incubated with S1 to remove the cohesive ends and to make DNA encoding domain III in the right reading frame. The 4.7-kilobase DNA fragment after S1 treatment was ligated and transformed into HB101. The plasmid pJH7 was then examined for its ability to express domain III of PE.

Isolation of Domains Ia and III of PE—BL21 (DE3) containing pJH7 or pJH14 were cultured in LB broth with 50 µg of ampicillin/ml at 37 °C. When absorbance at 650 nm reached 0.3, isopropyl-1-thio-β-D-galactopyranoside was added at a final concentration of 1 mM. Cells were harvested 120 min later. Domains Ia and III of PE were isolated from preparative gel electrophoresis under nonreducing conditions.

Generation of Antisera—Antisera to different domains of PE were generated in female New Zealand White rabbits. Animals were injected subcutaneously with 200 µg of purified domain Ia or III of PE, either as electroeluted protein or as a sodium dodecyl sulfate-polyacrylamide gel electrophoresis band, mixed with Freund's complete adjuvant. The animals were boosted at 10-day intervals with antigen in Freund's incomplete adjuvant. Sera were collected at 7 and 14 days after boosting. Antisera to native PE were generated by injection of

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¹ The abbreviations used are: PE, *Pseudomonas* exotoxin A; Ab^{PE}, anti-PE antibody; Ab^{Ia}, antidomain Ia antibody; EF-2, elongation factor 2.

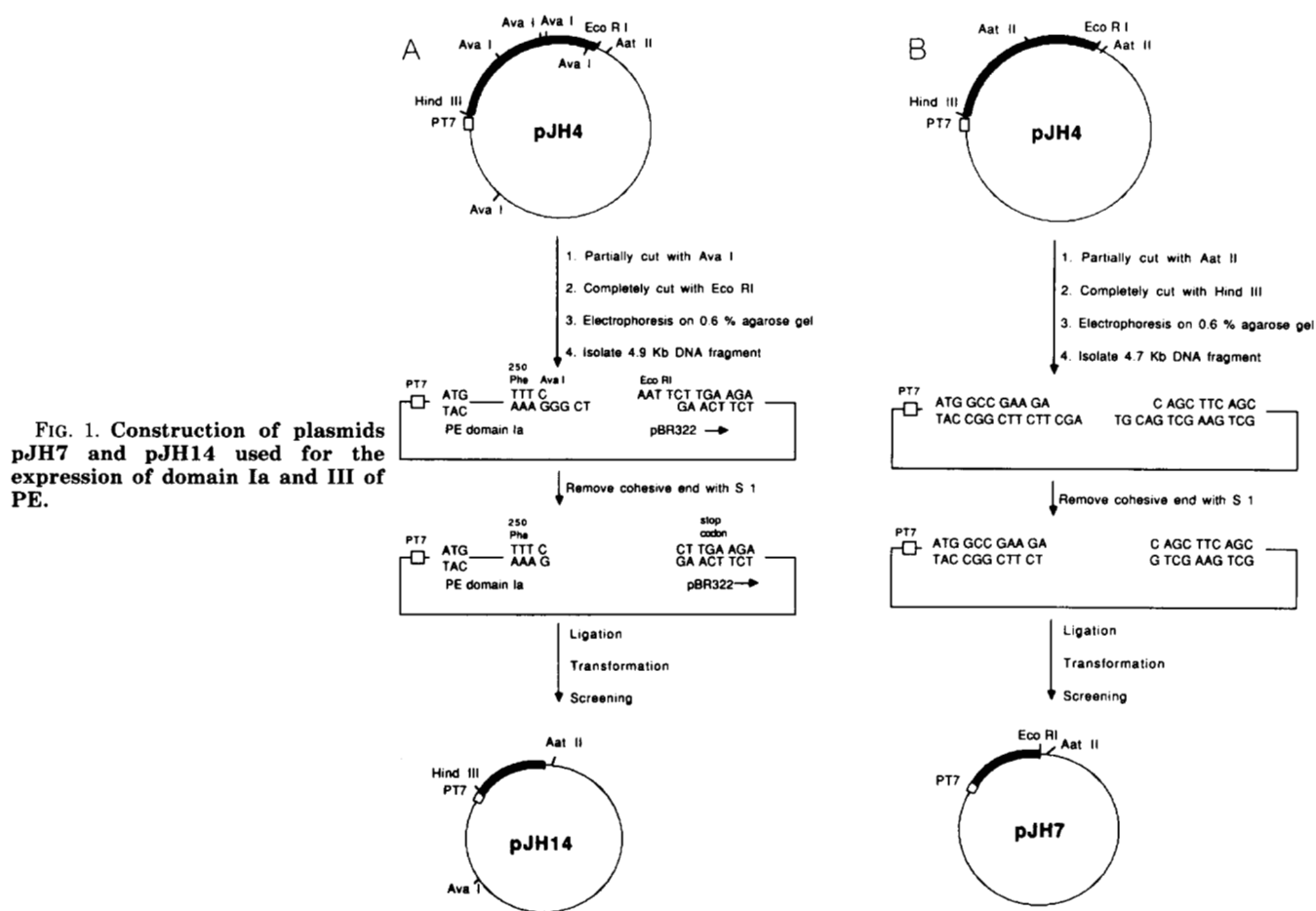


FIG. 1. Construction of plasmids pJH7 and pJH14 used for the expression of domain Ia and III of PE.

100 μ g of native PE into rabbits which had already been immunized with domain Ia of PE.

SDS-PAGE and Immunoblotting—Samples containing domain Ia, III, or PE were dissolved in Laemmli buffer and boiled for 5 min prior to application to a 0.1% sodium dodecyl sulfate, 10% acrylamide slab gel as described by Laemmli (10). After electrophoresis, the protein bands were transferred to nitrocellulose paper, followed by reaction with antisera against the fragments or full length PE. The bands were visualized by treatment with biotinylated goat anti-rabbit IgG, followed by avidin horseradish peroxidase H and stained with oxidizable peroxidase substrate, 0.02% hydrogen peroxide, and 0.1% diaminobenzidine. Procedures were carried out under conditions recommended by the supplier (Vector Laboratories).

Enzyme Assay—ADP-ribosyltransferase activity was measured according to the method described by Iglewski and Sadoff (11). Wheat germ extracts enriched for EF-2 were used as a source of EF-2. Assays (500 μ l total volume) contained about 10 pmol of EF-2, 37 pmol of [^{14} C]NAD (0.06 μ Ci), 2 μ g of PE, antisera against different domains of PE, and buffer (40 mM dithiothreitol, 1 mM EDTA, and 50 mM Tris, pH 8.1). After 30-min incubation at 37 $^{\circ}$ C, 0.5 ml of 12% trichloroacetic acid was added to each assay mixture. The assay mixtures were then placed on ice for 15 min, followed by centrifugation at 4 $^{\circ}$ C, 3000 $\times$ g for 10 min. The resulting pellet was washed with 1 ml of 6% trichloroacetic acid and centrifuged as above. The U- ^{14} C content of the pellet was measured in a liquid scintillation counter as an index of the ADP-ribosylation activity. The control group was done in the presence of normal rabbit serum.

NAD-glycohydrolase Assay—The NAD-glycohydrolase activity was measured as described by Kandel *et al.* (12). Five μ g of PE were incubated with or without 60 μ g of Ab^{PE} in a 200- μ l reaction mixture containing 50 mM Tris, pH 8.0, and 7 nmol of [carboxyl- ^{14} C]NAD (44 mCi/mmol). The reaction was carried out at 37 $^{\circ}$ C for 14 h. Two-hundred μ l of H₂O were added to the reaction mixture prior to extraction with 1 ml of water-saturated ethyl acetate. The extraction was repeated twice. The [^{14} C]nicotinamide which was extracted in ethyl acetate was measured in a scintillation counter as an index of NAD-glycohydrolase activity.

Inhibition of Protein Synthesis—Assays for the inhibition of protein synthesis by PE in the presence or absence of antisera against different domains of PE were carried out in Swiss 3T3 cell cultures. Swiss 3T3 cells were seeded 1 day before assay in 24-well tissue culture plates at a density of 10⁵ cells/well. Cells were washed once by replacing the medium with Dulbecco's modified Eagle's medium containing 0.2% bovine serum albumin before adding PE (100 ng/ml) alone or with antisera against different domains of PE. After 30 min at 37 $^{\circ}$ C, the medium was removed and replaced with fresh Dulbecco's modified Eagle's medium containing 0.2% bovine serum albumin. Four h later, [^{3} H]leucine was added to the medium at a final concentration of 3 μ Ci/ml, and cells were further incubated for 1 h to measure the rate of protein synthesis.

Cell Cytotoxicity Test—Tests of the inhibition of PE cytotoxicity by antisera were performed in Swiss 3T3 cell cultures. Swiss 3T3 cells were seeded 24 h prior to the cytotoxicity test in a 24-well tissue culture plate at a density of 2 $\times$ 10⁴ cells/well. After incubation for 72 h with 30 ng/ml of PE in the absence or presence of various concentrations of antisera against different domains of PE, the monolayers were stained with Methylene Blue to detect the surviving cells.

RESULTS

Generation and Characterization of Anti-PE, Antidomain Ia, and Antidomain III Antibodies—Domains Ia and III of PE were expressed in BL21 (DE₃) as described by Hwang *et al.* (7). Both domains Ia and III were isolated in large quantities by preparative electrophoresis under nonreducing conditions, followed by electroelution. As shown in Fig. 2A, domain Ia and domain III were purified to about 95% homogeneity. These materials and native PE were injected into rabbits to induce antibodies. The antibodies raised against full length PE, domain Ia, and domain III, respectively, were then examined for their antigen specificity. In Fig. 2, B–D, the results of immunoblotting of anti-PE (Ab^{PE}), antidomain III (Ab^{III}),

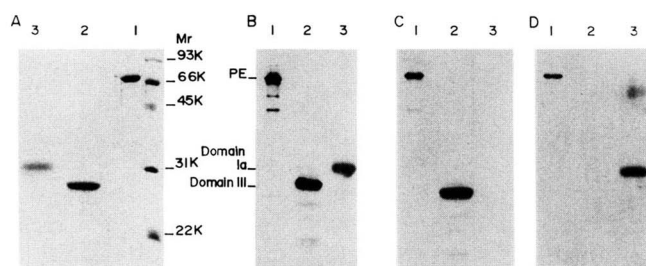


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of intact PE, domains Ia and III (panel A) and immunoblotting of intact PE, domains Ia and III by anti-PE antibody (panel B), antidomain III antibody (panel C) and antidomain Ia antibody (panel D). Lane 1, intact PE; lane 2, domain III of PE; lane 3, domain Ia of PE.

and antidomain Ia (Ab^{Ia}) antibodies, respectively, to native PE, domain III, and domain Ia are shown. As expected, all three antibodies recognized native PE. In addition, anti-PE antibody also recognized domain III and domain Ia of PE (Fig. 2B). However, antidomain III antibody did not cross-react with domain Ia (Fig. 2C). Neither did antidomain Ia antibody cross-react with domain III (Fig. 2D). These results demonstrated the antigen specificity of anti-PE, antidomain III, and antidomain Ia antibodies. Using intact PE, domains Ia and III as antigen, we have also titrated Ab^{PE} , Ab^{Ia} , and Ab^{III} by an enzyme-linked immunosorbent assay. The titer of antibody was defined as the reciprocal of the maximum dilution to give the half-maximum OD reading (0.5). When the ligand was bovine serum albumin, we failed to score all three antibodies in comparing with preimmune serum. However, when the ligand was PE, the titers of Ab^{PE} , Ab^{Ia} , and Ab^{III} were 16,000, 8,000 and 8,000, respectively. When the ligand was domain Ia, the titers of Ab^{PE} , Ab^{Ia} , and Ab^{III} were 12,000, 16,000, and <100, respectively. When the ligand was domain III, the titers of Ab^{PE} , Ab^{Ia} , and Ab^{III} were 16,000, <100, and 8,000, respectively. This result further attests to the antigen specificity of these antibodies. Although Ab^{Ia} has preferential affinity toward domain Ia, this may result from the fact that the antigen used to generate Ab^{Ia} contained some improperly folded domain Ia.

Immunochemical Studies on the ADP-ribosylation Domain—To examine the ADP-ribosylation domain, native PE was preincubated with various amounts of Ab^{PE} , Ab^{Ia} , and Ab^{III} . The ADP-ribosylation activity of PE was then examined. Ab^{PE} inhibited 54 and 90% of ADP-ribosylation activity of native PE at 10 and 20 $\mu\text{g}/\text{ml}$ of Ab^{PE} , respectively. At higher concentrations no further inhibition was observed. However, both Ab^{Ia} and Ab^{III} at all the concentrations used did not inhibit the ADP-ribosylation activity of native PE (Fig. 3). Since domain III and a portion of domain Ib were required for ADP-ribosylation activity (5), it was not unexpected that Ab^{Ia} does not inhibit the ADP-ribosylation activity of native PE. The inability of Ab^{III} to inhibit ADP-ribosylation activity was unexpected but could have been due to the inaccessibility of the ADP-ribosylational domain to the antibody. Thus, the area between domains Ia and III, which was accessible to Ab^{PE} but not to Ab^{Ia} and Ab^{III} , is important in ADP-ribosylation. This area may be involved either in the interaction with EF-2 or in the catalytic function. Since PE catalyzes the slow hydrolysis of the nicotinamide-ribose linkage of NAD in the absence of EF-2 (13), this may imply that the catalytic center for glycohydrolysis of NAD in PE is the same as that for the transfer of ADP-ribose to EF-2 in PE. Thus, the activity of NAD-glycohydrolyase may represent the catalytic function of PE. To examine whether the effect of

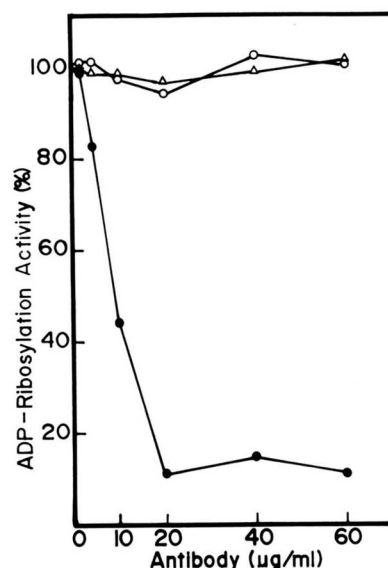


FIG. 3. Effect of Ab^{PE} , Ab^{Ia} and Ab^{III} on the ADP-ribosylation activity of native PE. Two μg of native PE was preincubated with various concentrations of Ab^{PE} , Ab^{Ia} , and Ab^{III} from 2 to 60 $\mu\text{g}/\text{ml}$. The ADP-ribosylation activity was then assayed as described under "Experimental Procedures." ●, Ab^{PE} ; △, Ab^{Ia} ; and ○, Ab^{III} .

TABLE I
PE-catalyzed hydrolysis of NAD

Toxin sample	NAD hydrolysis	
	−Dithiothreitol	+Dithiothreitol
	pmol/h	
5 μg PE	1.1	3.1
5 μg PE + 60 μg Ab^{PE}	2.6	5.1

Ab^{PE} on ADP-ribosylation of PE is due to the inhibition of catalytic function, the NAD-glycohydrolyase activity of PE was measured in the presence and absence of Ab^{PE} . As shown in Table I, Ab^{PE} did not inhibit the hydrolysis of NAD in the absence of EF-2. This result thus rules out the possibility that Ab^{PE} inhibits the catalytic function of PE. The inhibition of ADP-ribosylation of PE by Ab^{PE} is probably due to that binding of Ab^{PE} in the area between domains Ia and III which sterically hinders access of EF-2 to the catalytic site. Thus, the interface between domain Ia and III may be the entering gate for EF-2 during ADP-ribosylation. To further support this notion, the effects of Ab^{PE} and Ab^{III} were examined on PE in which domain Ia was deleted (PE(-Ia)). It was hoped that the junction of domain Ib and domain III, originally buried in native PE, would become exposed in PE(-Ia), and therefore, become accessible to Ab^{III} . If the interface of domain Ia and domain III is important for the entry of EF-2 into the active site of PE, Ab^{III} should inhibit the ADP-ribosylation activity of PE(-Ia). As shown in Fig. 4, Ab^{PE} inhibits 90% of the ADP-ribosylation activity of PE(-Ia) at a concentration of 2 $\mu\text{g}/\text{ml}$, while Ab^{Ia} does not inhibit the ADP-ribosylation activity of PE(-Ia) at all the concentrations used. Although Ab^{III} was able to inhibit the ADP-ribosylation activity, it was less effective than Ab^{PE} . It inhibited 42 and 85% of the ADP-ribosylation activity of PE(-Ia) at concentrations of 20 and 40 $\mu\text{g}/\text{ml}$, respectively. The difference in the inhibitory activities of Ab^{PE} and Ab^{III} may be due to the ability of Ab^{PE} to bind to domains II, Ib, and III, while Ab^{III} can only bind to domain III of PE(-Ia). In addition, the titer for Ab^{PE} was about 2-fold higher than Ab^{III} . This results in the higher efficiency of Ab^{PE} in blocking the interaction of PE(-Ia) with EF-2.

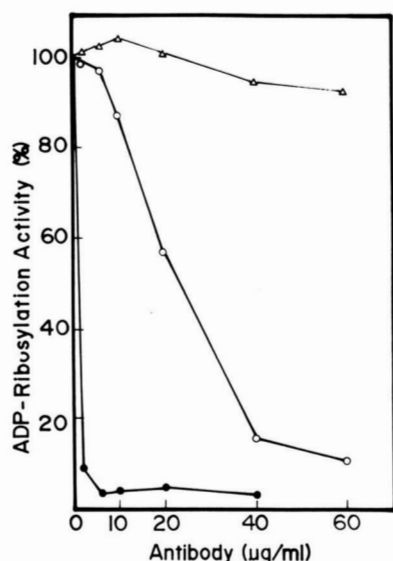


FIG. 4. Effect of Ab^{PE} and Ab^{III} on the ADP-ribosylation activity of PE with deletion of domain Ia. Half μ g of PE(-Ia) was incubated with various concentrations of Ab^{PE} and Ab^{III} at 37 °C, for 15 min. The ADP-ribosylation activity was then assayed as described under "Experimental Procedures." ●, Ab^{PE} ; △, Ab^{Ia} ; and ○, Ab^{III} .

TABLE II

Effect of anti-domain Ia, anti-domain III, and anti-PE antisera on reversing inhibition of cell protein synthesis by native PE

100 ng of PE and 100 μ l of antisera equivalent to 100 μ g of IgG were mixed prior to add to Swiss 3T3 cells. After 30 min of incubation at 37 °C, cells were washed and incubated 4 h in Dulbecco's modified Eagle's medium + 0.2% bovine serum albumin, then incubated with [^{3} H]leucine for 1 h. All these assays were duplicated.

Antisera tested	[^{3} H]leucine incorporation cpm
Control (no PE, no serum added)	1557 $\pm$ 70
100 ng PE	605 $\pm$ 70
100 ng PE + 100 μ l of normal rabbit serum	617 $\pm$ 41
100 ng PE + 100 μ l anti-domain Ia antisera	1568 $\pm$ 46
100 ng PE + 100 μ l anti-domain III antisera	528 $\pm$ 46
100 ng PE + 100 μ l anti-PE antisera	1515 $\pm$ 34
100 ng PE + 10 μ g anti-PE IgG	1398 $\pm$ 46

Immunochemical Studies on the Cell-binding Domain—It is well established that PE binds to cells as the first step in cell killing, although the cellular receptor for PE has not yet been identified (14–16). Since PE binding, followed by translocation, and ADP-ribosylation of EF-2 results in inhibition of protein synthesis and cell death, antibodies which recognize the cell binding domain should prevent PE binding and, therefore, prevent the toxic effects of PE. To examine the cell binding domain, the inhibition of protein synthesis by PE (100 ng/ml) alone or with antibodies against different domains of PE was carried out in Swiss 3T3 cells. As shown in Table II, both Ab^{PE} and Ab^{Ia} are able to reverse the inhibition of cell protein synthesis by native PE. Neither normal rabbit serum nor Ab^{III} prevented PE from inhibiting protein synthesis. In addition, the ability of Ab^{PE} , Ab^{Ia} , and Ab^{III} to block PE cytotoxicity was also examined. As expected, neither normal rabbit IgG nor Ab^{III} neutralized PE cytotoxicity. However, both Ab^{PE} and Ab^{Ia} were able to block PE cytotoxicity (Fig. 5). Since Ab^{Ia} did not affect the ADP-ribosylation activ-

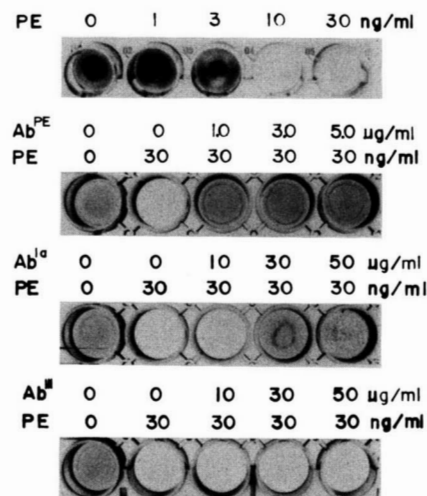


FIG. 5. Effect of Ab^{PE} , Ab^{Ia} , and Ab^{III} on the blockade of the cell-killing activity of PE. Swiss 3T3 cells were seeded in a 24-well plate at a density of 2×10^4 cells/well. Thirty ng/ml of PE without or with various concentrations of Ab^{PE} , Ab^{Ia} , and Ab^{III} was added to Swiss 3T3 cells. Three days later, the monolayers were stained with methylene blue to detect the surviving cells.

TABLE III

Vaccination against PE

Rabbits were injected subcutaneously with phosphate-buffered saline (PBS) or 100 μ g of purified domain Ia or III of PE, mixed with Freund's complete adjuvant. The rabbits were boosted at 10-day intervals with PBS or domain Ia or III in Freund's incomplete adjuvant. After rabbits generated antibodies against the injected antigens, rabbits were subcutaneously injected with 100 μ g of PE contained in 1.0 ml of sterile PBS. The animals were monitored daily for at least 2 weeks. All deaths occurred within 72 h.

Antigen injected	Ab generated before PE injection	Ab generated after PE injection	Deaths
PBS			3/3
Domain Ia of PE	Ab^{Ia}	Ab^{PE}	0/2
Domain III of PE	Ab^{III}		1/1

ity of PE, the reversal of both PE inhibition of cellular protein synthesis and its cytotoxic effects is most likely due to the inhibition of PE binding to the cell. These results clearly indicate that the cell binding domain is located in domain Ia of PE.

Vaccination against PE—To examine whether domain Ia of PE can be used for vaccination against PE-mediated disease, we have immunized rabbits with domains Ia or III, or injected animals with PBS, followed by examining their tolerance to PE. As shown in Table III, rabbits injected with phosphate-buffered saline or immunized with domain III died within 72 h after receiving 100 μ g of PE. However, rabbits immunized with domain Ia of PE acquired tolerance to 100 μ g of PE injected subcutaneously, and these rabbits generated antibodies against intact PE 2 weeks later. These data suggest that domain Ia of PE can be used for vaccination against PE-mediated disease.

DISCUSSION

Pseudomonas exotoxin A is the most potent virulence factor produced by *P. aeruginosa* (17, 18). PE is believed to bind to a specific receptor on the cell surface and then enters the cell. It is then translocated to the cytosol where it enzymatically inhibits protein synthesis by ADP-ribosylation of EF-2 (19,

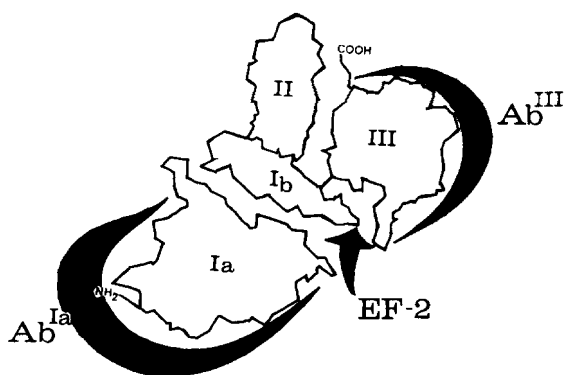


FIG. 6. Model for the interaction of pseudomonas exotoxin A and elongation factor 2 during the ADP-ribosylation reaction.

20). Thus, based on this proposed mechanism of action, PE should contain at least three functional domains. These are responsible for binding to cells, translocation across the endosome membrane, and ADP-ribosylation activity. Previous studies using recombinant DNA methods have shown that structural domain Ia is required for cell recognition, structural domain II for translocation of toxin across an intracellular membrane, and structural domain III with a portion of domain Ib for ADP-ribosylation (7). In this paper, we employed immunochemical methods to study further the binding and the ADP-ribosylation domains of PE.

Interaction of EF-2 to PE—We have examined the effect of Ab^{PE}, Ab^{Ia}, and Ab^{III} on the ADP-ribosylation activity of intact and modified PE. It has been shown that neither Ab^{Ia} nor Ab^{III} can block the ADP-ribosylation activity of native PE. Since this experiment was done by incubation of native PE with antibody first, followed by examination of the ADP-ribosylation activity of the PE-antibody complex in the presence of dithiothreitol, it may be argued that dithiothreitol reduced disulfide bonds resulting in the dissociation of PE and antibody. Therefore, we would not see the inhibition of ADP-ribosylation activity of native PE by Ab^{Ia} and Ab^{III}. However, under similar experimental conditions Ab^{PE} effectively inhibited the ADP-ribosylation activity of PE. The objection can therefore be ruled out. In addition, the titers of Ab^{PE}, Ab^{Ia}, and Ab^{III} were very close, within 2–3-fold. The inability of Ab^{Ia} and Ab^{III} to inhibit ADP-ribosylation of PE was not due to insufficiency of antibodies.

Previously Hwang *et al.* (7) found that domain III with 20 adjacent residues from domain Ib is required for ADP-ribosylation activity. In addition, the cleft in domain III in which Glu-553 is located was reported to be the NAD binding and catalytic center for ADP-ribosylation (21). However, in the present study we find that Ab^{III} does not affect native PE, while Ab^{PE} completely inhibits PE ADP-ribosylation activity. We also found that Ab^{PE} does not inhibit the hydrolysis of NAD in the absence of EF-2. Thus, antibodies binding to PE neither affect NAD diffusion to the active site nor inhibit the catalytic function of PE. The inhibition of ADP-ribosylation by Ab^{PE} is therefore due to steric effects, which result from Ab^{PE} binding to the area between domains Ia and III. This makes PE inaccessible to EF-2, thereby inhibiting ADP-ribosylation. The interaction of EF-2 with PE is, therefore, believed to be through the interface of domain I and domain III rather than on the surface of domain III. A model for the interaction of EF-2 with PE is proposed. As shown in Fig. 6, EF-2 enters into the active site of PE from the interface of domains Ia and III to the cleft in domain III where Glu-553 is located. This proposal is supported by the fact that Ab^{PE},

but neither Ab^{Ia} nor Ab^{III}, inhibits the ADP-ribosylation activity of native PE. From this model Ab^{PE} is able to bind to the interface of domain Ia and III and causes steric hindrance resulting in PE becoming inaccessible to EF-2. The ADP-ribosylation activity of PE is, therefore, inhibited by Ab^{PE}. On the other hand, domain Ia is away from the ADP-ribosylational domain. As expected, Ab^{Ia} does not inhibit the ADP-ribosylation activity of PE. Although Ab^{III} is able to bind to domain III, which is part of the ADP-ribosylational domain, it is unable to bind to the interface of domain Ia and III due to the steric hindrance from domain I of PE. Thus, Ab^{III} does not inhibit the ADP-ribosylation activity of PE.

Cell Binding Domain—Previous studies have shown that large amounts of domain Ia can reverse PE inhibition of protein synthesis in Swiss 3T3 cells (7). The blockade of PE cytotoxic activity by domain Ia is believed to be due to competition for binding. In this study, we have found that antidomain Ia antibody, but not antidomain III antibody can reverse the PE inhibition of protein synthesis and block the cell-killing activity of PE. Since Ab^{Ia} does not affect the ADP-ribosylation activity of PE, the blockade of PE cytotoxic activity by Ab^{Ia} is most likely due to the binding of Ab^{Ia} with the cell attachment domain of PE, thereby preventing PE binding to its receptor. This observation further confirms that the cell binding domain of PE is located in domain Ia.

Vaccination against PE—*Pseudomonas* exotoxin A is the most potent virulent factor produced by *P. aeruginosa* (17, 18). The development of a vaccine against PE-mediated disease has become urgent and important. In this study, we raised Ab^{PE} by first immunizing rabbits with domain Ia of PE, then subsequently injecting native PE. Our results showed that rabbits immunized with domain Ia of PE acquire tolerance against 100 µg of PE injected subcutaneously and that rabbits finally generate antibodies against intact PE rather than domain Ia alone. However, rabbits injected with phosphate-buffered saline or domain III die after receiving 100 µg of PE. These results suggest that domain Ia of PE can be used for vaccination against PE-mediated disease. In addition, since domain Ia of PE does not contain ADP-ribosylation activity, domain Ia should be nontoxic. Therefore, using domain Ia is safer than using PE toxoid for vaccination. It has been reported that PE toxoid can be prepared by photoaffinity inactivation (22). However, it is only inactivated 95% by photoaffinity inactivation. The residual 5% PE activity presents a risk factor to the recipient. Thus, domain Ia may be the better approach for vaccination against PE-mediated disease at the present time.

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Review

The Father, Son and Cholix Toxin: The Third Member of the DT Group Mono-ADP-Ribosyltransferase Toxin Family

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Abstract: The cholix toxin gene (chxA) was first identified in *V. cholerae* strains in 2007, and the protein was identified by bioinformatics analysis in 2008. It was identified as the third member of the diphtheria toxin group of mono-ADP-ribosyltransferase toxins along with *P. aeruginosa* exotoxin A and *C. diphtheriae* diphtheria toxin. Our group determined the structure of the full-length, three-domain cholix toxin at 2.1 Å and its C-terminal catalytic domain (cholix_c) at 1.25 Å resolution. We showed that cholix toxin is specific for elongation factor 2 (diphthamide residue), similar to exotoxin A and diphtheria toxin. Cholix toxin possesses molecular features required for infection of eukaryotes by receptor-mediated endocytosis, translocation to the host cytoplasm and inhibition of protein synthesis. More recently, we also solved the structure of full-length cholix toxin in complex with NAD⁺ and proposed a new kinetic model for cholix enzyme activity. In addition, we have taken a computational approach that revealed some important properties of the NAD⁺-binding pocket at the residue level, including the role of crystallographic water molecules in the NAD⁺ substrate interaction. We developed a pharmacophore model of cholix toxin, which revealed a cationic feature in the side chain of cholix toxin active-site inhibitors that may determine the active pose. Notably, several recent reports have been published on the role of cholix toxin as a major virulence factor in *V. cholerae* (non-O1/O139 strains). Additionally, FitzGerald and coworkers prepared an immunotoxin constructed from domains II and III as a cancer treatment strategy to complement successful immunotoxins derived from *P. aeruginosa* exotoxin A.

Keywords: cholix toxin; elongation factor 2; ADP-ribosylation; inhibitor development; computational chemistry

1. Introduction

Cholera disease continues to be a global health problem with thousands of victims each year, often occurring as a result of epidemics [1]. This disease is normally linked to secreted toxins (e.g., cholera toxin) produced by the O1 and O139 strains of *Vibrio cholerae*. However, it is known that other strains of *V. cholerae* also produce secreted toxins in their quest for food and colonization. In fact, a number of non-cholera toxin-producing strains of *V. cholerae* have been shown to display virulence, suggesting that other virulence factors participate in this organism's pathogenesis [2]. In certain cases, these toxins may allow colonization by non-pathogenic bacteria in symbiotic relationships with aquatic animal species [3].

Cholix toxin from *V. cholerae* was first identified in the *V. cholera* TP strain from aquatic samples in the U.S. [4]. Furthermore, a screen of *V. cholera* strains from both clinical and environmental sources revealed that the cholix *chxA* gene is present in nearly one-third of cholera environmental strains, being more common in non-O1/O139 strains [5]. Cholix toxin was recently characterized [6–10] and serves as a role model for structure-function characterization in the diphtheria toxin (DT) group of the mono-ADP-ribosyltransferase (mART) toxin family. Cholix is the third known member of the DT group within the mART toxin family, along with DT and *Pseudomonas aeruginosa* exotoxin A (ExoA). This family can be divided into two groups: CT (cholera toxin) and DT. Cholix is a 666-residue protein that possesses a signal peptide (residues 1–32) and a KDEL-like C-terminal sequence (KDELK), previously shown to be important for retrograde transport within the host cell endoplasmic reticulum [11]. The mature form of cholix is 71 kDa and contains four disulphide bonds. Although the cell biology for the intoxication process has not been well studied, it is likely that cholix follows a similar process in host cells to ExoA [12]. It is highly similar to ExoA (the catalytic domain III only) in three-dimensional structure, enzyme activity, target and inhibitor specificity [6,8]. However, despite the primary sequence similarity of domain III between cholix and ExoA toxins, the GC content of the *chxA* gene is different from genes found in *P. aeruginosa* (ExoA), suggesting that *chxA* was not the product of horizontal transfer between *P. aeruginosa* and *V. cholerae* [13]. Cholix is an A/B toxin that is comprised of a receptor-binding domain that is recognized by the low-density lipoprotein receptor-related protein (LRP-receptor), a membrane translocation domain for crossing the host cell membrane into the cytoplasm, and the enzymatic domain (Figure 1) [6].

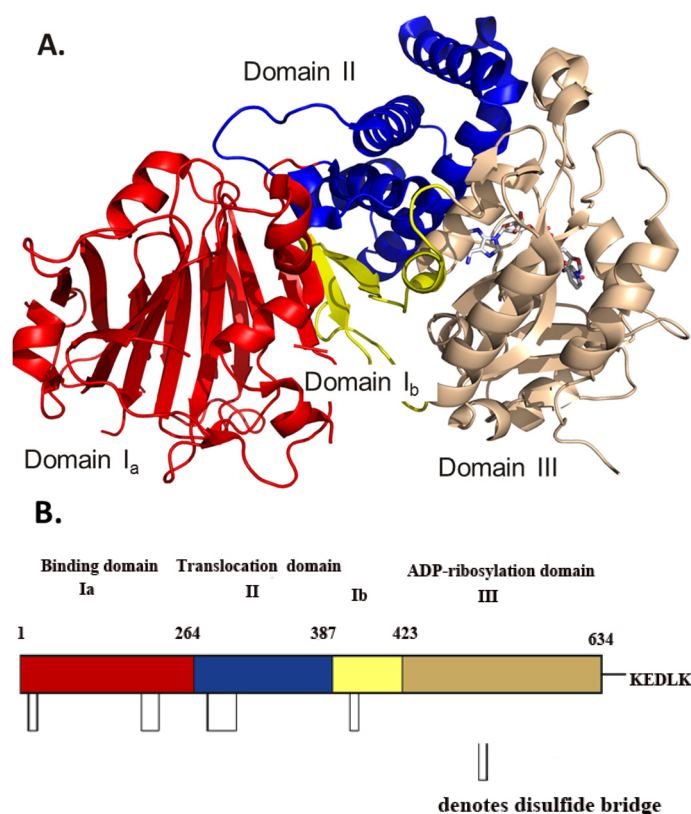


Figure 1. (A) Model of the 1.8 Å crystal structure of full-length cholix toxin in complex with NAD⁺. The cholix structure is shown as domain I_a (red), domain I_b (yellow), domain II (blue) and domain III (copper). The NAD⁺ moiety is shown in stick format with elemental colors. (B) A cartoon sequence of full-length cholix (leader sequence removed) is shown with the domains named and colored according to the scheme in (A) above. The approximate positions of the disulphide bridges are shown as open rectangles, and the C-terminal KDELK sequence is also shown (PDB: 3Q90).

V. cholerae is an aquatic organism that can often be found attached to the exoskeletons of crustaceans, and this behaviour may provide nutrients and protection against environmental stresses [14,15]. This organism may employ cholix as a tool to facilitate the ability of *V. cholerae* to colonize aquatic species, since cholix shows activity against eukaryotic cells by inhibiting protein synthesis in both mammals and crustaceans [6]. Cholix was also shown to be extremely toxic to yeast cells when expressed in the cytoplasm under the control of a copper-inducible system [8,16]. In this assay, it was shown that wild-type cholix caused a severe growth defect phenotype in yeast, whereas the catalytic signature variants, E574A and E581A, showed a significant recovery in the growth defect phenotype and a full recovery with the double variant, E574A/E581A [16]. Additionally, a yeast mutant of the elongation factor 2 (eEF2) target protein, G701R, conferred resistance to cholix, as well as to DT and ExoA and demonstrated that eEF2 is the cellular target for cholix in eukaryotic cells [16]. Cholix enters eukaryotic cells by receptor-mediated endocytosis through the LRP receptor in a similar fashion to ExoA [6]. It was suggested based on structural similarity to ExoA that activation of cholix occurs by reduction of a disulphide bond and cleavage by a furin-like protease in the endosome of the host cell [6]. The newly-formed catalytic fragment (residues 293–634) enters the cytoplasm and modifies eEF2 with

ADP-ribose at the unusual diphthamide residue [17] (Figure 2). This reaction involves the transfer of ADP-ribose from NAD^+ to the diphthamide residue on eEF2, leading to inhibition of protein synthesis and host cell death [6]. This reaction catalysed by DT group members has been well studied [18–20] and involves cleavage of the glycosidic bond (C–N) between nicotinamide and its linked ribose, as well as transfer of ADP-ribose to the imidazole group of the eEF2 diphthamide residue [20]. It was shown that a highly flexible loop 1 in cholix forms a solvent cover or water gasket to exclude the aqueous solvent from the reaction center and helps to stabilize the transition state for the reaction [20].

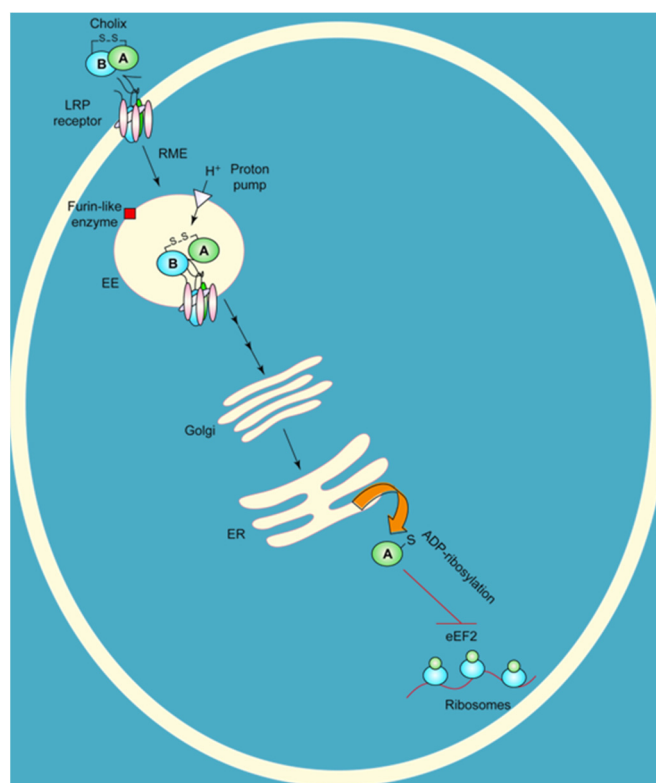


Figure 2. Target cell intoxication route of cholix toxin based on the known mechanism for *P. aeruginosa* exotoxin A. Cholix toxin binds to the LRP receptor protein on target eukaryotic cells, is internalized by receptor-mediated endocytosis (RME) and then is nicked to form the A and B fragments. The A fragment of cholix follows the retrograde pathway to the ER, where it is thought to translocate into the cytoplasm; it inhibits protein synthesis by modifying the diphthamide residue of eEF2 through its ADP-ribosylation activity. A furin-like enzyme is an endogenous protease that is believed to be responsible for nicking cholix in its Arg-rich loop during its intoxication mechanism.

2. Cholix Toxin as a Virulence Factor in *V. cholerae*

Purdy *et al.* [5] assessed the prevalence of the *chxA* gene among a collection of 155 diverse *V. cholerae* environmental strains that originated in various countries, including Bangladesh and Mexico. It was found that nearly one-half of 83 non-O1 and non-O139 strains and nearly 20% of 72 O1/O139 strains harboured the *chxA* gene. It was concluded from this study that cholix plays a major role in the fitness of *V. cholerae* and that it may promote parasitic or mutualistic associations with aquatic dwelling eukaryotes. It may also enhance virulence during inflammatory gastrointestinal diseases in

humans by combining with other virulence genes carried by non-O1 and non-O139 strains in which *chxA* is most prevalent. Although a role for cholix in causing diseases in man has not been clearly proven, at least two reports document diarrhea outbreaks in Peru and Kenya caused by non-O1/O139 *V. cholerae* isolates that were positive for cholix toxin [21,22]. In another study, the occurrence and genetic diversity of the *chxA* gene in both clinical and environmental *V. cholerae* strains that belong to O1 and O139, as well as non-O1/non-O139 serogroups were investigated. Three *chxA* toxinotypes were identified and correlated with their varied virulence patterns. Two toxinotypes, *chxA* I and *chxA* II, can cause extensive damage to the internal organs, especially the liver, of mice. Notably, the occurrence of the *chxA* gene among *V. cholerae* strains was found to be mostly independent of the presence of other major virulence genes. This observation along with the presence of the *chxA* gene among widely divergent non-O1 and non-O139 strains isolated from both patients and the environment highlights the potential of cholix toxin as an important virulence factor in non-O1/non-O139 strains of *V. cholera* [23].

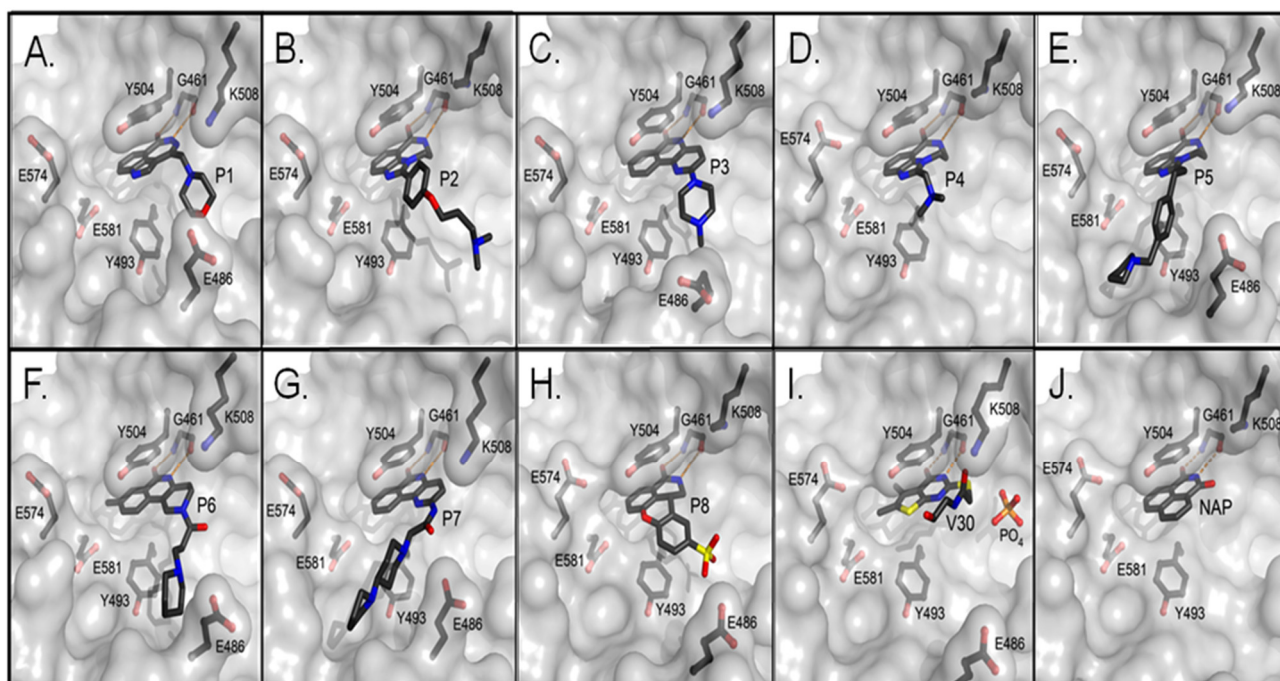


Figure 3. Crystal structures of the catalytic fragment of cholix_c bound to inhibitor compounds. (A) cholix-P1 (PDB: 3KI0), (B) cholix-P2 (PDB: 3KI1), (C) cholix-P3, (PDB: 3KI2), (D) cholix-P4, (PDB: 3KI3), (E) cholix-P5, (PDB: 3KI4), (F) cholix-P6, (PDB: 3KI5), (G) cholix-P7, (PDB: 3KI6), (H) cholix-P8, (PDB: 3KI7), (I) cholix-V30, (PDB: 3NY6), and (J) cholix-NAP (PDB: 3ESS). The inhibitors and nearby residues are shown in grey and black sticks, respectively. Hydrogen bonds are shown in orange dashed lines. All of the structures are from the Protein Data Bank with the PDB ID codes shown in parentheses.

3. Cholix Structure

Cholix is a useful model for ExoA and DT structure and function, because it is more amenable to crystallization; the three-dimensional structures of the catalytic domains of all three DT group toxins are highly superimposable and catalyse the same reaction with NAD⁺ and eEF2 as substrates. A total of

13 crystal structures of cholix have now been reported by our group, including the full-length cholix_{FL}, 2.1 Å apo and 1.8 Å NAD⁺ structures [6,7], and several structures of the catalytic domain, cholix_c, in complex with active-site inhibitors of the enzyme activity [6,8,16] (Figure 3). The cholix_{FL} structure consists of three domains that are quite similar to ExoA in structure, but not in sequence (only domain III shows significant sequence identity between these two toxins, 37%). These domains include a receptor-binding domain (domain Ia, 1–264; domain Ib, 387–423) that forms a 13-stranded antiparallel β-jellyroll, a translocation domain (domain II, 265–386) that is comprised of a bundle of six α-helices and a catalytic domain (domain III, 424–634) with an α/β fold topology (Figure 1). It is clear that domain III has the conserved ADP-ribosyltransferase (ADPRT) sequence pattern [24] and possesses a classic ADPRT fold domain (SCOP d.166.1.1, CATH 3.90.175.10) [25].

A furin-specific cleavage site protrudes from the surface of domain III (R²⁸⁷-S-R-K-P-R²⁹²) that is readily available for nicking by a furin-like protease [26]. The superposition of cholix onto the ExoA structure shows an RMSD of 2.04 Å for the C_α atoms with 1.70, 1.58 and 1.26 Å RMSD for domains I, II and III, respectively [6]. The positions of critical disulphides within the cholix structure align with those in ExoA. These include: Cys¹¹–Cys¹⁵, Cys²⁰⁸–Cys²²⁵, C²⁷⁸–Cys³⁰⁰ and Cys³⁹⁴–Cys⁴⁰⁰.

A comparison between the cholix apo and NAD⁺-bound structures did not show substantial changes in the backbone trajectory (RMSD of 0.33 Å for C_α), implying that NAD⁺ binding to cholix_{FL} did not trigger loop movements [4]. In contrast, the flexible loop 1 (L1: residues 458–463) is closed in the full-length ExoA, sterically blocks substrate binding and opens upon activation of the active-site structure (Figure 4) [27]. Indeed, the backbone structure of L1 residues along with the side chains of Arg⁴⁵⁸, Gln⁴⁶⁰ and Leu⁴⁶² sterically prevent NAD⁺ binding. Thus, cholix can bind NAD⁺ in the absence of structural activation, although the cholix_{FL} shows only weak catalytic activity compared to the recombinant catalytic fragment, cholix_c (see the later discussion). Interestingly, the C_α distance map of the apo cholix_{FL} structure revealed that the region in the catalytic domain corresponding to the R-loop (residues: 471–483, L1 in ExoA) is one of the main contact loops with domain II, along with the K-loop (residues: 503–512, L4 in ExoA) [9,10] (Figure 4); therefore, the dynamic properties of this loop for cholix in solution must account for important conformational changes required in its proposed role as an active-site gate. Indeed, a quantitative assessment on the backbone motion showed relatively high mobility for these active-site loops [9].

In regards to the cholix catalytic domain, crystal structures either uncomplexed or bound with NAD⁺ remain elusive, while 11 X-ray complexes with various bound inhibitors revealed a lack of atomic coordinates for the R-loop; hence, computational modeling was necessary to assess the structural and dynamic properties of cholix [10]. In this sense, an *in silico* catalytic domain obtained by truncation of the cholix_{FL} X-ray structure was employed as a starting structure in molecular dynamics simulation work, yielding a root mean square fluctuation profile comparable to the empirical calculation of backbone dynamics. The enhanced mobility of cholix_c, in comparison to the equivalent part of the cholix_{FL} structure relies on the lack of inter-domain contacts [9]. Surprisingly, the estimated mobility of the cholix K-loop “in solution” was higher than the R-loop (for both cholix_c and cholix_{FL}), which is contrary to the behavior “in crystals”, although compatible with its shifted location when compared with the full-length set (two structures) with the C-domain set (11 structures). Notably, L4 in ExoA provides the association with domain IV of its eEF2 target [19], by interacting with an α-helix proximal to the modified residues, Dip⁶⁹⁹ on eEF2 (yeast protein; Dip⁷¹⁵ for mammalian eEF2), the target of the ADP-ribose moiety.

Interestingly, L4 remains close to Tyr⁴⁸¹ in ExoA, which influences both the GH and ADPRT activities. Hence, the structure-encoded high mobility of the K/L4 loop in cholix_c/ExoA is concordant with its function in target protein recognition or binding.

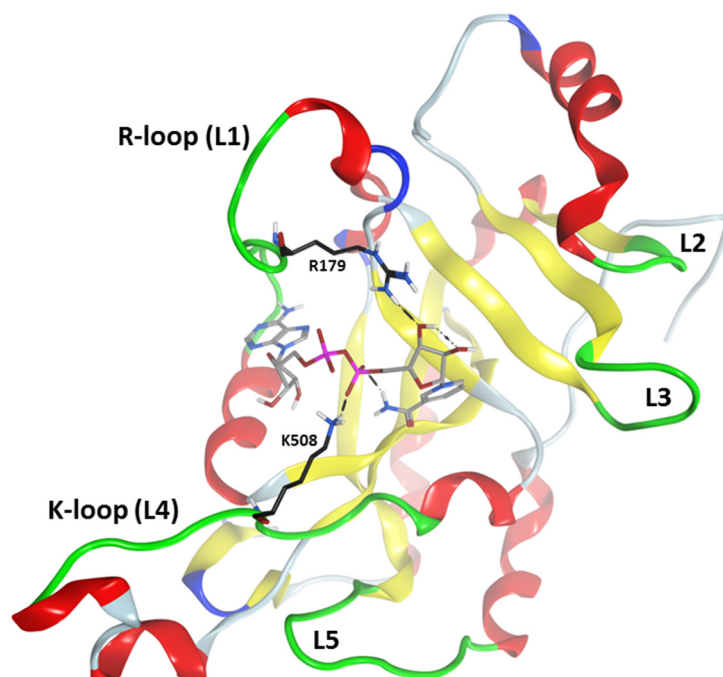


Figure 4. Active-site loops in cholix. Ribbon representation of cholix_c-NAD⁺ complex showing the active-site loops highlighted in green. The substrate binding (L1–L4) and target recognition (L4–L5) loops are shown. The L-nomenclature corresponds to the loop definition in exotoxin A (ExoA) [19]; for cholix, the active-site loops include: L1, Arg⁴⁷¹–Thr⁴⁸³; L2, Thr⁵⁴⁴–Pro⁵⁴⁷; L3, Glu⁵⁷⁴–Glu⁵⁷⁹; L4, Gly⁵⁰³–Gly⁵¹²; and L5, Gly⁶⁰¹–Asp⁶¹⁰. The C-atoms for Arg⁴⁷⁹ and Lys⁵⁰⁸ are depicted in black, and L1 and L4 are renamed in cholix as the R- and K-loops, respectively. Bound NAD⁺ is shown in grey C-atoms.

4. NAD⁺ Binding

In solution, the NAD⁺ conformational distribution is complex, because it can access a large conformational space due to the high number of rotatable bonds and intra-molecular contacts. However, NAD⁺ usually adopts a rather compact conformation in solution and a more extended conformation when bound to proteins. Bound to toxins, NAD⁺ conformation within their active sites has been extensively examined [28]. ADP-ribosyltransferases, such as cholix, cleave the NAD⁺ *N*-glycosidic bond and bind NAD⁺ with several possible adenine mononucleotide conformations. In fact, a comparison of a number of the mART toxin-NAD⁺ complexes reveals that the adenine orientation varies more than the rest of the NAD⁺ molecule [7]. The adenine orientation must not be crucial to the enzyme function, giving rise to such variation. However, variation in the adenine ring is commonly observed within the CT group toxins, but not for the DT group. Interestingly, in the cholix-NAD⁺ structure, the NAD⁺ adenine ring is rotated as compared to DT and ExoA (Figure 5). It has been suggested that most mART toxin structures reveal a strained *N*-glycosidic bond to promote cleavage of the nicotinamide. For cholix-NAD⁺, the torsional angle (χ_N dihedral defined by the atoms O^{4D}–C^{1D}–N^{1N}–C^{2N}) and length of the glycosidic

bond for bound NAD^+ are quite typical and do not support the idea of a highly strained *N*-glycosidic bond in the Michaelis complex [7].

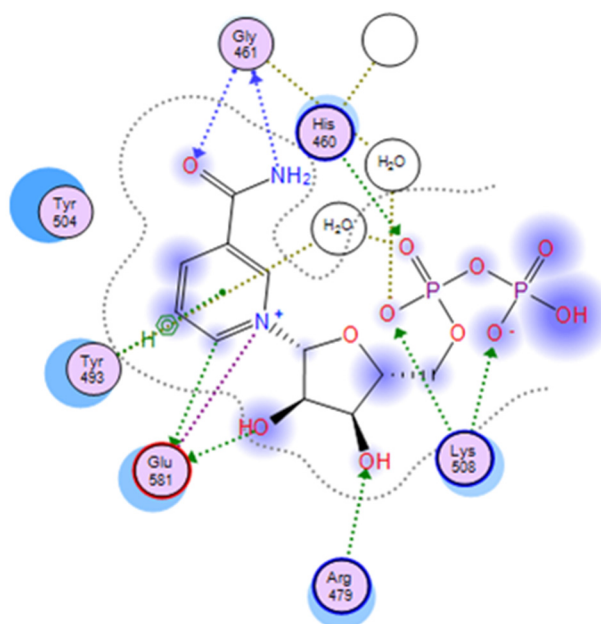


Figure 5. A two-dimensional interaction map of cholix_c with its bound NAD^+ . A 2D-representation of the $\text{PO}_4\text{-PO}_4\text{-ribose-nicotinamide}$ part of NAD^+ is shown in the active site of cholix_c. Crystallographic water molecules interacting with pocket residues are indicated (H-bonding with side-chains or backbone atoms are shown as green or blue arrows, respectively). The H-bond bridging with water molecules is shown as gold-dotted lines, and salt bridges are shown as purple lines.

The binding pocket of NAD is tight around the nicotinamide and adenine moieties, but wider and more exposed in the middle section, at the ribose-phosphate-phosphate-ribose linker. In the cholix_{FL}- NAD^+ crystal structure, 35 pocket residues, a glycerol and five crystallographic water (CW) molecules are part of the recognition surface, offering a rather complex interplay of non-bonded interactions (Figure 5). These events include electrostatic interactions with the negative pyrophosphate and the positive nicotinamide, hydrogen bonding between the carboxamide and *N*-ribose hydroxyl and π -stacking with the nicotinamide and adenine rings. The key residues Gly⁴⁶¹, Tyr⁴⁹³, Tyr⁵⁰⁴ and Glu⁵⁸¹ at the N-site, along with the ribose-phosphate-phosphate-ribose part of NAD^+ strongly interact with Arg⁴⁷⁹ (at the R-loop), Lys⁵⁰⁸ (at the K-loop) and His⁴⁶⁰, in addition to Ile⁴⁷⁰, Val⁴⁷⁷ and Gln³⁵⁶ at the A-site, and present the highest interaction energy (Figure 4). Accordingly, the ribose-nicotinamide portion of the NAD^+ molecule interacts more strongly with cholix residues than the ribose-adenine moiety, which dictates that the nicotinamide half of the molecule is mainly responsible for driving the energetics of NAD^+ binding to the cholix active site [9]. The three-dimensional reference-interaction-site model (3D-RISM) solvent analysis showed that NAD^+ binding does not alter the water organization, since the five in-pocket CW molecules are also present in the apo form. However, the binding of NAD^+ destabilizes these CW molecules, making them potentially displaceable by H-donor/acceptor ligand decorations (Figure 5). An exception is HOH⁷⁰² (PDB: 2Q9O), which appears to increase the binding energy by bridging the substrate to Gly⁴⁶¹ [9].

5. Kinetic Model and Substrate-Binding Properties

Cholix toxin shows both strong glycohydrolase (GH) and transferase (ADPRT) activities as a member of the DT group [6] (Table 1). Although cholix_{FL} only shows weak catalytic activity in the absence of activation, cholix_c (208 residues, 23 kDa) shows strong GH (K_M , 67 μ M; k_{cat} , 1.92 h⁻¹) and ADPRT (K_M , 45 μ M; k_{cat} 10 s⁻¹) activities (Table 1) [6], serving as a model of the fully-active cholix enzyme. In spite of the observation that the cholix_{FL}-NAD⁺ X-ray structure shows a single bound NAD⁺ molecule, for cholix_c, both (i) a biphasic NAD⁺ binding isotherm as monitored by Trp quenching and (ii) substrate inhibition of the ADPRT activity using the analog ϵ -NAD⁺ was recorded, suggesting the coexistence of two bound substrate molecules [7]. These observations are consistent with our previous report for ExoA showing a biphasic binding curve for NAD⁺ constituents, such as AMP and ADP [29]. Furthermore, the observation of dual NAD⁺ substrates binding to cholix_c appears to be variant dependent, where the variants E574A, E581A and E574A/E581A did not appear to bind a second NAD⁺ molecule, while the variants Y493A, Y504A/F and E579R exhibited the wild-type behavior. Accordingly, a minimum kinetic scheme was proposed that suggests a random binding of NAD⁺ and eEF2, with thermodynamics (dissociation constants, K_d 's; and interaction factors, α 's), kinetics (catalytic rate constants, k_i 's) and fluorescence (fractional quantum yields, η 's) parameters that account for the differential substrate curve of the fluorescence quenching and ADPRT activity among cholix_c wild-type and catalytic variants [7].

Table 1. Kinetic parameters of diphthamide-specific ADPRTs.

Parameter ^a	ExoA _c	DTA _c	Cholix _c
$K_{M(NAD)}$ (μ M)	121 $\pm$ 21	11 $\pm$ 1	45 $\pm$ 3
V_{max} (M s ⁻¹)	1.3 $\times$ 10 ⁻⁷	5.2 $\times$ 10 ⁻⁶	1.0 $\times$ 10 ⁻⁷
k_{cat} (s ⁻¹)	13 $\pm$ 2	5 $\pm$ 0.2	10 $\pm$ 0.5
^b k_{cat} (s ⁻¹) catalytic Glu→Ala	0.008 $\pm$ 0.0001	0.016 $\pm$ 0.002	0.004 $\pm$ 0.0003
k_{cat}/K_M (M ⁻¹ s ⁻¹)	1.1 $\times$ 10 ⁵	4.5 $\times$ 10 ⁵	2.2 $\times$ 10 ⁵

^a The kinetic parameters were determined under identical conditions for all three diphtheria toxin (DT) family members. The values represent the mean $\pm$ SD from three independent experiments. ^b The catalytic Glu to Ala mutations involved Glu⁵⁵³ (ExoA_c), Glu¹⁴⁸ (DTA_c) and Glu⁵⁸¹ (cholix_c).

In an attempt to reconcile the cholix kinetic data with the structural information, a pocket searching algorithm over apo cholix_{FL} reported two subcavities connected to the main NAD⁺ site. The 3D-RISM solvent analysis predicted a significant hydrophobic character (densities) of these subpockets [9]. Clearly, one site is occupied by a glycerol molecule in the cholix_{FL}-NAD⁺ structure, while the other site is filled with ethylene glycol in the apo cholix_{FL}. This latter subpocket is exposed and close to the N-site. In fact, the extended structure of the P5 and P7 inhibitors shows their terminal moieties occupying this subsite in the respective cholix_c-P5 and cholix_c-P7 complexes.

Furthermore, the more compact P1 and P8 inhibitors showed a biphasic binding isotherm with cholix_c, implying the coexistence of two bound molecules [8]. These results suggest that “effective” mutations involve the cholix_c N-site and indicate that the aforementioned subpocket might be part of the interaction surface for a second NAD⁺ molecule. If this is indeed the case, then the dynamic character of cholix_c (particularly the R-loop mobility) (Figures 4 and 5) might offer the required flexibility to contact a NAD⁺

molecule partially bound at this subsite, since cholix_{FL} does not exhibit this biphasic behavior. Nevertheless, the physiological relevance of this second binding site is unknown so far.

6. Cholix Inhibitors and Pharmacophore Model

The first inhibitor developed for cholix was based on the finding that in the presence of a known PARP inhibitor, PJ34, cholix_c produced well-formed and highly diffracting protein crystals [6], while the cholix_c-apo produced only poor quality crystals. Later, methods were devised to replace PJ34 with various inhibitors that were competitive for the nicotinamide moiety of NAD⁺ [8,16]. The inhibitors were drawn largely from two libraries of compounds. The first was a small library of known PARP inhibitors, and the second was a library of commercially available compounds generated from a virtual screen based on the 1.25 Å cholix_c-PJ34 structure (PDB: 2Q6M). The key features for most cholix active-site inhibitors include a benzamido group fused into a hetero-ring structure, which mimics the nicotinamide moiety of NAD⁺ [30]. There are four main inhibitor platforms (Figure 6), one consisting of a water-soluble phenanthridinone backbone (PJ34), and the second is a naphthalimide derivative that is more nonpolar (NAP). The third platform (e.g., P6) is based on PARP heterocyclic drugs, and the fourth (V30) is based on the virtual screen experiment of 500,000 compounds from a commercial library [8].

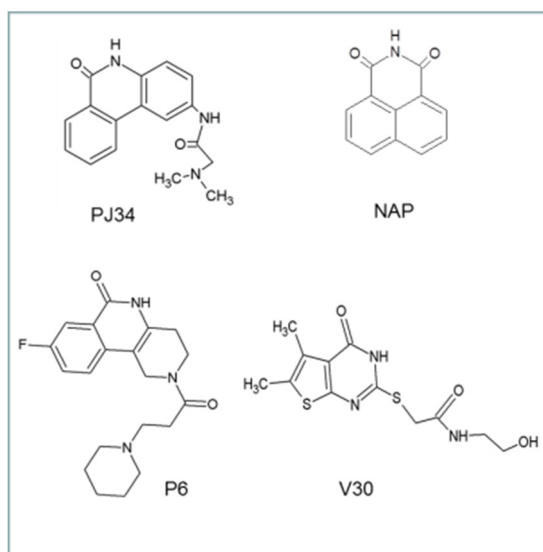


Figure 6. The four inhibitor platforms effective against cholix toxin. (1) Water-soluble phenanthridinones, such as PJ34; (2) naphthalimide derivatives, such as NAP, are more nonpolar than PJ34; (3) PARP-related inhibitors, such as P6, that are heterocyclics with a variety of substituents; (4) V30-related compounds; V30 was identified from a virtual screen against cholix toxin.

In all inhibitor compounds, a key interaction is the H-bonding between the cyclic amide of the inhibitor and the backbone groups of a Gly residue within the Y-H-G conserved motif within active-site region 1 for DT group toxins [8,16]. Importantly, since the DT group toxins bind NAD⁺ in a different conformation than dehydrogenases and most NAD⁺-binding enzymes, the inhibitor libraries show reduced cellular toxicity towards the host eukaryote [8,31]. It was shown that PJ34 does not protect cells from toxin-induced cell death, likely because of its high water solubility, whereas NAP, inhibitors V30

(virtual screen) and P6 (and other directed PARP library compounds) show excellent efficacy (EC_{50} values: 170 nM–4.5 μ M) in cell-based assays [8].

The binding modes (topology and energetic) of these inhibitors were addressed by using 11 high-resolution (better than 1.65 Å) X-ray structures of cholix_c complexed with PJ34 [6], NAP [16], V30 and the P1–P8 (P-series) compounds [8] (Figure 7). In 10 of 11 cholix-inhibitor complexes (all but V30), the inhibitor “core” superposes three pharmacophoric features of the nicotinamide moiety of NAD⁺ (*i.e.*, H-donor/acceptor by the amide group and an aromatic center by the pyridinium ring), in addition to a common hydrophobic-center definition that emerges at the N-site. However, the “side-chains” of these inhibitors do not share any other pharmacophore character of NAD⁺ (e.g., the adenine aromatic centers) (Figure 8); rather, eight inhibitors with a positive charge match their cationic features in defined locations, regardless of the core structure, attachment point or chemical group of the inhibitor “tail” [10].

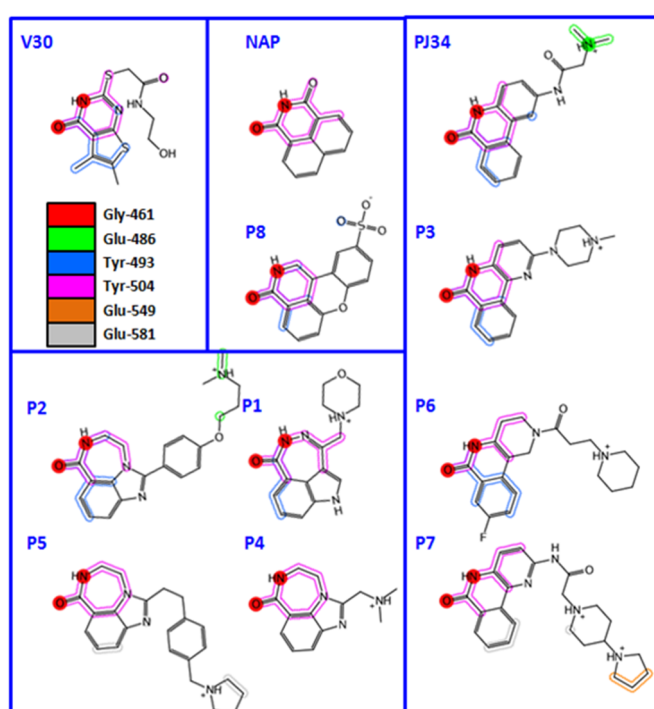


Figure 7. The 2D topological representations of V30, PJ34 and P-series inhibitor binding poses with cholix_c. The colored sections/moieties represent the interaction with the pocket residues shown in the legend. The two red circles show the common interaction with Gly⁴⁶¹. The 11 compounds were grouped according to common features of the inhibitor core region.

The conformational distribution of the flexible R-loop in cholix_c, along with constitutive subpockets, would dictate the accessibility and energetics of these locations (Figure 8). Indeed, a 3D-quantitative structure-activity relationship (3D-QSAR) analysis demonstrated that the binding energy is modelled mainly from differential interactions of Arg⁴⁷⁹ (R-loop), Lys⁵⁰⁸ (K-loop), Glu⁵⁸¹ and Gly⁴⁶¹ (core), among others [10]. The case of inhibitor V30 is unique, since several differences between this compound and other inhibitor platforms are evident; however, the most obvious difference can be attributed to the absence of the fused benzamide as part of the core structure. Thus, the cholix_c-V30 complex (PDB: 3NY6) presents features, such as (i) a rotated core ring-system in comparison to the NAD⁺ nicotinamide, (ii) out-of-plane atoms from two methyl groups into the flat N-subpocket and, more importantly,

(iii) a strong interaction between a sulfur tail-atom and the core-residue Gly⁴⁶¹ by a “sigma-hole” bond [32]. Currently, we are working to characterize several V30-derivatives based on the core structure of the inhibitor [33].

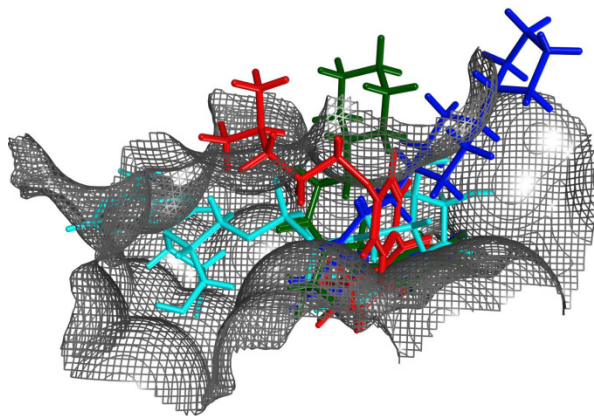


Figure 8. Ligands within the binding pocket of cholix. Superposition of the binding poses (after optimal overlapping of the pocket C α atoms) of NAD⁺ (cyan atoms) with representative inhibitors of the three classes of binding mode according to the spatial location of the terminal positive charge: P2 (red atoms), P6 (green atoms) and P7 (blue atoms).

7. Cholix Induces Host Cell Apoptosis

Cholix induces host cell death through the use of different apoptotic pathways depending on the cell type. Cholix was found to induce cell death in HeLa cells involving multiple apoptotic pathways, with caspase-1, -4 and -5 being responsible for the early-stage caspase-dependent apoptosis [34]. It was shown that Bak/Bax knockdown in HeLa cells significantly suppressed cholix-induced cytochrome c release and caspase-7 activation. However, the activation of caspases-3 and -9 was observed in Bak/Bax knockdown HeLa cells, as well as in control cells. Overall, the findings suggest that the cholix-induced apoptotic pathway uses both the mitochondria-dependent and -independent pathways [34]. It remains unclear which factors are required between the modification of eEF2 by cholix and the activation of caspases by cholix-induced apoptosis. Mcl-1 and other short-lived anti-apoptotic proteins are believed to be the apoptotic trigger in cholix-treated cells. It was proposed that cholix-induced apoptotic pathways in HeLa cells occurs as follows: (i) cholix-induced inhibition of protein synthesis initiates a stress response, which starts a caspase-dependent apoptotic cascade through inflammatory caspase activation during early-stage apoptosis; (ii) subsequently, caspases induce both mitochondria-dependent and -independent apoptotic pathways; (iii) the mitochondria-dependent pathway is started by Bak/Bax assembly, followed by the release of cytochrome c and activation of caspase-7; (iv) following caspase-3 and -7 activation, these two apoptotic signaling pathways lead to HeLa cell death [34].

8. Cholix Toxin as an Immunotoxin for Cancer Treatment

The gene region encoding domains II and III of cholix (CET40) was cloned into an expression vector routinely employed to produce ExoA-based immunotoxins [35]. It was demonstrated that CET40 is a potent cytotoxic protein that can be targeted using an antibody to antigens on the surface of cancer cells.

It was also shown that cell binding is mediated through the targeting antibody Fv and not by CET40 residues. Additionally, the immunological similarities of CET40 were compared with ExoA (PE40); the latter has produced a high rate of complete remissions in hairy cell leukemia and objective responses in other malignancies [36]. This is important because of the potential for using immunotoxins sequentially. Although PE40 and CET40 show high sequence homology and structural similarity, there was little immunological cross-reactivity shown between these two immunotoxins [35]. This implies that the major neutralizing epitopes of PE40 are not common with CET40, making it highly plausible to devise a strategy whereby PE40 immunotoxins are administered first, followed by a treatment with CET40 immunotoxins using the same targeting antibody. This treatment strategy has the potential to allow one or two additional cycles of immunotoxin cancer treatment.

9. Conclusions

Cholix toxin has emerged as a major virulence factor of non-cholera-producing strains of *V. cholerae*. It likely provides these strains with the ability to colonize the exoskeletons of crustaceans and related aquatic animals to provide the bacteria with a more stable food supply. The role of cholix toxin as a virulence factor leading to infection in humans has not yet been clearly established, although a role of cholix toxin in outbreaks in Kenya and Peru has not been ruled out. As the third member of the DT group of mART toxins, cholix now serves as a flagship for structure-function activities. The ability to readily obtain high-resolution crystal structures in the presence of substrates and inhibitors has provided powerful new insights into the mechanism of this notorious toxin family. Computational approaches have provided additional insights into the mART kinetic mechanism, and a pharmacophore model has been built to serve as the basis for the development of therapeutics for the treatment of infections caused by mART-producing pathogens. Finally, an immunotoxin consisting of cholix domains II and III has been prepared as an alternate treatment strategy in tandem with ExoA from *P. aeruginosa* for the treatment of certain human cancers.

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Author Contributions

Miguel R. Lugo and A. Rod Merrill both wrote the manuscript and read and approved the final draft.

Conflicts of Interest

The authors declare no conflict of interest.

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A deletion within the translocation domain of *Pseudomonas* exotoxin A enhances translocation efficiency and cytotoxicity concomitantly

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Summary

Pseudomonas exotoxin A (PE) is a cytotoxin composed of three structural domains. Domain I is responsible for cell binding, domain II for membrane translocation enabling access to the cytosol, and domain III for the catalytic inactivation of protein synthesis, which results in cell death. To investigate the role of the six α -helices (A–F) that form the translocation domain, we deleted them successively one at a time. All mutants showed native cell-binding and catalytic activities, indicating that deletions specifically affected translocation activity. This step of the intoxication procedure was examined directly using a cell-free translocation assay, and indirectly by monitoring cytotoxicity. Translocation activity and log(cytotoxicity) were highly correlated, directly indicating that translocation is rate limiting for PE intoxication. Deletion of B, C and D helices resulted in non-toxic and non-translocating molecules, whereas mutants lacking the A or E helix displayed significant cytotoxicity albeit 500-fold lower than native PE. We concluded that B, C and D helices, which make up the core of domain II, are essential, whereas the more peripheral A and E helices are comparatively dispensable. The last helix (F) is inhibitory for translocation because its deletion produced a mutant displaying a translocation activity 60% higher than PE, along with a three- to sixfold increase in cytotoxicity in all tested cell lines. This toxin is the most *in vitro* active PE mutant obtained until now. Finally, partial duplication of domain II did not give rise to a more actively translocated PE, but rather to a threefold less active molecule.

Introduction

One molecule, or a few molecules, of toxins such as *Pseudomonas* exotoxin A (PE), ricin or diphtheria toxin in the cytosol result in catalytic inactivation of protein synthesis and cell death (Eiklid *et al.*, 1980). These toxins are used to prepare immunotoxins (ITs) by coupling the toxic moiety to an antibody fragment or a growth factor. Production of specific ITs usually requires excision of the toxin cell-binding domain before conjugation with the ligand. ITs are promising pharmacological agents for cancer and AIDS therapy, and designing more potent targeted toxins is a major challenge (Pastan *et al.*, 1992; Thrush *et al.*, 1996).

Concerning the toxin portion of the IT, a few more active molecules have been obtained. One way to improve toxin efficacy is to fuse a second catalytic activity to the toxin (Li and Ramakrishnan, 1994). Nevertheless, a hybrid between a ribonuclease (barnase) and PE was less active than PE (Prior *et al.*, 1991), suggesting that, as far as PE is concerned, toxins with dual enzymatic activities might not always be more efficient than the native toxin.

PE is a 66.6 kDa protein secreted by *Pseudomonas aeruginosa* and is organized in three structural (Allured *et al.*, 1986) and functional (Hwang *et al.*, 1987) domains. Domain Ia mediates cell binding to the α_2 -macroglobulin receptor/low-density lipoprotein receptor-related protein (LRP) (Kounnas *et al.*, 1992), domain II is responsible for translocation into the cytosol and domain III catalyses the ADP-ribosylation of elongation factor 2 (EF2), thereby inhibiting protein synthesis and leading to cell death.

Internalization via receptor-mediated endocytosis is the first step of cell intoxication by PE (Kounnas *et al.*, 1992). Exposure to the low-pH environment encountered during endocytic uptake induces PE insertion into model membranes (Jiang and London, 1990), and is a prerequisite for PE transmembrane transport (Alami *et al.*, 1998). We showed that although PE is not processed during endocytosis by lymphocytes, it can directly translocate from endosomes to the cytosol (Alami *et al.*, 1998).

Lymphocytes are a major target for PE-based ITs (Pastan *et al.*, 1992), albeit they are, like most cells, moderately PE sensitive. Mouse fibroblasts such as Swiss 3T3 and L929 cells, are $\approx$ 200-fold more PE sensitive than other cell lines (Mucci *et al.*, 1995). In these highly PE-sensitive cells, 1–10% of endosomal PE is cleaved by endosomal furin-like

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proteases within the loop connecting helix A to helix B, at the beginning of the translocation domain. This cut generates an N-terminal 28 kDa fragment which contains the binding domain, and a 37 kDa active fragment composed of translocation and catalytic domains (Ogata *et al.*, 1990). After reduction of the disulphide bond (Cys-265–Cys-287) joining the two fragments, the 37 kDa active fragment could be transported further to the *trans*-Golgi network (TGN) and the ER before translocation (Pastan *et al.*, 1992). Accordingly, replacing the REDLK carboxyl-terminal sequence of PE by KDEL, the ER retention sequence signal for soluble proteins, which is recognized by a receptor cycling between the TGN and the ER (Miesenböck and Rothman, 1995), resulted in a two- to threefold increase in cytotoxicity (Seetharam *et al.*, 1991).

Fusion of the alkaline-phosphatase lysosomal targeting signal to the amino terminus of a chimeric protein obtained by coupling interleukin 2 to a truncated PE lacking its cell recognition domain enhanced cytotoxicity to lymphocytes two- to threefold (Fishman *et al.*, 1994).

Altogether, these studies show that it is possible to produce a more powerful toxin by fusing a targeting sequence enabling preferential intracellular localization to a translocation site. These motifs are recognized by receptors with expression levels that vary according to the cell types, and this approach is not always successful (Seetharam *et al.*, 1991).

Translocation is the rate-limiting step of the PE intoxication procedure (Hudson and Neville, 1987), and several studies have examined involvement of the PE domain II in cytotoxicity. This translocation domain (residues 253–364) contains six consecutive α -helices (A–F) (Allured *et al.*, 1986). The single disulphide bridge of the domain, connecting A and B helices, was found to be moderately important for PE cytotoxicity because substitution of both cysteines (265 and 287) by serines decreased the toxicity to mouse fibroblasts by 80-fold (Madshus and Collier, 1989).

Point mutations in domain II enabled identification of a large number of amino acids implicated in PE transmembrane transport. Replacement by Ala of any of the 27 residues exposed at the surface of domain II resulted in less active PE, except for Pro-268→Ala which was slightly (20–100%) more active than PE in the tested cell lines (Kasturi *et al.*, 1992). Similar results were obtained when all Arg within domain II were individually changed: all of the resulting point-mutated PE, except Arg-274→His, were less active than native PE (Jinno *et al.*, 1989). Hence, point mutations did not enable identification of a portion within the domain which could be the most closely implicated in the translocation process.

Deletion mapping studies based on the primary structure of the protein showed that the amino-terminal segment encompassing A and B helices (residues 254–308) (Hwang *et al.*, 1987), especially amino acids 254–263 (within the A

helix) (Siegall *et al.*, 1989), are required for PE translocation. At the carboxyl-terminal end, residues 339–345 within the E helix were found to be critical (339–345) (Siegall *et al.*, 1989), whereas residues 346–364 containing the remaining part of the E helix and the entire F helix could be deleted without apparent loss of translocation activity (Siegall *et al.*, 1991).

In these earlier reports, translocation activities were deduced from cytotoxicity measurements and not examined directly using a direct translocation assay. The respective involvement in the translocation process of the different helices that make up the basic structural units of this domain has never been investigated.

In this study, we examined this structure–activity relationship of PE translocation domain, using both moderately and highly PE-sensitive cell lines. Six mutants, each lacking one of the α helices of domain II, were prepared. We monitored their translocation activity both directly, using a cell-free assay (Alami *et al.*, 1998), and indirectly using conventional cytotoxicity measurements. The mutant lacking the smallest and last α -helix (F) of the domain exhibited both enhanced translocation activity and superior cytotoxicity compared with native PE.

Results

Effects of the deletion on the toxin structure

We constructed six PE mutants, each lacking one of the helices that form PE domain II (Fig. 1A). Details of the constructions are given in Fig. 1B. The cloning procedure led to replacement of the deleted α -helix by the dipeptide Pro–Gly. PE and mutant toxins were overexpressed in *E. coli* and purified from the periplasm to at least 80% homogeneity (Fig. 2A). They all migrated on SDS–PAGE with the expected mobilities, indicating that the signal peptide has been correctly removed by *E. coli* signal peptidase.

Because PE-delA and PE-delB both bear an unpaired cysteine, we examined the possibility of the formation of disulphide-linked homodimers during purification. Analysis by non-reducing SDS–PAGE (Fig. 2B) showed that PE-delA was a monomer, indicating that the odd cysteine was presumably buried and unavailable to promote dimerization, whereas a minor fraction ($\approx 20\%$) of PE-delB molecules were dimers. These dimers were not observed within cells (data not shown) and were presumably reduced during uptake. Among the mutants, PE-delC, PE-delD and especially PE-delE migrated more slowly than PE on these non-reducing gels, indicating a slightly less compact tertiary structure. This migration modification on non-reducing gels was not found to be related to any biological or spectral property of the molecules (see below).

The structure of the translocation domain in the deletion mutants was assessed using circular dichroism (CD). The

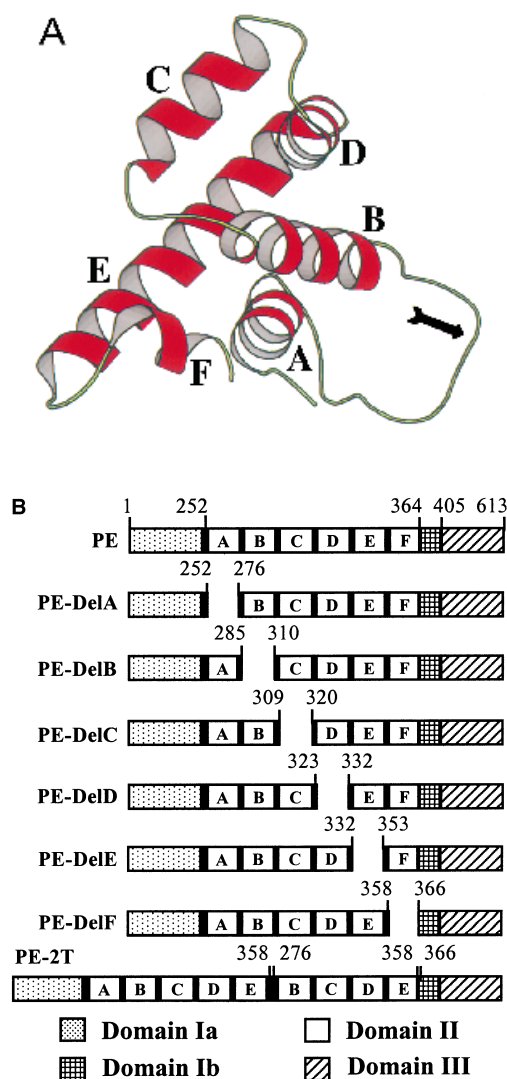


Fig. 1. A. Ribbon representation of PE translocation domain (Allured *et al.*, 1986). The figure was generated using MOLSCRIPT (Kraulis, 1991). α -helices are lettered (from the amino to the carboxyl terminus) and the position of the furin cleavage site is indicated by an arrow. B. Schematic representation of the various PEs modified in domain II used in this study. The coding sequence was obtained by separate PCR amplification of fragments upstream and downstream from the deletion before ligation into Pet3d behind proOmpA signal sequence. The deleted helix was replaced by Pro-Gly as a result of this procedure.

mean residue ellipticity at 222 nm indicated that the percentage contribution of α -helix (Chen and Yang, 1971) decreased from 23% (PE) or 24% (PE-delC and PE-delF) to 22% (PE-delD), 20% (PE-delA and PE-delB) and 18.5% (PE-delE). Because the translocation domain contains $\approx 50\%$ (6 out of 13) of the PE α -helices (Allured *et al.*, 1986), these CD data indicate that the secondary

structure of this domain (i.e. the formation of the remaining helices) was preserved despite the deletion of one helix. They also suggest that the overall secondary structure of the toxin was not affected by the mutation. We then examined the effects of the deletion within domain II on the other domains by monitoring their biological activities.

Deletion mutants retain native PE internalization efficiency and catalytic activity

The first step of the PE internalization procedure is endocytosis, which results from binding of PE domain I to the LRP (Kounnas *et al.*, 1992). We monitored the uptake of PE mutants by BW lymphocytes using a conventional internalization assay. As shown in Fig. 3, none of the deletions within PE domain II interfered with cell binding and internalization, indicating that domain I function was not affected by the mutation.

We then examined whether ADP-ribosylation activity, which lies in domain III, was altered by a deletion within domain II. All mutants retained complete enzymatic activity (Fig. 3), indicating that the catalytic domain was also folded correctly in all engineered PE. Altogether, these data show that deletion within PE domain II did not affect the biological activity and presumably the structure of the flanking domains.

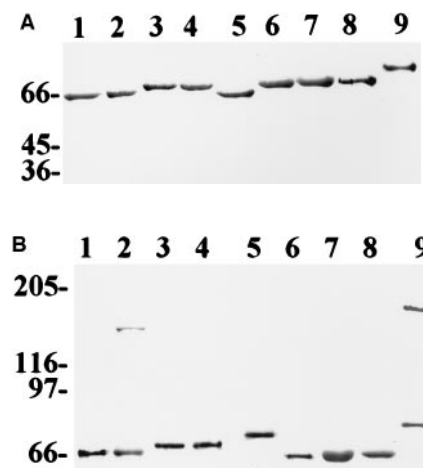


Fig. 2. Analysis of purified PE and mutant proteins by SDS-PAGE under reducing (A) or non-reducing (B) conditions. Toxins at 0.5 mg ml^{-1} were separated on 12% (A) or 10% (B) gels, which were then stained with Coomassie blue. Lane 1, PE-delA (64.3); lane 2, PE-delB (64.0); lane 3, PE-delC (65.7); lane 4, PE-delD (65.8); lane 5, PE-delE (64.5); lane 6, PE-delF (66.1); lane 7, PE (66.6); lane 8, PE343Q (66.6); lane 9, PE-2T (75.3). The predicted sizes of the mature toxins are indicated (kDa).

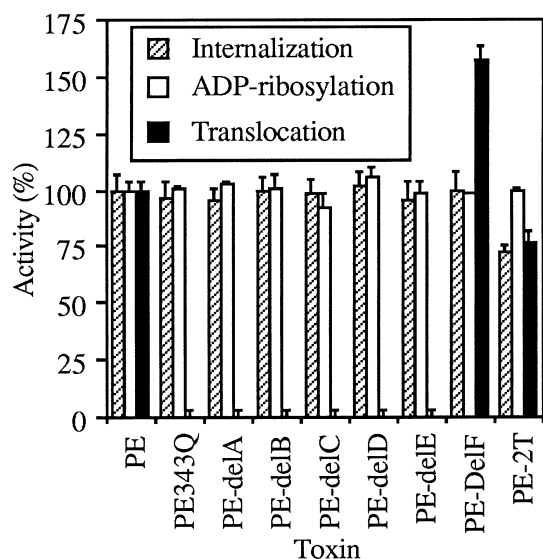


Fig. 3. Biological activities of PE mutants modified in the translocation domain. Assays were performed as described in *Experimental procedures*. The activity of wild-type PE expressed in *E. coli* and purified from the periplasm was set as 100%. Values are means of three independent experiments ($n=4$) $\pm$ s.e.m.

Membrane translocation activity of PE deletion mutants

We recently described a cell-free assay to specifically examine PE transmembrane transport from lymphocyte endosomes. Transferrin, horseradish peroxidase and a PE point mutant (Ala-343 $\rightarrow$ Gln), PE343Q, were used as negative controls (Alami *et al.*, 1998). Mutant PE deleted of the A, B, C, D or E helix did not show any measurable translocation activity (Fig. 3). When F, the smallest helix (five residues) was excised from PE, the resulting mutant was much more actively translocated than PE (+60%). In agreement with our previous observation for PE (Alami *et al.*, 1998), PE-delF was not processed within lymphocyte endosomes and, accordingly, full-length PE-delF was transported across the endosome membrane (data not shown).

PE deletion mutants are normally processed in highly PE-sensitive cells

Transports of less than 5–10% of the native PE translocation activity cannot be observed using the cell-free translocation assay. Hence, even though this test enables specific examination of this critical step of the toxin intoxication procedure, its moderate sensitivity hampered discrimination among poorly translocated mutant PEs, such as those obtained by deletion of all helices but F. To obtain further information on the respective translocation activities of the deletion mutants, it was necessary to test their

toxicity to highly sensitive mouse L929 fibroblasts which, with an IC_{50} of 3 pM for PE, enable measurement of cytotoxic activities as low as 10^{-5} -fold that of native PE. Nevertheless, a translocation assay is not available for highly PE-sensitive cells such as L929 fibroblasts. Contrary to the lymphocytes used in the previous part of this study, L929 cells can process PE to produce a 37 kDa carboxyl-terminal active fragment which is probably involved in the intoxication procedure (Ogata *et al.*, 1990).

This processing might have been altered by the deletions we performed. To be certain that the results of the cytotoxicity assays would reflect translocation activity, we examined whether deletion mutants were processed to the same extent as PE by L929 cells.

No PE cleavage products were seen when the toxin was incubated in the absence of cells (Fig. 4A and B). A large proportion (25–40%) of endocytosed PE molecules were proteolysed by L929 cells to a $\approx$ 55 kDa protein (Fig. 4A), which is reminiscent of the N-terminal fragment generated by trypsin-mediated cleavage after Arg-490 (Ogata *et al.*, 1990). Consistent with previous observations (Chiron *et al.*, 1996), less than 1% of cell-associated PE was proteolysed by furin to produce the active 37 kDa fragment, which required longer film exposure for detection by ECL (Fig. 4B). The sizes of this fragment vary in the 34–37 kDa range (Fig. 4B), as predicted from the size of the mature toxins (Fig. 2A). Under these conditions, the 28 kDa fragment was difficult to visualize (Ogata *et al.*, 1990).

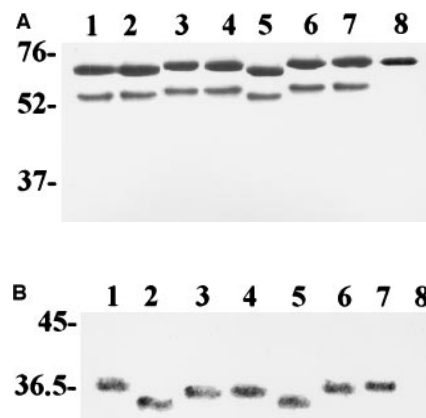


Fig. 4. Cell-mediated processing of PE and domain II deletion mutants. Mouse L929 cells were incubated with $2 \mu\text{g}$ toxin ml^{-1} at 37°C for 4 h before washing and chasing for 1 h. After cell lysis, PE immunoprecipitation, SDS-PAGE and transfer to nitrocellulose, PE was detected using affinity-purified goat anti-PE antibodies followed by recombinant protein G-peroxidase and ECL detection. Films were exposed for detection of (A) the $\approx$ 55 kDa fragment (3–5 s), or (B) the 34–37 kDa active fragment (50–60 s). Lanes 1–6, deletion mutants from PE-delA to PE-delF; lane 7, PE; lane 8, PE incubated in the absence of cells.

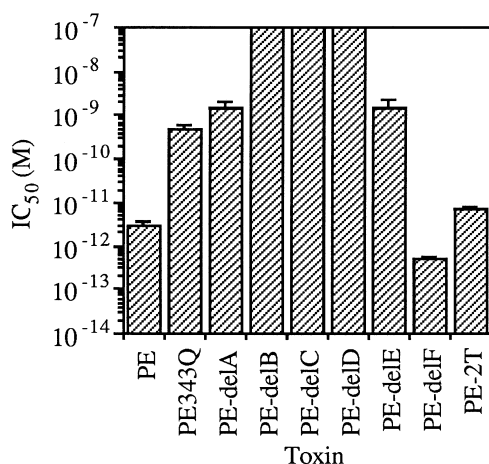


Fig. 5. Cytotoxic activity of native PE and mutant proteins on L929 cells. IC₅₀ is the concentration of toxin required to inhibit 50% of cell protein synthesis as measured by [³⁵S]-methionine incorporation. Mean values of three independent experiments ($n=4$) $\pm$ s.e.m.

PE and deletion mutants were all processed to the same extent by these trypsin- and furin-like activities in L929 cells (Fig. 4). We concluded that none of the deletions generated in domain II significantly affected toxin processing by these cells through trypsin or furin activities. Together with the CD spectra indicating that the remaining helices folded correctly and data demonstrating that activities of the neighbour domains were maintained, these results provide compelling evidence that the general structure of the molecule was essentially preserved in the deletion mutants.

Cytotoxic activity of the PE mutant proteins

The respective translocation activities of these mutants could then be assessed through cytotoxicity measurements using L929 cells because we showed that neither upstream steps in the intoxication procedure (endocytosis and processing) nor downstream stages (catalytic activity) were significantly altered by a deletion within domain II. The ability of the deletion mutants to intoxicate L929 cells is shown in Fig. 5.

These assays allowed us to sort PE domain II mutants into three groups. The first one included PEs in which the B, C or D helix was excised, resulting in complete innocuity of the protein (IC₅₀ > μ M). The second set of PE mutants contained toxins devoid of A or E helices which, albeit 500-fold less active than PE, were significantly cytotoxic. In contrast, the mutant lacking the F helix was sixfold more toxic than PE on L929 cells.

According to the schematic three-dimensional structure of the PE domain II (Fig. 1A), we conclude from this deletion

mapping study that B, C and D helices form the essential core region of the domain and that the flanking A and E helices are less intimately associated with the translocation process, whereas the F helix inhibits translocation.

The superior activity of the PE-delF mutant over PE was also observed (Fig. 6) on three other cell lines: mouse (BW) and human (CEM) lymphocytes as well as human epidermoid carcinoma cells (A431). Nevertheless, with these less PE-sensitive cells, the superiority of the PE-delF mutant was only three- to fourfold.

Partial duplication of domain II did not result in enhanced translocation activity

PE domain II is small (110 residues), and its crystal structure (Fig. 1A) shows two long loops (more than 10 residues), one connecting helix A to helix B and the other between helix E and helix F. We considered that, to improve PE translocation activity, partial duplication of this domain to obtain a translocation domain made up of ABCDE-BCDE was sterically conceivable. The resulting mutant (PE-2T) was purified to homogeneity (Fig. 2A). According to its mean residue ellipticity at 222 nm, this construct had an helical content of 35%, indicating that both native and additional α -helices formed correctly. Nevertheless, PE-2T was found to dimerize to a significant extent ($\approx$ 50%; Fig. 2B). The additional B helix contains an unpaired cysteine which, contrary to the delA mutant, is apparently accessible and promotes dimerization of the molecule via a disulphide.

PE-2T was found to be slightly less efficiently endocytosed than PE (minus 28%). It is unlikely that this defect is related to dimerization because a much higher degree of ligand crosslinking is required to significantly affect internalization, e.g. transferrin¹⁰ is internalized only twofold more slowly than transferrin (Marsh *et al.*, 1995). Partial duplication of domain II did not enhance translocation

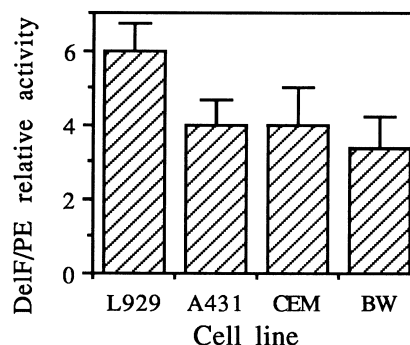


Fig. 6. Relative cytotoxic activity of PE-delF on four different cell lines compared with PE. IC₅₀ were determined as described in Fig. 5. The results are expressed as the IC₅₀ (PE)/IC₅₀ (PE-delF) ratio. For L929, A431, CEM and BW cell lines, IC₅₀ (PE) were 3 pM, 200 pM, 12 nM and 20 nM respectively.

activity, which was even slightly reduced (minus 23%) compared with native PE (Fig. 3). As a result of these reduced endocytosis and translocation activities, cytotoxicity of this chimera was approximately threefold lower than native PE (Fig. 5).

Translocation and cytotoxic activity of PEs modified within the translocation domain are correlated

Using the three toxins with translocation activity high enough to allow measurement using the cell-free assay, i.e. PE, PE-delF and PE-2T, and despite this low number of mutants, a direct correlation ($P < 0.005$ using the correlation coefficient test) between translocation activities and log(cytotoxicity) was observed (data not shown). This shows that translocation activity is limiting for PE intoxication. Because of this semilog relationship, a moderate increase in translocation activity (+60% for PE-delF) sharply increased cytotoxicity ($\times 6$).

Discussion

In this report, we examined the involvement of each α -helix within PE domain II in the translocation process by performing deletion mapping, and demonstrated for the first time that cytotoxicity of a toxin with a cytosolic target can be improved by enhancing its translocation activity.

We also directly showed that the translocation step is limiting for PE toxicity because a more actively translocated toxin was more cytotoxic than native PE, whereas poorly translocated mutants were only slightly toxic; when PEs mutated in domain II displayed measurable translocation activity, a direct relationship between translocation and log(cytotoxicity) was observed. Our data confirm earlier indirect evidence that the translocation step is the rate-limiting step during cell intoxication (Hudson and Neville, 1987).

The CD spectra analysis indicated that none of the deletions significantly affected the secondary structure of the domain. No mutant aggregation was detected by gel filtration (not shown), and only a minor fraction (20%) of one deletion mutant (PE-delB) was found to dimerize. Biological activities of PE-delB were unlikely to be affected by dimerization because PE-2T, which has a much greater propensity to dimerize ($\approx 50\%$ of PE-2T molecules were dimers), was only 23–28% less active than native PE in internalization and translocation assays and had native ADP-ribosylation activity. Moreover, PE-delB dimers were not detected within cells. Some mutants nevertheless appeared to be less compact than native PE on non-reducing gels (Fig. 2B). This was especially the case for PE-delE, which was the least tightly folded in this test even though it was significantly cytotoxic to L929 cells, whereas PE-delB, which is apparently as compact as PE, was inactive. These

data indicate that the defect in translocation activity was not due to the slight loss of molecule compactness detected on non-reducing gels. It is more likely that this conformation modification influences other characteristics of the molecule, such as stability.

None of the deletions within this domain II interfered with folding of the flanking domains Ia and III as assessed by using their respective biological functions, i.e. endocytic uptake and ADP-ribosylation activity. These data are in agreement with the crystallographic (Allured *et al.*, 1986) and genetic (Hwang *et al.*, 1987) data, indicating independent folding and functions of these domains.

As for most toxins, the exact site of PE translocation has not yet been clearly identified. In moderately PE-sensitive cells such as lymphocytes which are unable to process PE, the toxin intracellular pathway is essentially restricted through its interaction with the LRP (Kounnas *et al.*, 1992) to endocytic elements involved in the transferrin cycle (Alami *et al.*, 1998). In agreement with this localization, we observed cell-free PE translocation from endosomes (Alami *et al.*, 1998) and signals targeting PE chimeras to the lymphocyte endocytic pathway enhanced toxicity to this cell type (Fishman *et al.*, 1994).

The intoxication procedure appears to be different in highly PE-sensitive mouse fibroblasts, which exhibit endosomal furin-like activity that produces a 28 kDa amino-terminal fragment linked to a 37 kDa carboxyl-terminal active fragment via a disulphide (Ogata *et al.*, 1990). After reduction of this disulphide, the active fragment could then be subjected to retrograde transport and reach the TGN or ER for translocation (Pastan *et al.*, 1992).

The PE-delA mutant provided information concerning the involvement of PE processing in the intoxication procedure in highly PE-sensitive cells. Because processing takes place between A and B, and PE-delA is as efficiently endocytosed and processed as PE, the same number of identical active fragments is generated from either toxin within cells. Then why is the PE-delA mutant ≈ 500 -fold less toxic than PE? It is unlikely that this difference results from the removal of the disulphide between the two helices because elimination of both cysteines (Cys-265–Cys-287) only decreased cytotoxicity 80-fold (Madshus and Collier, 1989).

Only Cys-265 is absent in the PE-delA mutant. The remaining Cys-287 within the B helix did not promote the formation of disulphide-linked homodimers (Fig. 2B). It, thus, appears that the PE-delA mutant was less active than PE because the A helix takes part in translocation, which therefore could involve the entire toxin in highly PE-sensitive cells. This result is in agreement with data indicating that there is no relationship between processing and toxicity of PE mutants because mutants which were more efficiently processed than native PE were less toxic (Chiron *et al.*, 1996).

Partial duplication of the PE translocation domain did not produce a toxin with better cell-killing activity. Nevertheless, no dramatic reduction in translocation or cytotoxic activity was obtained, highlighting the possibility of performing relatively large insertions in this area of the molecule. This could be useful for future work.

Apart from translocation and toxicity, the behaviour of the PE-delF mutant and of PE was the same in all the tests we performed to examine the structure and activities of these toxins. The enhanced translocation activity of PE-delF is therefore not the result of extensive remodelling of the molecule. This helix is the smallest in the domain (five amino acids, 360–364). The improvement in both translocation and cytotoxic activity upon its replacement by Pro–Gly (because of the cloning procedure) probably did not result from proline insertion in this flexible region (Fig. 1A), but rather from the deletion *per se*. Substitution of the RDELK carboxyl-terminal sequence of PE by KDEL or (KDEL)₃ enhanced cytotoxicity to L929 cells by threefold (Seetharam *et al.*, 1991). The PE-delF mutant, sixfold more toxic than PE on L929 cells and three- to fourfold more toxic on moderately PE-sensitive cell lines, is the most active PE mutant obtained until now and could give rise to more efficient ITs. The enhanced cytotoxicity of PE-delF, regardless of whether highly (L929) or moderately (lymphocytes) PE-sensitive cell lines were used, and the correlation between translocation activity (measured using lymphocyte endosomes) and cytotoxicity to L929 cells indicate that there are some PE translocation mechanism similarities in both classes of cell lines.

The superior translocation activity of the delF mutant could be due to the ability of this molecule to insert more easily into the endosome membrane upon acidification, or to an advantage expressed at a later stage, during the translocation procedure *per se*. Our translocation assay did not enable us to discriminate between these possibilities.

Replacement of three negative (two Asp and one Glu) by three positive (Lys) charges within the first α -helix of the DT translocation domain resulted in a toxin which was 10-fold more actively translocated than DT, even though this was not associated with increased cytotoxicity (Madshus, 1994). There are many differences between DT and PE translocation domains (Parker and Pattus, 1993); the former remains in the endosome membrane, whereas at least the major part of the latter is transferred to the cytosol with the catalytic domain upon translocation (Pastan *et al.*, 1992; Alami *et al.*, 1998). Nevertheless, with DT (Madshus, 1994) and PE (this study), an increase in translocation efficacy was obtained by modification of the translocation domain within the edge region which is connected to the catalytic domain (albeit indirectly in the case of PE). This critical portion of the translocation domain might be involved in the correct positioning of the enzymatic domain for transmembrane transport.

Experimental procedures

DNA manipulations

Pet3d expression vectors containing the coding sequence of PE or PE343Q behind the proOmpA signal sequence were provided by Dr J. M. Lord (University of Warwick, UK). This signal sequence enables PE secretion into the periplasm (Chaudhary *et al.*, 1988). UpPE and UpX primers were used for PCR amplification of a PE fragment containing domain I and the portion of domain II upstream from helix X, whereas the downstream PCR fragment, containing the portion of domain II after the deleted helix X and domain III, was obtained using primers DoX and DoPE. UpX and DoX primers contained a *Xma*I cloning site. UpPE and DoPE were complementary to the region of the PE gene containing the *Nco*I site and the *Bam*HI site respectively. Each PCR was for 28 cycles (95°C for 1 min, 60°C for 30 s and 1 min at 72°C).

Upstream PCR fragments were cut with *Nco*I and *Xma*I and downstream PCR fragments were digested with *Xma*I and *Bam*HI before cloning into *Nco*I- and *Bam*HI-restricted Pet3d-proOmpA-PE to produce proOmpA-PE-delX (PE deleted of helix X).

Deletion was confirmed by sequencing the amplified sequences for each mutant.

To construct the PE2T chimera in which the translocation domain was ABCDEBCDE, a PCR was performed using proOmpA-PE-delF as template and DoA and DoPE as primers. The resulting fragment was cloned into *Xma*I–*Bam*HI restricted Pet3d-proOmpA-delF and sequenced. Diagrams of the different PE constructions are given in Fig. 1B.

Toxin expression and purification

E. coli BL21 (λ DE3) cells carrying the recombinant plasmid were grown at 37°C in L-broth containing ampicillin (100 μ g ml⁻¹). Isopropyl- β -D thiogalactoside (1 mM) was added when OD₆₀₀ reached 0.6 and bacteria were harvested 2 h later. The periplasmic fraction (Chaudhary *et al.*, 1988) was dialysed against buffer A (20 mM tris-HCl, pH 7.6) and applied to a Q-Sepharose column. After washing with buffer A, a NaCl linear gradient (0–1 M) was applied. PE mutants were eluted at 0.35–0.42 M NaCl. Native PE, PE343Q and PE-delF were already >90% pure after this step. Other PE mutants were further purified on a Mono-Q column (Pharmacia) and eluted with a NaCl linear gradient (0–0.7 M) in sodium phosphate buffer (20 mM, pH 7.4). Pure toxins eluted at 0.22–0.30 M NaCl and were at least 80% homogeneous (cf. Fig. 2).

Protein analysis and labelling

Far-UV CD measurements were performed at 25°C using a toxin concentration of 0.1 mg ml⁻¹ ($\approx$ 1.5 μ M) in 5 mM sodium phosphate buffer (pH 7.2). A Superdex 200 column (Pharmacia) eluted with PBS was used for gel filtration analysis. Proteins were labelled with ¹²⁵I as indicated (Alami *et al.*, 1998).

ADP-ribosylation assays

Frozen and thawed toxin (10 μ l; 1 pmol) was added to 50 μ l buffer (10 mM DTT, 4 mM EDTA, 100 mM tris-HCl, pH 8.2),

20 µl purified EF2 (Alami *et al.*, 1998) and 20 µl [^{32} P]-NAD (50 ng, 100 000 c.p.m.), whereas H₂O was added to control tubes. After 2 h at 30°C, the reaction was stopped by 1 ml of ice-cold 10% trichloroacetic acid before filtration onto glass-fibre filters and scintillation counting.

Cell lines and cytotoxicity assays

BW 5147 (mouse lymphocytes), CCRF-CEM (human lymphocytes) (Beaumelle *et al.*, 1992), A431 (human epidermoid carcinoma) and L929 (mouse fibroblasts) (Ogata *et al.*, 1990) were cultured as indicated. The cytotoxic activity of PE mutants was assayed on various cell lines seeded into 96 well plates at the appropriate density: L929, 7500 per well; A431, 15 000 per well; BW, 30 000 per well; and CEM, 60 000 per well. L929 and A431 cells were allowed to attach for 2 h at 37°C before toxin addition, whereas BW and CEM cells directly received toxin dilutions. After 24 h at 37°C, [^{35} S]-methionine (500 000 c.p.m. per well) was added and cells were further incubated for 20 h before lysis in 0.1 N NaOH and collection of trichloroacetic acid-precipitable material. Control values were obtained in the absence of toxin. The results are expressed as IC₅₀.

Internalization assay

Mouse BW lymphocytes in DMEM supplemented with 0.1 mg ml⁻¹ bovine serum albumin (DMEM/BSA) were labelled with [^{125} I]-toxin (1 µg ml⁻¹) at 37°C. They were then diluted in ice-cold PBS, washed, resuspended in 0.3% Pronase[®] in PBS and left for 40 min at 2°C before centrifugation through dibutylphthalate (Beaumelle and Hopkins, 1989). Endocytosis efficiency was expressed as the Pronase-resistant percentage of cell-associated [^{125} I]-toxin after 30 min of uptake.

Endosome fractionation and cell-free assay for PE translocation

Mouse BW lymphocytes were resuspended in DMEM/BSA containing 0.15 mg ml⁻¹ low-density lipoproteins and 1 µg ml⁻¹ [^{125} I]-toxin. After 30 min at 37°C, they were washed, cooled on ice, then fractionated for endosome purification using a discontinuous sucrose gradient as detailed previously (Beaumelle and Hopkins, 1989). The assay for PE translocation is described elsewhere (Alami *et al.*, 1998).

Briefly, purified endosomes were resuspended in translocation buffer containing 10 mM ATP/10 mM MgCl₂. After the indicated period of time at 37°C, translocation was stopped by cooling on ice. Translocated toxin was then separated from endosomal toxin by ultracentrifugation on a 17% sucrose cushion. The supernatant/endosomes c.p.m. ratio was plotted as a function of time. Translocation was linear with time over at least 2.5 h for all mutants, and the translocation rate was calculated using linear regression.

Processing of PE mutants by highly sensitive cells

Subconfluent L929 cells were cultured at 37°C for 4 h in medium containing 2 µg ml⁻¹ toxin, washed and further incubated for 1 h to allow internalization of plasma membrane-bound toxin (Ogata *et al.*, 1990). After scraping and washing with PBS,

cells were lysed in 1 ml of immunoprecipitation buffer (20 mM tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride). The lysate was cleared by centrifugation before adding purified goat anti-PE antibodies (2 µg). After 2 h at 4°C, 15 µl Pansorbin (Calbiochem) precoated with 10 µg of rabbit anti-goat purified IgG (Sigma) was added. Immuno-precipitates were washed and finally resuspended and boiled in reducing SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, then transferred to nitrocellulose which was incubated with goat anti-PE antibodies (0.8 µg ml⁻¹ blocking buffer). Membrane-bound native IgG was labelled using recombinant protein G-peroxidase (Sigma, 1 µg ml⁻¹ blocking buffer), and, after 1 h, revelation was performed using an ECL kit (Amersham). Films were exposed within their linear range of detection and scanned for quantification using ImageQuant (Molecular Dynamics).

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Healing of Intestinal Inflammation by IL-22

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An interleukin (IL)-10 family cytokine, IL-22 is characterized by several unique biological properties, including 1) the target restricted to innate cells; 2) the distinct expression pattern between large and small intestines; 3) alteration of the cellular source depending on several factors; 4) the dual abilities to serve as protective versus proinflammatory mediators in inflammatory responses; and 5) the close association with some major inflammatory bowel disease (IBD) susceptibility genes. The major functions of IL-22 in the intestine are the stimulation of epithelial cells to produce a wide variety of antibacterial proteins, the reinforcement of mucus barrier through stimulation of mucin 1 production under intestinal inflammatory conditions, and the enhancement of epithelial regeneration with goblet cell restitution. Through these beneficial functions, IL-22 contributes to the improvement of some types of experimental chronic colitis, which are mediated by T helper (Th)1 or Th2 responses. Most important, studies using both loss-of-function and gain-of-function approaches have clearly demonstrated the ability of IL-22 to promote intestinal wound healing from acute intestinal injury. These findings highlight IL-22 as an attractive and promising target for future IBD therapy. Alternatively, the enormous progress in the field of IL-22 biology has also suggested more complicated mechanisms with the IL-22 pathway than previously predicted. This review article briefly summarizes previous and current knowledge on IL-22 particularly associated with intestinal inflammation.

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Key Words: epithelial defense, mucin 1, antibacterial proteins, epithelial regeneration, susceptibility genes, inflammatory bowel disease

Interleukin (IL)-22 is an IL-10 cytokine family member that was originally discovered in 2000 from a mouse T cell line stimulated with IL-9.¹ Enormous progress in this area has since been seen particularly during last 5 years.^{2–6} The growing body of evidence highlights several characteristic properties with IL-22. The first characteristic is the cellular targets. IL-22 can specifically target innate cells such as intestinal and respiratory epithelial cells, keratinocytes, and hepatocytes—this is due to the restricted expression of IL-22 receptor (IL-22R) on these innate, but not adaptive immune, cells.⁷ The second characteristic is the unique expression pattern in the intestine. IL-22 expression in the large intestine of mice and humans is elicited under inflammatory conditions,^{8–10} whereas IL-22 is constitutively expressed in the normal small intestine.^{11,12} The third characteristic is the dual abilities to serve as protective versus proinflammatory factors—presumably depending on the tissue targeted, the disease mechanism involved,

or the local cytokine environment exposed. For example, IL-22 contributes to the improvement of experimental colitis and hepatitis,^{9,10,13} but it also causes psoriasis.^{14,15} Therefore, IL-22 has been metaphorically called “a sheep in wolf’s clothing”¹⁶ or “a two-headed cytokine.”² The fourth characteristic is the close association of IL-22 with inflammatory bowel disease (IBD) susceptibility genes. Several IBD susceptibility genes such as IL-23R, IL-10R2, and signal transducer and activator of transcription (STAT)3 are functionally associated with IL-22 directly or indirectly.¹⁷ Therefore, these findings suggest that further work on IL-22 would have the potential to not only dissect the fundamental mechanism of IBD, but also provide an important rationale to develop novel therapeutic measures for this disorder.

DISTINCT EXPRESSION PATTERN OF IL-22 IN LARGE VERSUS SMALL INTESTINES

IL-22 is constitutively expressed in the small intestine of humans and mice to preserve the intestinal homeostasis against enteric microorganisms.^{11,12,18} In contrast, IL-22 expression is hardly detectable in the normal colon of humans^{8,9,19,20} and mice.^{9,10,21} Diverse intestinal inflammatory conditions, which range from IBD to infectious colitis, elicit the expression of IL-22 in the colon.^{8–10,19–21} Interestingly, the expression level of IL-22 in the inflamed colon tends to be higher in Crohn’s disease (CD) patients as compared with ulcerative colitis (UC) patients.^{8,9,19} In CD, serum IL-22 levels are significantly higher in patients

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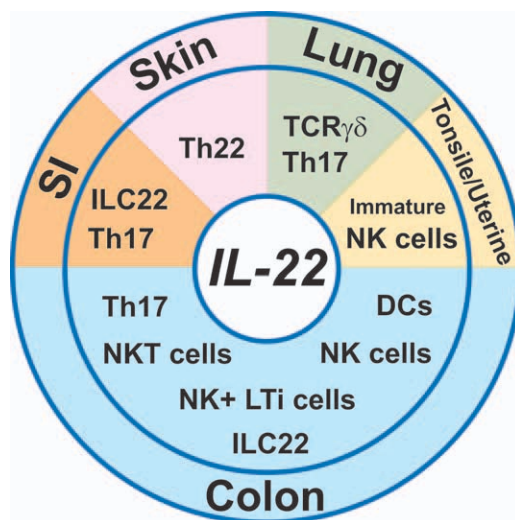


FIGURE 1. Cellular sources of IL-22. Potential major cellular sources in the small intestine (SI, orange), skin (pink), lung (green), tonsil and uterine (yellow), and colon (blue) are shown. Some innate cells in the colon may represent a same lineage with different differentiation stages.

with risk-increasing *il23r* variants than in those with risk-decreasing *il23r* variants.²²

In mice, IL-22 is highly expressed in the inflamed colon of T helper (Th)1-mediated colitis model (CD45RB model) as compared with Th2-mediated colitis model (T-cell receptor [TCR] α knockout [KO] mice).⁹ The CD45RB model has widely been used for the study of human CD,²³ and the TCR α KO model shares some characteristic features with human UC.²⁴ These findings suggest that colonic IL-22 expression, which can be elicited by inflammatory insults, is further conditioned by the local cytokine environments in the inflamed colon (e.g., Th1 vs. Th2). Indeed, in skin, atopic dermatitis with significant contribution from Th2 responses exhibits lower levels of IL-22 expressions as compared with psoriasis characterized by enhanced Th1/Th17 responses.²⁵

CELLULAR SOURCES OF IL-22 IN THE SMALL INTESTINE

IL-22 can be produced by a wide variety of innate and adaptive immune cells, including Th17 CD4⁺ T cells, Th22 CD4⁺ T cells, CD8⁺ T cells, TCR $\gamma\delta$ T cells, dendritic cells (DCs), natural killer (NK) cells, lymphoid tissue inducer (LTi) cells, and innate lymphoid cells (ILCs).^{2–6} Interestingly, the major cellular source of IL-22 seems to differ depending on the tissue involved⁴ (Fig. 1). For example, Th22 cells, which express skin homing receptors (CCR4 and CCR10) and produce IL-22 but not IL-17, represent the major source of IL-22 in the skin.^{26,27} In pulmonary immune response, TCR $\gamma\delta$ T cells and Th17 cells primarily contribute to the production of IL-22.^{28,29} In

human tonsil, stage-3 immature conventional NK cells serve as the source of IL-22.³⁰ IL-1 β is required to retain the expression of aryl hydrocarbon receptor (AHR) and thus IL-22 in the immature NK cells.³⁰ Similarly, such immature NK cells are also the source of IL-22 in human uterine mucosa.³¹

In the small intestine under healthy steady state and also under an inflammatory condition caused by *Citrobacter rodentium* infection, RAR-related orphan receptor (ROR) γ t-dependent innate lymphoid cells (ILCs), which share some characteristics with NK cells and LTi cells (Lin[–]Thy1⁺cKit⁺CD4⁺CD3[–]), are primarily responsible for the production of IL-22.^{11,12,18,32,33} These IL-22-producing ILCs are referred as “ILC22 cells.”³² The ILC22 cells include NK receptor-expressing LTi cells, ILCs (Lin[–]Thy1⁺CD127⁺cKit⁺CD3[–]) expressing a natural cytotoxicity receptor NKp46 with low to no NK1.1 expression, and human NK22 cells expressing NKp44, CD56, and CCR6 with low expression of NKp46.³² Recent studies suggest that NKp46⁺ ILCs are the progeny of LTi cells.³³ Gut commensals promote the activation of ROR γ t in ILC22 cells to enhance their development,¹¹ but microbiota are not strictly required for the development of ILC22 cells, as indicated by the presence of these cells in germ-free mice.^{18,34} In response to *Toxoplasma gondii* infection, a major source of IL-22 in the small intestine may shift from ILC22 cells to CD4⁺ T cells.³⁵ Commensals may be required for the shift, as IL-22 becomes undetectable in the small intestine of germ-free mice after a monoassociated infection with *T. gondii*.³⁵

CELLULAR SOURCES OF IL-22 IN THE LARGE INTESTINE

As compared with the other tissues mentioned above, cellular sources of IL-22 in the inflamed colon seem to differ depending on the type of inflammation. NK cells and Th17 CD4⁺ T cells have been reported to produce IL-22 in the inflamed colon of UC patients.²⁰ In contrast, CD4⁺ T cells expressing NK group 2 member D (NKG2D) have been proposed to produce IL-22 in the inflamed intestine of CD patients.³⁶ In addition, intestinal NKp46⁺ NKp44[–], but not NKp46[–] NKp44⁺, NK cells have also been demonstrated to produce IL-22.³⁷ Interestingly, IL-22-producing NKp46⁺ NKp44[–] NK cells are more increased in the inflamed colon of CD patients as compared with that of UC patients.³⁷ In addition to such NK cells, ILCs, which are characterized by lineage marker (Lin)[–]CD45⁺, CD3[–], IL-7R α ⁺ phenotype, have recently been identified to exist in human colon.³⁸ These ILCs are further classified into two subsets depending on the expression pattern of neural cell adhesion molecule (NCAM, CD56). CD56⁺ ILCs produce IL-22 and IL-26 in response to IL-23, while CD56[–] ILCs produce IL-17A and IL-17F. Interestingly, an

increased number of both IL-22-producing CD56⁺ ILCs and IL-17-producing CD56⁺ ILCs are observed in the inflamed colon of CD patients as compared with UC patients.³⁸ These findings indicate that innate cell populations (NK cells and CD56⁺ ILCs) producing IL-22 expand more in CD as compared with UC. The enhanced expansion of these NK cells and ILCs may contribute to maintaining higher levels of IL-22 expressions in CD mucosa as compared with UC mucosa.

As observed in IBD patients, major cellular sources of IL-22 in the inflamed colon of mouse IBD models also tend to alter depending upon the fundamental disease mechanisms. In the Th1-mediated experimental colitis model (CD45RB model), both NK cells and Th17 cells are primarily responsible for the production of IL-22 in the inflamed colon.¹⁰ The NK cells are further classified into CD27^{high} versus CD27^{low} NK cell subsets. Interestingly, IL-23 can induce the IL-22 expression in both NK subsets, whereas IL-12 and IL-18 induce IL-22 expression only in the CD27^{high} NK cell subset.¹⁰ CD11c⁺ dendritic cells (DCs) may represent a major source of IL-22 in an acute intestinal injury model induced by oral administration of dextran sulfate sodium (DSS).²¹ In the *C. rodentium*-induced infectious colitis model, DCs may also serve as a major source of IL-22.³⁹ Alternatively, a recent study demonstrates in the *C. rodentium* infectious model that ROR γ ⁺ LTi cells, but not DCs, produce IL-22 in the inflamed colon, and DCs rather contribute to the activation of the naïve LTi cells through production of IL-23.⁴⁰ Similarly, IL-23-dependent CD4⁺ LTi cells, which are characterized by the expression of NKp46 and CD90 and by the localization within the intraepithelial compartment of colon, have been proposed to represent the dominant source of colonic IL-22 in the context of *C. rodentium* infection.⁴¹

SIGNALING MACHINERY TO INDUCE IL-22 EXPRESSION

IL-22 production by Th17 T cells is induced by IL-23 or IL-6.¹⁴ Notably, TGF- β , although critical for Th17 differentiation, inhibits the IL-22 production.¹⁴ The inhibition of IL-22 production by TGF- β is mediated by a transcription factor, c-Maf.⁴² As observed in Th17 cells, IL-23 also acts on ILCs to induce the expression of IL-22.⁴³ AHR, which serves as a cytosolic sensor of aromatic hydrocarbons and plays critical roles in the metabolism of benzopyrene as well as in the acute toxicity of halogenated dioxins, is also required for the ability of Th17 T cells to produce IL-22.⁴⁴ Significantly decreased expression of AHR is observed in CD patients as compared with UC patients.⁴⁵ In addition, AHR sustains the expression of NKp46 in small intestinal ILCs through activation of Notch pathway and consequently drives the development of IL-22-producing ILCs.¹⁸ AHR is also required for the devel-

opment of colonic ILCs.⁴⁶ In the DSS-induced colonic injury model, IL-22 expression in the colon may be induced through TLR4 (LPS), TLR9 (CpG), or to a lesser extent TLR2 (lipoteichoic acid).²¹

RESPONDER CELLS TO IL-22

IL-22 is recognized by a heterodimeric receptor consisting of IL-22R1 and IL-10R β .²⁻⁶ IL-10R β is ubiquitously expressed by a majority of cell types, while the expression of IL-22R1 is restricted to innate cells such as epithelial cells, hepatocytes, and keratinocytes.²⁻⁶ Therefore, this expression pattern of IL-22R1 allows IL-22 to specifically target innate, but not adaptive immune, cell populations.⁷ In the intestine of mice and humans, IL-22R1 is expressed by epithelial cells and myofibroblasts.^{8,9,20} However, the responsiveness of myofibroblasts to IL-22 may be much weaker as compared with that of epithelial cells, as indicated by the requirement of high (200 ng/mL) versus low (5 ng/mL) doses of IL-22 for the activation of myofibroblasts versus epithelial cells, respectively.^{8,9}

ACTIVATION OF STAT3 BY IL-22

IL-22 stimulation can activate STAT3, to a lesser extent STAT1, and in certain cells STAT5.^{47,48} In addition, the ability of IL-22 to activate Erk1/2, JNK, and p38 MAP kinase pathways has been proposed in a rat hepatoma cell line.^{47,48} A high dose of IL-22 also induces a transient activation of Erk1/2 in colonic cancer cell line HT29.¹⁹ However, the concept of the Erk1/2 activation by IL-22 has been challenged by some reports showing the inability of IL-22 to activate Erk1/2 in an HepG2 cell line⁴⁹ and in primary epithelial cells from human and mouse colons.⁹ In contrast, the strong activity of IL-22 to stimulate STAT3 has been well confirmed using human colonic biopsies, human colonic cancer cell lines, and primary mouse colonic epithelial cells.^{9,10,20,21} Most importantly, epithelial STAT3 activation under DSS-induced colitis has been demonstrated to depend more on IL-22 rather than IL-6, a well-known STAT3 activator.²¹ This observation may be unexpected but consistent with a recent discovery that IL-22R1 uses a novel mechanism to “constitutively” activate STAT3 through constitutive interaction of its C-terminal tail with the coiled-coil domain of STAT3.⁵⁰ Indeed, IL-22 has recently been shown to have a strong ability to activate STAT3 as compared with IL-6.⁵¹

IL-22 AND ANTIBACTERIAL PROTEINS

IL-22 has been implicated in several important functions in innate cells, including the host defense against microorganisms and wound healing.²⁻⁶ One of the major functions of IL-22 is the induction of antibacterial protein expressions. IL-22 stimulates keratinocytes to produce S100A7 and S100A8.⁵² In collaboration with IL-17, IL-22

also synergistically promotes the expression of β -defensin 2 and S100A9 in keratinocytes.⁵³

Interestingly, even though the expression levels of S100A8 (also called MRP8), S100A9 (also called MRP14), regenerating gene (Reg) III γ and RegIII β are different, they are significantly enhanced and commonly observed in the colonic epithelial cells of different types of experimental “chronic” colitis models, including Th1-mediated and Th2-mediated colitis models.⁵⁴ Alternatively, in the DSS-induced “acute” colonic injury model, the expression pattern of these molecules differs in the acute versus recovery phases.⁵⁴ RegIII γ was initially thought to play a role in cell regeneration, but subsequent studies have identified the ability to serve as an antibacterial protein against Gram(+) bacteria.⁵⁵ Interestingly, IL-22 stimulates epithelial cells to produce RegIII γ and RegIII β in the context of infection with a Gram(-) bacteria, *C. rodentium*,³⁹ whereas *Listeria monocytogenes* stimulates the production of RegIII γ in an MyD88-dependent manner.⁵⁶ In the DSS-induced colonic injury model, IL-22-dependent induction of RegIII γ and β expressions through STAT3 has been proposed,²¹ but a recent study suggests that IL-23 (the inducer of IL-22 expression) rather than IL-22 directly induces the expression of RegIII γ and β .⁵⁷ An IL-17 family member, IL-17C, which is produced by intestinal epithelial cells, has recently been shown to act in synergy with IL-22 to induce the expression of antibacterial proteins, including S100A8, S100A9, RegIII β , and RegIII γ .⁵⁸ The ability of IL-22 to promote the expression of β -defensin 2 has also been shown using a colonic cancer cell line, HT29.¹⁹ In CD patients, IL-22 induces the production of lipopolysaccharide (LPS)-binding protein by hepatocytes, which may contribute to the prevention of LPS-induced systemic responses.⁴⁹

IL-22 AND INTESTINAL MUCUS BARRIER

Intestinal mucus functions as a lubricant and a physiological barrier between luminal contents and the mucosal surface.⁵⁹ Mucin 1 (Muc1), which is a heavily O-glycosylated membrane-bound mucin, represents one of the major components in the intestinal mucus.⁵⁹ Bacteria-derived adenosine 5'-triphosphate (ATP) stimulates the development of intestinal Th17 T cells,⁶⁰ and a segmented filamentous bacterium also leads to the appearance of intestinal Th17 cells.⁶¹ Interestingly, a recent study proposes the ability of Muc1 to suppress the expansion of both Th17 cells and IL-17-producing ILCs, presumably by blocking the translocation of bacterial products from intestinal lumen into intestinal lamina propria.⁶²

The protective roles of Muc1 in both Th2-mediated colitis of TCR α KO mice and Th1-mediated colitis of the CD45RB model have recently been shown using loss-of-function systems (Muc1 KO mice).⁶² Alternatively, forced overexpressions (Muc1 transgenic mouse system) of “hypo-

glycosylated” Muc1 make breaches in the static mucus barrier⁶³ and exacerbate Th1/Th17-mediated colitis in IL-10 KO mice,⁶⁴ suggesting that complete glycosylation of Muc1 is required for eliciting the protective function in colitis. Indeed, impaired glycosylation in the mucus exacerbates or causes colitis.^{65,66} Interestingly, the ability of IL-22 to promote the production of functional Muc1 through activation of STAT3, but not STAT1, has been demonstrated using human colonic cancer cell lines (T84 and HT29) and primary colonic epithelial cells from mice.^{9,67} Consistent with these findings, binding of STAT3 within the promoter region of the *muc1* gene has been demonstrated.⁶⁸ Most notably, Muc1 expression was abolished in a patient with the early onset form of IBD, who had a functional polymorphism within *il10rb*, a receptor for IL-22.⁵¹

IL-22 AND EPITHELIAL REGENERATION

IL-22 has been demonstrated to promote the epithelial cell regeneration with goblet cell restitution under intestinal inflammatory conditions, but it may play no obvious role in normal colonic epithelial homeostasis in the healthy state.^{9,21} Consistent with these observations, STAT3 activation through IL-22 enhances the transcription of antiapoptotic and pro-proliferative genes such as *birc5*, *plag5*, *smo*, *myc*, and *mcl1* under inflammatory conditions.⁶⁹ In addition, IL-22 stimulates a colonic cancer cell line to express a molecule termed “deleted in malignant brain tumor 1 (DMBT1)” that may play a role in epithelial cell differentiation.⁷⁰ RegI α , which serves as a trophic and antiapoptotic factor, is also reported to be induced in the inflamed colon of UC patients by IL-22.²⁰

PROTECTIVE VERSUS PROINFLAMMATORY ROLES OF IL-22 IN INFLAMMATORY RESPONSES

Recent accumulating evidence indicates dual abilities of IL-22 to serve as protective versus inflammatory mediators depending on several factors (Fig. 2). The protective role of IL-22 has been shown in ConA-induced hepatitis,¹³ alcoholic fatty liver and liver damage,⁷¹ GVHD associated with heart allografts,⁷² autoimmune myocarditis,⁷³ allergic airway inflammation,⁷⁴ and uveitis.⁷⁵ In contrast, the proinflammatory role of IL-22 has also been demonstrated in collagen-induced arthritis.⁷⁶ In skin, IL-22 causes psoriasis,¹⁵ but it may also suppress acne vice versa.⁷⁷ IL-22 may play no role in experimental autoimmune encephalomyelitis.⁷⁸ In infectious models, deficiency of IL-22 makes mice highly susceptible to a broad spectrum of pathogens, including *Klebsiella pneumoniae*,²⁹ *Mycobacterium tuberculosis*,⁷⁹ and cutaneous *Staphylococcus aureus*.⁸⁰

ROLES OF IL-22 IN INTESTINAL INFLAMMATIONS

In addition, IL-22 plays dual roles in intestinal infection, presumably depending on the type of pathogen. IL-22

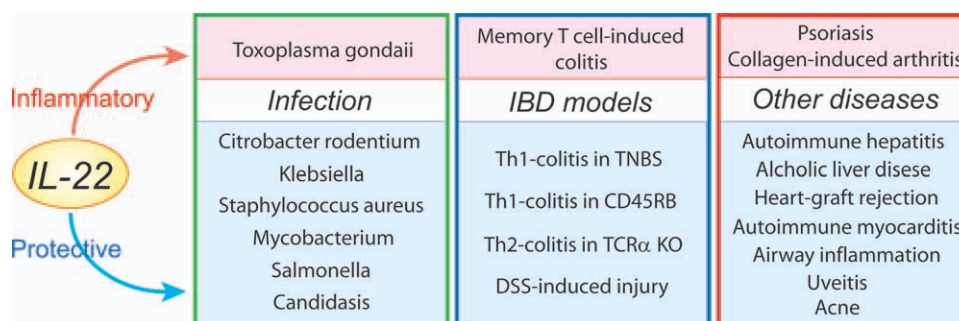


FIGURE 2. Role of IL-22 in inflammatory responses. Experimental diseases, in which IL-22 has been shown to play a proinflammatory role, are indicated in pink boxes. Experimental diseases, in which IL-22 has been demonstrated to play a protective role, are listed in blue boxes.

provides a protective role against *C. rodentium*-induced colitis,^{18,39,41,46} systemic infection with *Salmonella enterica*,⁸¹ and gastrointestinal *Candidiasis* in the absence of IL-17R.⁸² In contrast, IL-22 is required for the development of *T. gondii*-induced immunopathology in the small intestine.³⁵

The potential role of IL-22 in IBD has also been explored using mouse model systems (Fig. 2). In a Th2-mediated chronic colitis model (TCR α KO mice), supplementation of IL-22 expressions in the inflamed colon through a local gene delivery system improves colitis by reinforcing intestinal mucus barrier function.⁹ In a Th1-mediated colitis model (CD45RB model), there is no significant difference in colitis severity when IL-22-deficient versus IL-22-intact CD4⁺ CD45RB^{high} T cells are transferred into recipient recombination activation gene (RAG)1 KO mice that have endogenous IL-22 productions by innate cells.^{10,83} In contrast, IL-22-deficient CD4⁺ CD45RB^{high} T cells cause more severe colitis as compared with IL-22-intact CD4⁺ CD45RB^{high} T cells when IL-22-deficient and RAG1-deficient double knockout mice are used as the recipient.¹⁰ These findings suggest that both donor T cell-derived and recipient (NK cell)-derived IL-22 participate in the suppression of this colitis, but host innate cell-derived IL-22 has a more prominent role in this regard.¹⁰ In contrast, the proinflammatory role of memory CD4⁺ T cell-derived IL-22 in a new colitis model has also been proposed.⁸⁴ The authors propose that adoptive transfer of CD25⁺ cell-depleted CD45RB^{low} memory CD4⁺ T cells can induce colitis in recipient RAG1 KO mice, but IL-22-deficient CD25⁺ cell-depleted CD45RB^{low} memory CD4⁺ T cells are unable to do it.

Unlike the IBD models mentioned above, expression of IL-22 is not elicited in the trinitrobenzene sulfonic acid (TNBS)-induced colitis model, and thus a strategy to induce the expression of IL-22 can be applied for the treatment of this colitis.⁴⁵ Administration of an AHR agonist, Ficz, augments the colonic IL-22 expression and suppresses TNBS colitis.⁴⁵ Neutralization of the IL-22 activity by treatment with anti-IL-22 monoclonal antibodies (mAbs) abolishes the beneficial effect of Ficz on this colitis. Ficz

also has a therapeutic effect on Th1-mediated colitis of the CD45RB model.⁴⁵

ROLE OF IL-22 IN INTESTINAL WOUND HEALING

DSS-induced acute colonic injury spontaneously recovers after termination of DSS treatment, allowing us to closely examine the intestinal wound healing process.^{24,85} In this model, the ability of IL-22 to promote intestinal wound healing has been well proven using different experimental approaches.^{9,10,21,45,57} IL-22 KO mice exhibit impaired recovery from DSS-induced acute colonic injury.²¹ Administration of neutralizing anti-IL-22 Abs also delays the recovery in wildtype mice.⁹ No recovery is observed in IL-22-deficient and RAG1-deficient double knockout mice lacking both T and B cells.¹⁰

In addition to these loss-of-function systems, the role of IL-22 in intestinal wound healing after exposure to DSS has been explored using gain-of-function systems that may be applicable to IBD patients. Supplementation of IL-22 expressions in the colon through a local gene delivery system promotes the recovery with enhanced goblet cell restitution.⁹ In addition, treatment with Ficz, which is capable of augmenting the IL-22 expression, suppresses the development of DSS-induced chronic colitis that is induced by repeated administrations of DSS at each interval.⁴⁵ Interestingly, recovery from DSS-induced acute colonic injury is significantly impaired in IL-23R-deficient and RAG2-deficient double knockout mice that lack IL-22 expression, and administration of recombinant IL-22 rescues the recovery in these mice.⁵⁷

REGULATION OF IL-22 FUNCTION

The function of IL-22 is positively or negatively regulated by some other factors. In psoriasis, the cooperative role of IL-22, IL-17, and IFN- γ may be required to fully establish the pathogenesis.⁸⁶ IL-22 can induce psoriasis-like skin alteration, including acanthosis and hypogranularity, and IL-17 and IFN- γ are also necessary to induce the recruitment of inflammatory cells such as T cells and neutrophils into the region.⁸⁶ In bleomycin-induced airway

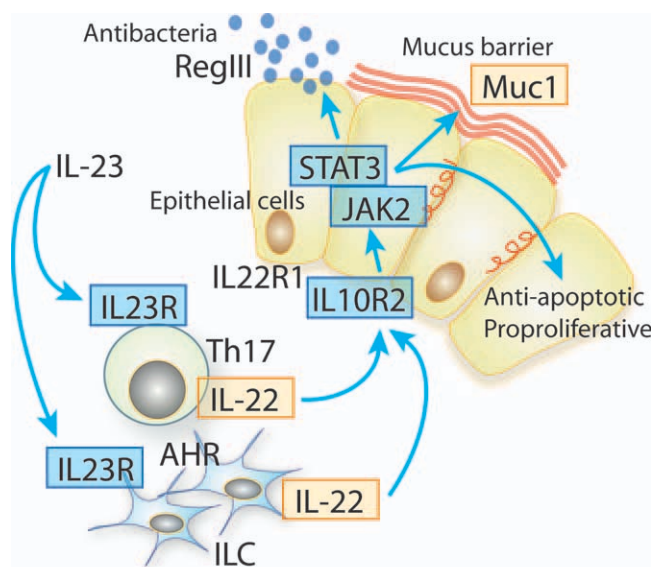


FIGURE 3. Functional linkage of IL-22 to some IBD susceptibility genes. Ligations of IL-23R by IL-23 maintain the expansion of IL-22-producing Th17 cells and induce the development of IL-22-producing ILCs. Subsequent ligation of IL-10R2/IL-22R1 receptor complex by IL-22 activates JAK2/STAT3 pathway in epithelial cells. The activated STAT3 then stimulates epithelial cells to produce RegIII and Muc1 and also promotes epithelial cell regeneration. Well-defined IBD association genes are indicated in blue boxes, and potential IBD association genes indicated in yellow boxes.

inflammation, IL-22 plays a proinflammatory role in the presence of IL-17A and, in turn, plays a protective role in the absence of IL-17A.⁸⁷ This finding suggests that IL-17A governs the proinflammatory versus tissue-protective properties of IL-22.⁸⁷ In addition, the lymphotoxin (LT) pathway, which is induced by the interaction of LT β R with LT β , but not with LIGHT, controls the protective function of IL-22 against *C. rodentium*.⁴⁰

Importantly, the soluble class II cytokine receptor, designated IL-22Ra2 (also called IL-22 binding protein, IL-22BP, or CRF2-10), exists in our bodies.^{88–90} IL-22BP has high affinity binding capability for IL-22 and serves as an endogenous inhibitor of IL-22 activity.^{88–90} The cellular source of IL-22BP has not been fully established, but IL-22BP is highly expressed in placenta, spleen, skin, and lung and to lesser extent in large and small intestine of humans.⁸⁸ IL-22BP expressions are also detectable in normal colon of mice.⁹ Interestingly, distinct expression patterns of IL-22 versus IL-22BP are observed in DSS-induced acute colonic injury—IL-22 expressions increase whereas IL-22BP expressions decrease.⁹ Supplementation of IL-22BP expressions in the inflamed colon through a local gene delivery system significantly delays the recovery from the DSS-induced acute colonic injury and also suppresses goblet cell restitution.⁹ These findings indicate that IL-22 activity in the colon is controlled by its endogenous inhibitor IL-22BP.

ASSOCIATION OF IL-22 WITH IBD SUSCEPTIBILITY GENES

An attractive biological property with IL-22 is the functional association with some major IBD susceptibility genes (Fig. 3). Interaction of IL-23 with IL-23R has been implicated in the maintenance of IL-22-producing Th17 cells^{91,92} and in the development of IL-22-producing innate cells, including ILCs, LT α i cells, and NK cells.^{10,37,38,41,43} Functional polymorphisms within the *IL23r* gene are negatively associated with the development of both CD and UC.^{91,92} IL-22 binds to a heterodimeric receptor composed of IL-10R β and IL-22R1. Polymorphisms of *il10rb* are positively associated with both CD and UC.⁹³ In addition, *il22* is located within a UC-risk locus on chromosome 12q15.⁹⁴ The receptor ligation by IL-22 induces rapid activation of STAT3 through Janus kinase (JAK)2 and TYK2. These *stat3*, *jak2*, and *tyk2* all are well-defined susceptibility genes of CD and to lesser extent UC.^{91,92} The STAT3 activation then stimulates epithelial cells to produce Muc1.^{9,67,68} A recent genome-wide association study proposes *muc1* as a potential candidate gene associated with CD.⁹⁵

CONCLUSION

Despite only 10 years of history since the discovery, enormous efforts and rapid progression in the field of IL-22 have been seen. Indeed, as mentioned above, several unique biological properties of IL-22 have been defined. Importantly, the ability of IL-22 to promote intestinal wound healing in mice has been reproducibly confirmed by independent groups using different experimental approaches, and recent advances in genome-wide association studies have suggested the close association of the IL-22 pathway with some major IBD susceptibility genes. These facts clearly highlight IL-22 as a promising target for IBD therapy. However, given the potential of IL-22 to serve as both protective and proinflammatory mediators, depending on several factors, further extensive work on IL-22 would be necessary to bring novel and practical intervention for improving the lives of patients with IBD in an effective and safe manner.

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Active Site Mutations of *Pseudomonas aeruginosa* Exotoxin A

ANALYSIS OF THE HIS⁴⁴⁰ RESIDUE*

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Pseudomonas aeruginosa exotoxin A (ETA) is a member of the family of bacterial ADP-ribosylating toxins which use NAD⁺ as the ADP-ribose donor. By analogy to diphtheria and pertussis toxins, the His⁴⁴⁰ residue of ETA has been proposed to be one of the critical residues within the active site of the toxin. In this study the role of the His⁴⁴⁰ residue was explored through site-directed mutagenesis which resulted in the production of ETA proteins containing Ala, Asn, and Phe substitutions at the 440 position. The His⁴⁴⁰-substituted ETA proteins were purified and analyzed. All substitutions at the 440 site displayed severely reduced ADP-ribosylation activity (>1000-fold). However, NAD glycohydrolase activity remained intact and in the case of ETAH440N actually increased 10-fold. NAD⁺ binding is not affected by substitutions at the 440 site as indicated by similar *K_m* values for the ETA variants tested. Conformational integrity of the mutant toxins appears to be largely unaffected as assessed by analysis with a conformation-sensitive monoclonal antibody as well as sensitivity to proteinase digestion. In view of the location of His⁴⁴⁰ residue within or close to the proposed NAD⁺-binding site, these results suggest that His⁴⁴⁰ may be a catalytic residue involved in the transfer of the ADP-ribose moiety to the EF-2 substrate.

Pseudomonas aeruginosa exotoxin A (ETA)¹ and diphtheria toxin (DT) are two well characterized members of a group of bacterial protein toxins known as ADP-ribosyltransferases, based upon their enzymatic mode of action. Both toxins catalyze the transfer of the ADP-ribose moiety of NAD⁺ into covalent modification of the eucaryotic protein elongation factor 2 (EF-2). The result is paralysis of the function of the EF-2 protein and the resultant inhibition of protein synthesis in the target cell which the toxin has entered (1, 2).

The three-dimensional structure of the 613-amino acid ETA protein has been resolved to the 3-Å level (3). This study and others (3–6) have led to an assignment of the functional domains within the ETA structure. According to this scheme, residues 405–613 (domain III) and 365–404 (domain Ib) form the catalytic domain responsible for the ADP-ribosyltrans-

ferase activity as well as associated NAD glycohydrolase activity. The N-terminal domains Ia (residues 1–252) and II (residues 253–364) are involved in binding to the ETA receptor and for translocation across the endosomal membrane of sensitive cells, respectively.

Recently the three-dimensional structure of diphtheria toxin has been resolved (7). Although immunological cross-reactivity has not been demonstrated between the intact ETA and DT molecules, considerable sequence homology exists within the enzymatic domains of these two toxins (8, 9). The NAD-binding site in both ETA and DT have been proposed on the basis of computer modeling studies (9, 10). Essentially, the NAD site consists of a hydrophobic cavity composed of residues with aromatic side chains, such as Tyr⁴⁸¹ (Tyr⁶⁵, DT), Tyr⁴⁷⁰ (Tyr⁵⁴, DT), Trp⁴⁶⁶ (Trp⁵⁰, DT), Trp⁵⁵⁸ (Trp¹⁵³, DT), and His⁴⁴⁰ (His²¹, DT). The hydrophobicity of the pocket favors the binding of the nicotinamide and adenine portions of NAD⁺ due to aromatic ring-stacking as well as hydrogen bonding interactions. Two alternative NAD⁺ binding arrangements have been proposed. In one arrangement the nicotinamide ring of NAD⁺ faces Tyr⁴⁸¹ while the adenine ring faces Trp⁴⁶⁶, or vice versa in the alternative arrangement. The His⁴⁴⁰ residue which is positioned at the bottom of the cavity projects its imidazole ring into the cavity and rotates freely in order to facilitate its involvement in hydrogen bonding. Several hydrophilic residues such as Glu⁵⁵³ (Glu¹⁴⁸, DT), Glu⁵⁴⁶ (Glu¹⁴², DT), Glu⁵⁴⁷, Asp⁴⁶¹ (Asn⁴⁵, DT), and Asp⁴⁶³ (Asp⁴⁷, DT) form the edge of the NAD⁺ binding cavity and participate in H-bonding with the two ribose moieties of the NAD⁺ molecule.

This inspection of the ETA active site residues suggests an important role for the His⁴⁴⁰ residue. The importance of His⁴⁴⁰ is also implicated by its strict conservation in the active sites of other ADP-ribosylating toxins, e.g. DT (His²¹), pertussis toxin (His³⁵), cholera toxin (His⁴⁴), and *Escherichia coli* heat-labile enterotoxin (His⁴⁴). The role of His²¹ in DT has been analyzed recently in two independent studies using site-specific substitution with a variety of amino acids (11, 12). Results of both studies indicate that His²¹ is involved in NAD⁺ binding, NAD glycohydrolysis, and ADP-ribosylation of EF-2. Likewise, the analogous His³⁵ residue of pertussis toxin has been found to be critical for NAD⁺ binding and glycohydrolysis (13).

In order to further define and establish the function of the ETA His⁴⁴⁰ residue, we utilized site-directed mutagenesis to insert various amino acids in place of the histidine at position 440. The mutated toxins (ETAH440A, ETAH440N, and ETAH440F) exhibit dramatic reductions of ADP-ribosyltransferase activity as well as a severe reduction of *in vitro* cytotoxicity. However, NAD glycohydrolase activity appears to be undiminished in the case of the His⁴⁴⁰ substitutions studied thus far.

EXPERIMENTAL PROCEDURES

Oligonucleotide-directed Mutagenesis and Toxin Production—An overlap extension polymerase chain reaction (PCR) method (14, 15) was

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¹ The abbreviations used are: ETA, *P. aeruginosa* exotoxin A; DT, diphtheria toxin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EF-2, elongation factor 2; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NAD, nicotinamide adenine dinucleotide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

TABLE I

Overlap extension PCR for oligonucleotide primer-directed mutagenesis at the 440 site within the exotoxin A sequence

Numbers denote nucleotide sequence number of *tox*A and base pairs is denoted by bp. For the PCR no. 1 reaction the *tox*A gene within pGW601 served as template. PCR no. 2 represents the overlap extension of the initial PCR products and includes two far-end primers.

Primer	PCR No. 1	PCR No. 2
His 440 (CAC) to Ala 440 (GCA), Phe 440 (TTC) or Asn 440 (AAC)		
1901 GGCGCGGCCAACGCCGAC	1918	
	1901–2147, 247 bp	
2147 AGGTGCCNNNGTAGCCGACGAAC	2125	
		1901–2400, 500 bp
2131 CGGCTACNNNGGCACCTTCCTCG	2153	
	2131–2400, 269 bp	
2400 ACCTCGCCCGCCGCTCCG	2381	

used to introduce oligonucleotide primer-directed substitutions at position 440 within the ETA sequence. The wild-type ETA gene (*tox*A) in plasmid pGW601 (16) served as a template for the PCR process. Two pairs of primers were used to direct the synthesis of two fragments with a CAC (His⁴⁴⁰) to GCA (Ala⁴⁴⁰), TTC (Phe⁴⁴⁰), or AAC (Asn⁴⁴⁰) mutation in each (Table I). Both fragments overlapped by 17 base pairs and in the presence of two far end primers (primers 1901–1918 and 2400–2381), the PCR process produced a 500-base pair fragment encoding the desired His substitutions (Table I). A 380-base pair *Apa*I/*Xho*I fragment of the 500-base pair PCR product was introduced into pGW601 by substitution with the corresponding wild-type sequence. The resultant plasmids, pXH102 (ETAH440A), pXH105 (ETAH440N), and pXH106 (ETAH440F) were sequenced in order to verify the presence of the correct substitutions (Sequenase version 2.0, U. S. Biochemicals Corp.).

In order to obtain suitable amounts of the altered toxins for study, the *tox*A gene in each plasmid was subcloned in the following manner for expression in the *Tox*A-negative *P. aeruginosa* host strain PA103A which carries an insertionally inactivated *tox*A gene.² A 1.3-kilobase pair *Eco*RI/*Bgl*II fragment from each plasmid was subsequently cloned into pGW580 (16) by fragment replacement, resulting in plasmids pXH202, pXH205, and pXH206 which carry the *tox*A gene under control of its own promoter. In each case, the *tox*A gene was subsequently extracted using a 2.7-kilobase pair *Eco*RI/*Hind*III fragment and inserted into pRO1614, a broad-host range plasmid (18). The resultant plasmids, pXH302 (ETAH440A), pXH305 (ETAH440N), and pXH306 (ETAH440F) were used to transform *P. aeruginosa* strain PA103A, a non-toxicogenic derivative of strain PA103. Transformation of PA103A was carried out according to a previously described procedure (18) and transformants were selected on *Pseudomonas* Isolation Agar in the presence of 300 µg ml⁻¹ carbenicillin (Sigma). Procedures such as plasmid isolation, endonuclease digestion, transformation of *E. coli* cells, and agarose gel electrophoresis of DNA fragments or plasmids, were carried out according to the methods described by Sambrook *et al.* (19).

Wild-type ETA was purified from *P. aeruginosa* strain PA103 transformed with a plasmid (pGW28) containing the *toxR* gene which encodes for a transcriptional activator known to enhance toxin production, as described previously (20). The point-mutated ETA proteins were purified from the *Tox*⁻ PA103A strain transformed with the appropriate plasmid carrying the mutated *tox*A gene. All toxins were purified to homogeneity as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Coomassie Blue staining (21).

Conformation-sensitive ELISA—An enzyme-linked immunosorbent assay format (ELISA) was used to determine conformational changes in the mutant exotoxin proteins. The procedure was carried out at 25 °C according to a published protocol (22) with modifications. Purified monoclonal antibody (TC-1, IgG₂) which has previously been shown to be sensitive to conformational changes in toxin (23) was coated onto Immulon II plates (Dynatech, Alexandria, VA) in 10 mM phosphate-buffered saline (PBS), pH 7.3, for 20 h. Following washing and blocking with 1% bovine serum albumin, purified exotoxin A in PBS containing 1% bovine serum albumin and 0.1% Tween 20 was added and incubated with the immobilized mAb for 2 h. The exotoxin samples were either pretreated with 8 M urea in the presence of 130 mM dithiothreitol (DTT) in order to activate the toxin prior to incubation with the immobilized mAb or left untreated. Toxin molecules captured by the immobilized mAb were detected following the sequential additions of rabbit anti-toxin followed by goat anti-rabbit IgG-horseradish peroxidase-conju-

gated antibody. *ortho*-Phenylenediamine served as the substrate for the horseradish peroxidase conjugate and the resulting absorbance was recorded at 490 nm.

In Vitro Protein Synthesis Assay—The cytotoxicity of wild-type and mutant ETA was determined by measuring the inhibition of protein synthesis using mouse L-929 cells cultured in the presence of trace amounts of [³H]leucine (ICN Radiochemicals, Irvine, CA), as described previously (20). 10⁵ cells were seeded into 24-well culture plates and incubated overnight in RPMI medium (Whittaker Laboratories, Walkersville, MD) supplemented with 10% fetal calf serum. Following incubation, the cells were washed once in leucine-free medium and then incubated in medium to which toxin had been added. Following a 1-h exposure to toxin, the cells were washed once again with leucine-free medium and incubated for an additional hour in leucine-free RPMI. Finally, [³H]leucine (0.3 µCi) was added during the last hour of incubation in order to allow [³H]leucine uptake into the cells and incorporation into newly synthesized protein. In the last step, cells were washed thoroughly and lysed in the presence of 0.1 M NaOH. The cell lysates were collected by filtration and protein-incorporated [³H]leucine was measured by liquid scintillation counting. Lack of [³H]leucine incorporation into cellular proteins is indicative of the inhibition of protein synthesis as a result of exposure to functional toxin.

ADP-ribosyltransferase Assay—The ability of ETA to carry out the ADP-ribosyltransferase reaction was evaluated as described originally by Iglewski and Kabat (1) and modified by Galloway *et al.* (20) using [adenine-¹⁴C]NAD⁺ (Amersham Corp., Arlington Heights, IL) and wheat germ EF-2. Briefly, 0.1 µg of wild-type ETA or 1 µg of mutated ETA was diluted to a final volume of 10 µl in either 10 mM PBS, pH 7.3, or in activation buffer consisting of PBS containing 3 M urea, 30 mM DTT. The diluted toxin was then added to 42 µl of assay buffer (80 mM Tris-HCl, pH 8.0, 70 mM DTT, 1 mM EDTA) containing 41 pmol of radiolabeled NAD⁺ and approximately 1 nmol of wheat germ EF-2. The reaction mixture was allowed to incubate at a specified temperature between 0 °C (ice bath) and 37 °C for 1 (ETA) or 4 h (mutated ETA). The reaction was stopped by the addition of 100 µl of 10% trichloroacetic acid. The precipitated ADP-ribose-EF-2 complex was collected onto Whatman (Whatman Inc.) glass microfilter membranes which were washed with 5% trichloroacetic acid, dried and counted using liquid scintillation methodology.

NAD Glycohydrolase Assay—The NAD glycohydrolase activity of toxin was determined according to a method described by Barbieri *et al.* (24). Toxin (1 µg) was diluted in 50 mM Tris-HCl, pH 8.0, buffer containing a final concentration of 3 M urea plus 30 mM DTT (activated) and incubated in a 25-µl reaction mixture containing 80 pmol of [nicotinamide-³H]NAD (1.26 Ci/mmol), 100 mM Tris-HCl, pH 8.0, 20 mM DTT, and 1 mg/ml egg albumin. The reaction was extended to 4 h at 15 °C and stopped by the addition of 10 µl of 1 M boric acid. Free radioactive nicotinamide was extracted using 250 µl of water-saturated ethyl acetate and counted. Urea/DTT buffer controls were included in order to determine background levels of radioactivity. For steady-state kinetic analysis, serial [³H]NAD⁺ concentrations were used in the assay.

Proteinase Sensitivity Analysis—Native ETA or His⁴⁴⁰-substituted ETA (30 µg, in PBS, pH 7.3) were subjected to limited digestion by trypsin (2 µg, EC 3.4.21.4, Sigma) at 25 °C for varying periods before the reaction was stopped by the addition of soybean trypsin inhibitor (2 µg, Sigma). The resulting digests were evaluated using 15% SDS-PAGE analysis and Coomassie Blue staining.

RESULTS

ADP-ribosyltransferase Analysis—Using the purified wild-type and His⁴⁴⁰-substituted toxins (see Fig. 4) we first evalu-

² D. J. Wozniak, X.-Y. Han, and D. R. Galloway (1994) submitted for publication.

TABLE II
ADP-ribosyltransferase activities of purified toxins with substitutions at site 440

The ADP-ribosylation reaction was carried out using 0.8 μ M [¹⁴C]NAD⁺, crude extract of wheat germ EF-2, and 0.1–1 μ g of toxin. Data presented represents the average of four to five separate determinations.

Residue at site 440	Urea/DTT activated	Nonactivated	Relative activity
	$\text{pmol } \mu\text{g}^{-1} \text{ h}^{-1}$		%
His (wild-type)	214.6 $\pm$ 12.6	64.0 $\pm$ 8.2	100
Ala	0.16 $\pm$ 0.07	0.17 $\pm$ 0.09	0.07 (1/1338)
Phe	0.10 $\pm$ 0.05	0.05 $\pm$ 0.05	0.05 (1/2140)
Asn	0.05 $\pm$ 0.02	0.11 $\pm$ 0.07	0.02 (1/4280)

ated the effect of the His⁴⁴⁰ substitutions on the primary enzymatic activity associated with exotoxin A, namely the ability to ADP-ribosylate eucaryotic EF-2. Our initial results indicated a substantial loss in ADP-ribosyltransferase activity associated with substitution at the His⁴⁴⁰ site (data not shown). The assay was repeated under conditions of lower temperature (15 °C) and a longer incubation period (4 h) in order to obtain measurable counts for evaluation. We have recently observed that 15 °C is an optimum temperature for this assay.³ As shown in Table II, each of the 440-substituted toxins demonstrated a substantial reduction (>1000-fold) in ADP-ribosyltransferase activity, regardless of whether the toxins had been previously activated or not. These results establish that His⁴⁴⁰ occupies a critical position within the active site of the toxin.

An alternative method for the evaluation of ADP-ribosyltransferase activity involves measuring the ability of toxin to inhibit protein synthesis in toxin-sensitive cells. When functional ETA is incorporated into susceptible mouse L-929 fibroblast cells, ADP-ribosylation of EF-2 results in the inhibition of protein synthesis which can be observed by a reduction in [³H]leucine incorporation into newly synthesized protein. As an example, ETAH440A was analyzed in this fashion and it can be seen by the results presented in Fig. 1 that a substantial reduction in the inhibition of protein synthesis occurs, similar to that observed for the His⁴²⁶ mutant (ETAH426L) as described previously (20). The His⁴²⁶ residue has previously been shown to occupy a critical site which may be involved in the interaction with the EF-2 substrate (20, 25).

NAD Glycohydrolase Activity Analysis—In the absence of EF-2, ETA hydrolyzes the NAD⁺ substrate into nicotinamide and ADP-ribose at a slow, but measurable rate (26, 27). In order to determine whether the 440-substituted mutant toxins contain a functional defect in their ability to interact with NAD⁺ or EF-2, or both, the NAD glycohydrolase activities of the wild-type and mutant toxins were compared (Fig. 2). According to published protocols the NAD glycohydrolase activity is generally measured at 37 °C (24, 28). However, we have observed that 15 °C is the optimal temperature for this assay (data not shown) and therefore our assays have been performed at this temperature. The results of our analysis are shown in Fig. 2 and kinetic analysis of the data is presented in Table III. In comparison with the wild-type toxin, all 440-substituted toxins show similar K_m values (<2-fold), implying functional NAD⁺ binding capability. Furthermore, the K_{cat} value of the wild-type toxin is similar to those of ETAH440A and ETAH440F. However, K_{cat} for ETAH440N shows a significant (10-fold) increase. The catalytic efficiency (K_{cat}/K_m) for ETAH440N is approximately 5-fold higher than that of the wild-type toxin. These data indicate that while substitutions at site 440 have severely reduced ADP-ribosyltransferase activity,

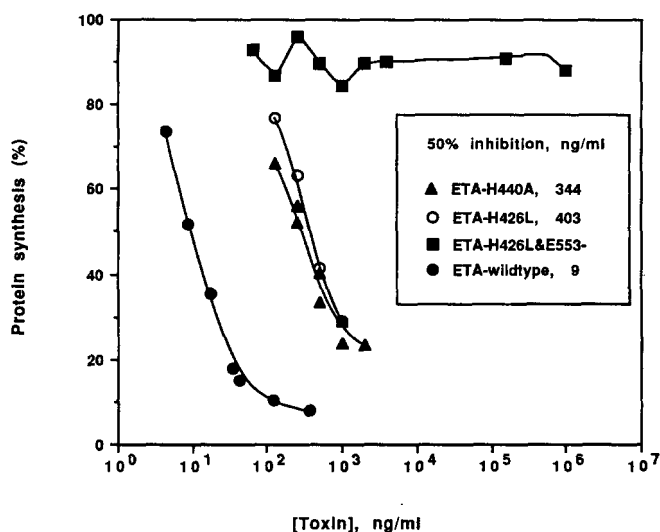


FIG. 1. Inhibition of protein synthesis in mouse L-929 fibroblasts. Mouse L-929 fibroblast cells (10^5 cells/well/24-well plate) were cultured in the presence of trace amounts of [³H]leucine in RPMI medium followed by a 1-h exposure to ETA or mutated ETA at the indicated concentrations. The cells were placed in fresh medium to which 0.3 μ Ci of [³H]leucine had been added and incubated an additional hour. The cells were harvested, washed repeatedly, and lysed in the presence of 0.1 N NaOH. Lysates were collected by filtration and counted using liquid scintillation methodology. The inset indicates the concentration of ETA that resulted in 50% inhibition of [³H]leucine incorporation into protein.

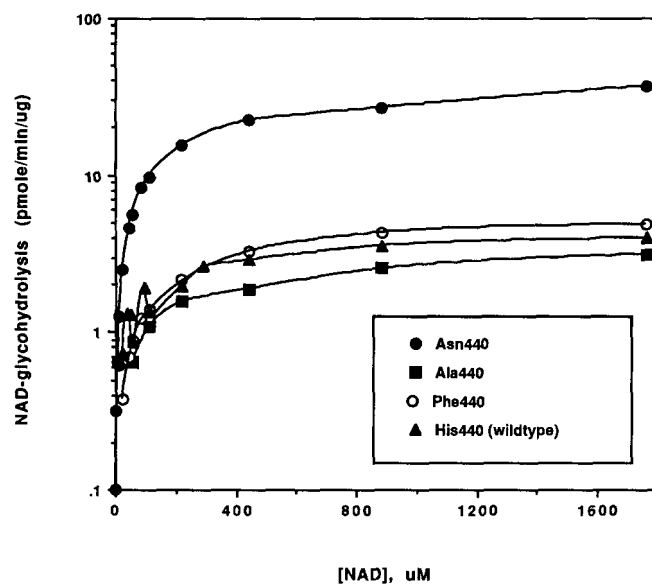


FIG. 2. Comparative analysis of the NAD glycohydrolase activities of wild-type and His⁴⁴⁰-substituted ETA. In each case 1 μ g of purified ETA was activated by treatment with urea/DTT and then assayed for NAD⁺ hydrolysis as described under "Experimental Procedures." Assays were carried out at 15 °C for a 4-h period using the concentrations of [³H]NAD⁺ indicated (10–1800 μ M).

NAD glycohydrolase activity is not adversely affected. Since there is no evidence to suggest that ADP-ribosyltransferase and NAD glycohydrolase activities are catalyzed by separate sites within the ETA structure, it is likely that the inefficient ADP-ribosyltransferase activity associated with these substitutions at the 440 position are caused by defects in the catalytic mechanism or by inefficient interactions with the EF-2 substrate. The increased NAD glycohydrolase activity associated with ETAH440N may be caused by a number of mechanisms, including regional conformational changes within the proposed

³ X.-Y. Han, unpublished observation.

TABLE III
Kinetic analysis of NAD glycohydrolase activity from the data presented in Fig. 2

	Residue at site 440			
	His (wild-type)	Phe	Ala	Asn
K_m (μ M)	156	245	191	300
V_{max} (μ mol min ⁻¹ μ g ⁻¹)	3.7	4.8	2.9	37.5
K_{cat} (h ⁻¹)	15	19	11	150
relative K_{cat}	1.0	1.3	0.7	10.0
K_{cat}/K_m (μ M ⁻¹ h ⁻¹)	0.1	0.08	0.06	0.5
relative K_{cat}/K_m	1.0	0.8	0.6	5.1

NAD⁺-binding site. A conformational assessment of the toxins was therefore carried out.

Conformational Analysis of ETA—It has previously been shown that ETA is produced in a nonfunctional configuration which must be activated in order to demonstrate ADP-ribosyl-transferase activity (29). We have previously described the use of a monoclonal antibody designated TC-1 which is able to distinguish between the inactive and active configurations of ETA by binding to an epitope within the 419–432 sequence which assumes an α helical conformation within domain III of the toxin (23). The TC-1 mAb binds to the epitope which is apparently exposed when ETA is in the active configuration and fails to bind to the inactive configuration of ETA. Therefore the TC-1 antibody can be used as a probe of unfolding or conformational change of the toxin molecule. In this study mAb TC-1 was used to analyze possible conformational changes resulting from substitutions at the 440 site. Immunoblotting analysis indicated similar binding to the unfolded toxin molecules regardless of substitution at site 440 (data not shown). Using a capture ELISA format we analyzed the ability of the conformation-sensitive TC-1 mAb to bind to both the activated (unfolded) and non-activated forms of wild-type and mutant ETA, as shown in Fig. 3. Our results indicate that all the activated configurations of toxin bind the TC-1 mAb equally well (as supported by the immunoblotting analysis). The non-activated toxins do not bind the TC-1 mAb as readily, as expected. However, non-activated ETAH440F exhibits an intermediate degree of TC-1 binding (Fig. 3), suggesting possible destabilization or conformational alteration of the ETA structure as a result of placement of a phenylalanine residue at position 440. The presence of a bulky aromatic ring contributed by the Phe residue within the 440 site may result in local conformational shifts or destabilization which produces a conformational change at the 419–432 helix.

In a separate analysis of conformational differences among the 440-substituted toxins we used proteinase sensitivity as an indication of conformational integrity. Our analysis of trypsin sensitivity (Fig. 4) of the modified ETA proteins indicates that the His⁴⁴⁰ substitutions do not render ETA more sensitive to trypsin digestion than the wild-type ETA. These results suggest that the substitutions at the 440 site do not result in destabilization of the ETA structure.

DISCUSSION

In this study we have demonstrated that the His⁴⁴⁰ residue of ETA plays a critical role in the ADP-ribosylation of elongation factor 2. Previous studies have proposed that His⁴⁴⁰ of exotoxin A, His²¹ of DT, and His³⁵ of pertussis toxin are functionally homologous residues within the NAD⁺-binding sites of their respective proteins (8, 9). Analysis of the three-dimensional structures of ETA and DT reveal that ETA His⁴⁴⁰ and DT His²¹ occupy essentially identical positions within the active sites of these toxins (3, 7). Using a site-directed mutagenesis approach in which the ETA His⁴⁴⁰ residue is replaced by Ala, Asn, or Phe provides evidence that substitution of the

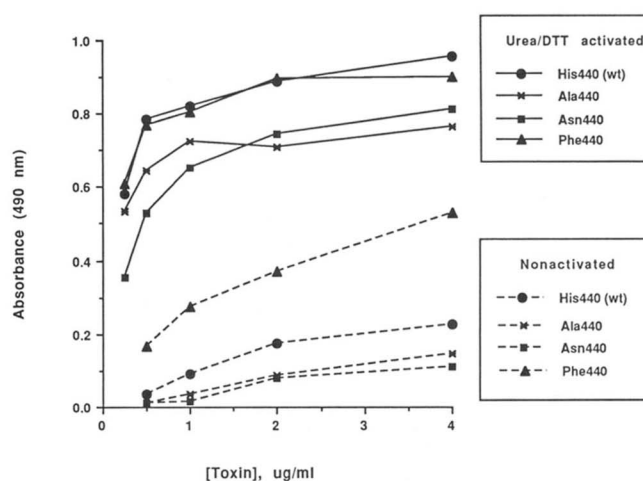


FIG. 3. ELISA-based capture assay for conformational analysis of activated and non-activated ETA and His⁴⁴⁰-substituted ETA. Individual wells of Immulon II (Dynatech, Alexandria, VA) 96-well microtiter plates were coated with purified IgG of the TC-1 mAb (2 μ g/ml). Activated and non-activated toxins were added and incubated for 2 h at 25 °C. TC-1-bound toxin was subsequently detected using high-titered rabbit anti-ETA antibody and donkey anti-rabbit IgG-horseradish peroxidase conjugate as described under "Experimental Procedures."

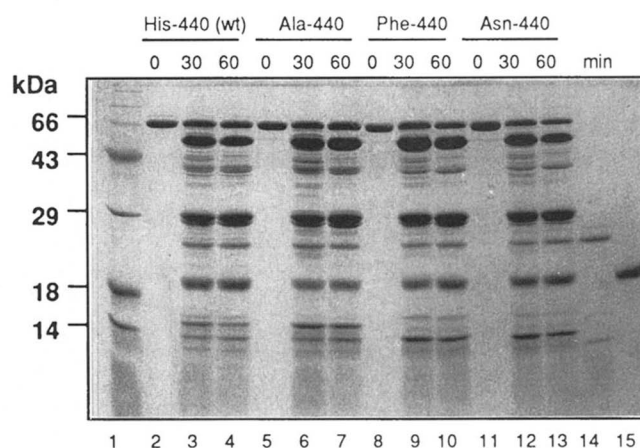


FIG. 4. Trypsin digestion profiles of purified ETA and His⁴⁴⁰-substituted ETA. Purified toxins (1.5 μ g/ μ l) were digested in the presence of trypsin (0.08 μ g/ μ l) for either 30 or 60 min at 25 °C. The reactions were stopped by the addition of soybean trypsin inhibitor and the resulting digests analyzed using 15% SDS-PAGE and Coomassie Blue staining. Lanes 2, 5, 8, and 11 represent undigested toxin; lanes 3, 6, 9, and 12 represent 30-min digests; and lanes 4, 7, 10, and 13 represent 60-min digests; lanes 14 and 15 represent trypsin (3 μ g) and soybean trypsin inhibitor (3 μ g), respectively.

histidine at the 440 position reduces the ADP-ribosyltransferase activity of the toxin more than 1000-fold (Table II). This result is substantiated by the further observation that a replacement of the histidine at the 440 position substantially reduces the ability of the toxin to inhibit protein synthesis in an *in vitro* assay (Fig. 1). In view of the apparent analogy of ETA His⁴⁴⁰ with His²¹ of DT these results are not surprising.

Significantly, and in contrast to the findings recently reported for both DT and PT (11–13) alteration of the ETA His⁴⁴⁰ residue does not appear to eliminate or reduce the NAD glycohydrolase activity associated with the toxin. In fact, substitution of an Asn residue at this position results in an apparent 10-fold increase in the NAD glycohydrolase activity (Fig. 2, Table III). Analysis of the kinetics of the NAD glycohydrolase reaction indicates that the catalytic efficiency (K_{cat}/K_m) of this reaction is similar for both the Ala⁴⁴⁰ and Phe⁴⁴⁰ substitutions,

but increases 5-fold in the case of Asn⁴⁴⁰ substitution (Table III). However, similar K_m values for both the wild-type and mutant toxins implies that the NAD⁺ binding function remains intact (Table III). These results are supported by fluorescence quenching experiments which indicate that NAD⁺ binding in the mutant toxins is unaffected by substitutions at the 440 position (data not shown). The increased NAD glycohydrolase activity associated with the Asn⁴⁴⁰ substitution in ETA may result from the effects of the polar side chain of the residue. It seems unlikely that the Asn side chain is directly involved in the formation of hydrogen bonds with NAD⁺ during cleavage of the *N*-glycosidic bond, as appears to be the case in DT, since by analogy with DT substitution with Asn at this position should result in some retention of ADP-ribosyltransferase activity. Similarly, the nonpolar Ala⁴⁴⁰ and Phe⁴⁴⁰ substitutions in ETA should result in reduced NAD glycohydrolase activities as observed in the case of DT if the same hydrogen bonding model applies. Our findings suggest that ETA His⁴⁴⁰ does not appear to follow the model proposed for DT His²¹. Rather, Asn⁴⁴⁰ substitution may facilitate NAD glycohydrolysis in some indirect fashion. One possibility is that the polar side chain may allow the ready access of water molecules in order to accept the ADP-ribose moiety following cleavage of the *N*-glycosidic bond through the involvement of as yet unidentified residues. Another possibility is regional conformational changes which could allow more efficient NAD glycohydrolysis to occur.

Similar site-directed mutagenesis studies have recently been reported for the analysis of the His²¹ residue of diphtheria toxin (11, 12). In these studies it was reported that substitution of His²¹ with Arg, Leu, Ala, and Gln significantly reduces both ADP-ribosyltransferase and NAD glycohydrolase activities. The reduction in these activities was shown to be due to reduced NAD⁺ binding as revealed by fluorescence quenching analysis. Interestingly, an Asn²¹ substitution only reduced the ADP-ribosyltransferase activity 3-fold. This conservation of function may be due to the ability of the amide δ -nitrogen to form hydrogen bonds. Consequently Blanke *et al.* (11) proposed that the primary function of His²¹ is to form a hydrogen bond with the nicotinamide carboxymide of NAD⁺. This interaction would facilitate formation of the initial DT-NAD complex, orient the *N*-glycosidic bond of NAD⁺ for nucleophilic attack by the incoming diphthamide residue of EF-2, and stabilize the transition state intermediate of this reaction. Similar mutagenesis studies of the His³⁵ residue of pertussis toxin also indicate the involvement of this residue in NAD⁺ binding and glycohydrolysis (13).

However, the results of the present study do not support a direct role for the ETA His⁴⁴⁰ residue in NAD⁺ binding or hydrolysis. Our results do indicate that His⁴⁴⁰ is required for the ADP-ribosyltransferase reaction and therefore suggest that the His⁴⁴⁰ residue must either be involved in the interaction with EF-2 or participate in the catalysis of the reaction. It is difficult to measure the interaction with EF-2 directly. However, direct contact of His⁴⁴⁰ with the incoming EF-2 substrate is unlikely since His⁴⁴⁰ is located on the floor of a hydrophobic cavity within the active site of ETA (3). Furthermore, a role for the His⁴⁴⁰ residue in maintaining an appropriate conformation for interaction with EF-2 seems unlikely in view of the fact that sterically conservative substitutions of Ala and Asn do not maintain ADP-ribosyltransferase activity. In view of these considerations it may be that His⁴⁴⁰ plays a catalytic role in the ADP-ribosylation reaction. This hypothesis is supported by the location of the residue within the active site (30) and by its close spatial relationship with Glu⁵⁵³, a crucial catalytic residue (27). Glu⁵⁵³ was initially identified using an NAD⁺-photolabeling technique (31)

and upon further analysis a catalytic function was suggested for this residue (32, 33). These studies demonstrated that deletion of Glu⁵⁵³, or substitutions with Asp or Cys at this site all abolished ADP-ribosyltransferase activity and cytotoxicity, yet produced little or no change in NAD⁺ binding or glycohydrolysis. In conjunction with studies of Glu¹⁴⁸ of DT, a functional homologue of Glu⁵⁵³, it was found that both the negative charge and the chain length of the carboxyl group of ETA Glu⁵⁵³ (or DT Glu¹⁴⁸) are crucial with respect to catalytic function (17, 27). Therefore it was proposed that the carboxylate serves to deprotonate the incoming diphthamide on EF-2, thereby activating the nitrogen at position 1 of the diphthamide imidazole ring which then imposes a nucleophilic attack on the *N*-glycosidic bond of NAD⁺ in a direct displacement mechanism (30). In some respects the functional features of Glu⁵⁵³ appear to be similar to those of His⁴⁴⁰, as demonstrated in the present study. The fact that the Asn⁴⁴⁰ substitution severely reduces catalysis of the ADP-ribosyltransferase reaction suggests that it is not the hydrogen bond-forming ability of His⁴⁴⁰, but rather the acid-base properties which may be important for catalysis. The aromatic nature of the His⁴⁴⁰ imidazole ring seem less important since substitution of a Phe residue at this position does not maintain function. Although the data presented in this study is not sufficient to propose a detailed catalytic mechanism, we speculate that the acid-base properties of His⁴⁴⁰ may contribute to the interaction with the incoming EF-2 diphthamide residue, stabilize the transition state intermediate, and facilitate the transfer of the ADP-ribose moiety to the diphthamide site.

The functional differences between His⁴⁴⁰ of ETA and His²¹ of DT have encouraged us to search for additional active site residues which may be critical for ADP-ribosyltransferase activity. However, an inspection of the active site structure of ETA failed to indicate potential residues which might serve a function homologous to the His²¹ of DT (data not shown).

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Mutational Analysis of Domain I of *Pseudomonas* Exotoxin

MUTATIONS IN DOMAIN I OF *PSEUDOMONAS* EXOTOXIN WHICH REDUCE CELL BINDING AND ANIMAL TOXICITY*

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Pseudomonas exotoxin (PE) is a single polypeptide chain that contains 613 amino acids and is arranged into three structural domains. Domain I is responsible for cell recognition, II for translocation of PE across membranes and III for ADP ribosylation of elongation factor 2. Treatment of PE with reagents that react with lysine residues has been shown to lead to a reduction in cytotoxic activity apparently due to a modification of domain I (Pirker, R., FitzGerald, D. J. P., Hamilton, T. C., Ozols, R. F., Willingham, M. C., and Pastan, S. (1985) *Cancer Res.* 45, 751–757). To determine which lysine residues are important in cell recognition, all 12 lysines in domain I were converted to glutamates by site-directed mutagenesis. Also, two deletion mutants encompassing almost all of domain I (amino acids 4–252) or most of domain I (amino acids 4–224) were studied. The mutant proteins were produced in *Escherichia coli*, purified, and tested for their cytotoxic activity against Swiss 3T3 cells and in mice. The data indicate that conversion of lysine 57 to glutamate reduces cytotoxic activity towards 3T3 cells 50–100-fold and in mice about 5-fold. Deletion of amino acids 4–224 causes a similar reduction in toxicity towards cells and mice. Deletion of most of the rest of domain I (amino acids 4–252) causes a further reduction in toxicity toward cells and mice indicating this second region between amino acids 225 and 252 of domain I is also important in the toxicity of PE. Competition assays indicated that the ability of PE^{Glu57} to bind to 3T3 cells was greatly diminished, accounting for its diminished cytotoxic activity.

Pseudomonas exotoxin A (PE)¹ is a potent toxin that is secreted by *Pseudomonas aeruginosa* and may have an important role in the pathogenesis of *Pseudomonas* infections. The injection of as little as 0.2 µg of PE into mice results in death due to massive destruction of the liver. PE is made up of a single polypeptide chain with a molecular mass of 66,000 daltons. Crystallographic studies have shown that PE contains three distinct domains (1). Expression of different portions of the PE gene in *Escherichia coli* has revealed that domain I is responsible for cell recognition, domain II is responsible for translocation across membranes and domain III for ADP ribosylation of elongation factor 2, the cytoplasmic target of the PE molecule (2).

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¹ The abbreviation used is: PE, *Pseudomonas aeruginosa* exotoxin A.

Cell recognition plays a key role in toxin-mediated cytotoxicity, since toxins that lack a binding domain do not kill cells. Previously, we demonstrated that the removal of domain I of PE rendered the resulting protein, termed PE40, virtually harmless for cells in culture (3). However, the removal of such a large (27 kDa) portion of the toxin does not help locate individual regions or key amino acids that may be important for binding.

The treatment of PE with iminothiolane and similar reagents that react with lysine residues results in a molecule with diminished cytotoxicity toward Swiss 3T3 cells and other cell types but full ADP ribosylation activity (4). Within the PE molecule there are 12 lysine residues in domain I, none in domain II, and 3 in domain III. Because iminothiolane treatment did not diminish the ability of PE to ADP ribosylate elongation factor 2 when measured in cell-free extracts of wheat germ, it seemed likely that iminothiolane was reacting with 1 or more lysine residues present in domain I and by doing so decreased the ability of PE to bind to cultured cells. To test this hypothesis, we have used site-directed mutagenesis to convert the lysine residues in domain I to glutamate residues. Our studies indicate that modification of lysine 57 to glutamate greatly decreases the toxicity of PE toward cultured cells and also has a small effect on the toxicity of PE in mice.

We have also studied the activity of two other modified forms of PE in which most of domain I, amino acids 4–224, or virtually all of domain I, amino acids 4–252, were deleted. Deletion of amino acids 4–224 produces a molecule with the same toxic activity as PE^{Glu57}. However, a larger deletion, amino acids 4–252, produces a molecule with very low toxicity toward mice and cultured cells indicating amino acids 225–252 are very important for the toxic activity of PE in animals.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Bacteriophage—*E. coli* strain JM101 (r_K⁺, m_K⁺, Δ(lac, proA,B)/F⁺, supE, thi, traD36, proA,B, lacI ZM15) was used as the host for propagation of M13 mp19 or M13 mp18. Uracil-containing single-stranded DNA was made from phage propagated in RZ1032 (dut[−], ung[−]). Plasmids pVC4, 45, and 205 have been described previously (5). pVC4 carries gene for full length PE. pVC45 encodes full length PE with OmpA signal sequence. The signal sequence is processed away, causing secretion of the protein in the periplasm. pVC205 encodes PE43 with signal sequence. All the plasmids express the recombinant protein under a T7 promoter-based expression system. HB101 (F[−], recA13, hsdS20(r_B[−], m_B[−]) ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl-5, mtl[−], supE44, λ[−], from Bethesda Research Laboratories (BRL) was used for propagations of plasmids. BL21λ (DE3) which carried T7 RNA polymerase gene in lysogenic and inducible form (6) was used as host for recombinant PE expression. M13 mp19 and M13 mp18 double-stranded DNAs were from BRL.

The strategy used to obtain the desired mutants is outlined in Fig.

1. Oligonucleotide-directed mutagenesis was carried out by a modification of the method of Kunkel (7). Oligonucleotides with one or more mutation(s) were synthesized using an Applied Biosystems DNA synthesizer. A 0.9-kilobase *Hind*III-*Sal*I fragment of pJH4 (2) was subcloned into M13mp19. Single-stranded DNA prepared from second cycle phage in uridine-containing medium was used as a template for *in vitro* mutagenesis. Mutants were identified by DNA sequencing (8). After the mutation was confirmed, the appropriate DNA fragment was prepared from replicative form DNA with appropriate restriction enzymes and ligated with vector DNA prepared from pVC4.

To make PE mutants with negligible ADP-ribosylating activity (9), a 1-kilobase *Sal*I-*Eco*RI fragment from pVC4 was subcloned into M13mp18, in which a codon for Glu at position 553 was changed to one for Asp with the same strategy described above. After identification of the mutations, a 0.5-kilobase *Bam*HI-*Eco*RI fragment of mutant replicative form DNA was subcloned back into pVC45.

Mutant proteins were screened for their killing activity on Swiss 3T3 cells. Cells were plated at a density of 5×10^4 /well in 24-well dishes. One day after plating, 50 μ l of serial dilutions of bacterial extracts from isopropyl-1-thio- β -D-galactopyranoside-induced cultures was added to the cells. Cells were stained with 0.5% Methylene Blue dissolved in 1:1 methanol:H₂O (v/v) 3 days after the addition of toxin.

Protein synthesis inhibition assays were performed as described (10). Swiss 3T3 cells were used to test the biological activity of various mutant PE molecules. For competition assays, Swiss 3T3 cells were plated at a density of 1×10^5 /well in 24-well dishes one day before use. Purified competitor, either PE^{Asp553} or PE^{Glu57,asp553}, was added to cells for 5 min prior to the addition of various amounts of PE. Cells were initially incubated for 15 min at 37 °C, then washed twice and further incubated for 4 h. Protein synthesis was measured after a 1-h incubation with [³H]leucine (2).

ADP ribosylation activity of bacterial extracts and purified PE was measured by the procedure of Collier and Kandel (11) using wheat germ extracts enriched in elongation factor 2.

Expression of PE was carried out as described previously (10) with minor modifications. The cell pellet from 500 ml of culture was disrupted by sonication in 5 ml of 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 10 mM EDTA, 10 mM dithiothreitol. The pellet obtained by centrifugation at $10,000 \times g$ for 10 min was dissolved in 5 ml of a solution containing 6 M guanidine HCl, 0.1 M Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol, pH 8.0, left at 20 °C for 10 min, and recentrifuged at $10,000 \times g$ for 10 min. The supernatant was diluted 20-fold with a solution containing 6 M guanidine HCl, 50 mM Tris-HCl, 1 mM EDTA, 1.25 mM reduced glutathione, 1.0 mM oxidized glutathione, pH 8.5, and dialyzed against 20 mM Tris-HCl,

1 mM EDTA, pH 8.0. The clear supernatant after centrifugation was applied on a Mono Q column (HR 10/10 attached to a Pharmacia LKB Biotechnology Inc. fast protein liquid chromatography system) at a flow rate of 3 ml/min and washed with Buffer A (20 mM sodium phosphate, pH 7.0) until the absorbance at 254 nm came down to the base line. Proteins were eluted with a 240-ml linear gradient of NaCl (0–350 mM in Buffer A) and finally with 80 ml of 1 M NaCl in Buffer A.

PE43 was prepared from the periplasm fraction of *E. coli* BL21 carrying the pVC205 plasmid as described previously (5). The cell pellet from an isopropyl-1-thio- β -D-galactopyranoside-induced culture was gently washed with 100 ml of 20% sucrose in 20 mM Tris-HCl mM EDTA pH 8.0, and then suspended in 100 ml of prechilled water. After keeping on ice for 10 min, the cell suspension was centrifuged at 8000 rpm for 10 min. The supernatant was applied to a Mono Q column in a similar manner as described above. The fraction containing ADP ribosylation activity was further purified by using a TSK 250 column (0.75 $\times$ 60 cm) attached to a Pharmacia LKB Biotechnology Inc. fast protein liquid chromatography system and eluted with 0.2 M sodium phosphate, pH 7.0, containing 1 mM EDTA at a flow rate of 0.5 ml/min. In this procedure, 8.7 mg of purified PE43 was obtained from a 2-liter culture.

Animal toxicity tests were performed using either BALB/c mice (6–8 weeks) or NIH mice (6–8 weeks). Samples were mixed with sterile pyrogen-free 0.9% NaCl containing 10 mg/ml human serum albumin, and 0.5 ml was injected intraperitoneally.

RESULTS

Cell-killing Activity—Because we suspected that 1 or more lysine residues in domain I were responsible for the extreme cytotoxicity of PE on Swiss 3T3 cells, we converted the lysine residues to glutamate residues. Fig. 2 shows the various mutants obtained. Some contain single mutations, whereas others have multiple mutations. A summary of the ability of the mutant molecules to kill Swiss 3T3 cells is shown in Table I.

In each experiment, extracts from three clones each containing the same mutation were tested along with three controls: (i) extract from a clone making full length PE; (ii) extract from a clone making PE40, which is a mutant protein missing domain I (2); and (iii) an appropriate amount of extraction buffer. To cover a wide range of activity, dilutions of 1:100, 1:500, and 1:4500 were made for each mutant extract and 50- μ l aliquots of each dilution added to cells.

Two mutants with multiple glutamate substitutions were

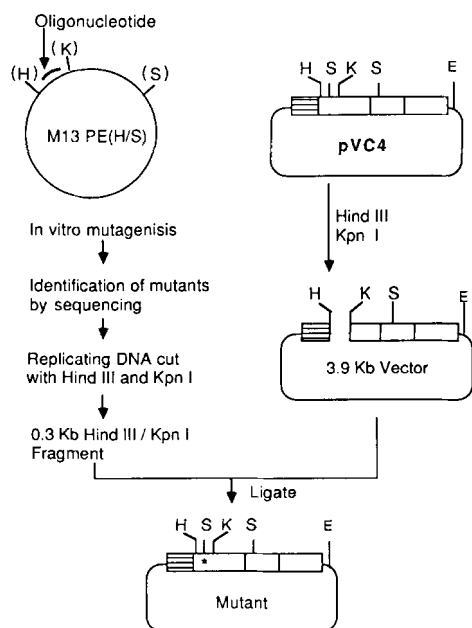


FIG. 1. The strategy used to make and express the PE mutants. E, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Sal*I. $\square$, T7 promoter; $\square$, PE gene; *, mutation. kb, kilobase(s).

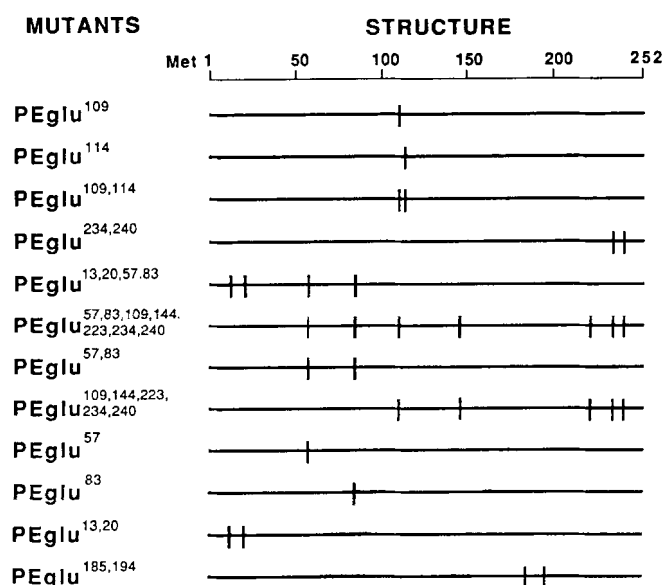


FIG. 2. Structure of PE mutants. Domain I of PE is drawn as a horizontal line. The lysine residues in domain I of PE were changed to glutamate residues. The location of each changed residue is shown by a vertical line.

TABLE I

Cell killing and ADP-ribosylating activity of various mutant toxins

Aliquots (50 μ l) of serially diluted total bacterial cell extracts were added to Swiss 3T3 cells plated at a density of 5×10^4 cells/well in 24-multiwell dishes 1 day after seeding. Three days after addition of extracts, cells were stained with Methylene Blue. For ADP-ribosylating activity assays, 5 μ l of 100-fold dilution of 8 M urea, 0.1 M dithiothreitol-pretreated extract was used in a 250- μ l reaction mixture containing 2.4 μ M [^{14} C]NAD (6×10^4 cpm) and 10 μ l of wheat germ extract.

Mutants	Cell-killing activity	ADP-ribosylating activity
		% of PE
PE ^{Glu109}	100	ND
PE ^{Glu114}	100	ND
PE ^{Glu109,114}	100	ND
PE ^{Glu234,240}	100	ND
PE ^{Glu13,20,57,83}	~2	101
PE ^{Glu57,83,109,144,223,234,240}	<2	95
PE ^{Glu57,83}	<2	99
PE ^{Glu109,144,223,234,240}	50–60	95
PE ^{Glu57}	<2	102
PE ^{Glu83}	100	ND
PE ^{Glu13,20}	100	ND
PE ^{Glu185,194}	100	ND

found to have reduced killing activity. One of these was PE^{Glu57,83,109,144,223,234,240}, and the other was PE^{Glu13,20,57,83}. With the former, no cell-killing activity was evident with 50 μ l of a 1:100 dilution, and this was equivalent to 50 μ l of a 1:4500-fold dilution of a clone-making native PE. If this reduced activity resulted from a single mutation, then either Glu-57 or Glu-83 could be implicated since both were present in each of the constructs. Furthermore, a double mutant PE^{Glu57,83}, also had very low cytotoxic activity. When constructs with single mutations were examined, PE^{Glu57} had very low cytotoxic activity, whereas PE^{Glu83} was fully active. These results strongly implicate lysine 57 has having an important role in the cytotoxic activity of PE.

The only mutant not containing Glu-57 that had diminished cell-killing activity was PE^{Glu109,144,223,234,240}. Mutations in positions 109, 234, and 240 produced some loss in cytotoxicity. It seemed possible that lysines 144 and 223 also contributed to the high cytotoxic activity of PE, but more quantitative assays failed to suggest this possibility (see below).

The ADP-ribosylating activity of various mutant PE molecules was also examined where appropriate, and all of the molecules were found to be fully active (Table I). Furthermore, all the mutant molecules were intact and had a M_r of approximately 66,000 when detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting (data not shown).

Protein Synthesis Inhibition Assays—To confirm the results from cell-killing assays, protein synthesis inhibition assays, which are somewhat more quantitative, were carried out using cell extracts (Fig. 3 and Table II). The mutants which had lysine 57 replaced by glutamate showed a 50–100-fold decreased ability to inhibit protein synthesis. PE^{Glu109,144,223,234,240} had almost the same ID₅₀ value as that of wild type PE, although its cell-killing activity seemed to be weaker than PE (Table I). These results strongly suggested that lysine 57 is important for the cytotoxic effect of PE on Swiss 3T3 cells.

To eliminate the possibility that factors present in *E. coli* extracts were affecting the results, we purified PE, PE^{Glu57}, and PE^{Glu234,240} from extracts of *E. coli* expressing the appropriate plasmids.

After guanidine extraction and renaturation, the extracts were applied to a Mono Q column and the toxins eluted with

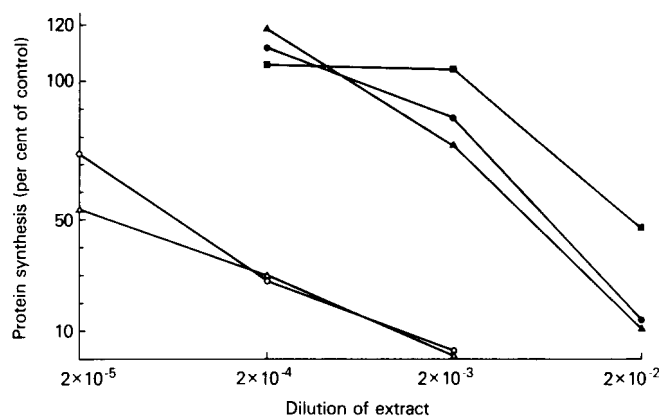


FIG. 3. Protein synthesis inhibition in Swiss 3T3 cells by various mutant PE molecules. 50 μ l of serially diluted cell extracts was added to cells. After an overnight incubation, the cells were labeled with [^{3} H]leucine for 1 h. $\circ$, PE; Δ , PE^{Glu109,144,223,234,240}; $\bullet$, PE^{Glu57}; $\blacktriangle$, PE^{Glu57,83}; and $\blacksquare$, PE^{Glu57,83,109,144,223,234,240}.

a gradient of NaCl. As shown in Fig. 4, each of the toxins eluted in close to the same position, with PE^{Glu234,240} eluting later than PE^{Glu57} which, in turn, eluted slightly later than PE. Their position reflects the additional negative changes present in the mutant proteins. The purity of the three purified toxin molecules is shown in the inset to Fig. 4.

These purified proteins were then examined for their ability to inhibit protein synthesis in 3T3 cells. In agreement with the assays using crude extracts, PE^{Glu57} had greatly reduced cytotoxicity when compared with PE or PE^{Glu234,240} (Fig. 5 and Table II).

Competition Assay—To demonstrate that the change of lysine 57 to glutamate 57 affected cell binding and not some other function of PE, various competition binding assays were performed. To carry out these competitions, the ADP-ribosylating activity of each molecule used for competition was reduced to a negligible level by changing Glu-553 to an Asp residue (9). Cells were pretreated with either PE^{Asp553} (which was defective in ADP-ribosylating activity but had full binding activity) or PE^{Glu57,Asp553} (which was defective in ADP-ribosylating activity and had unknown binding activity). Following a 5-min pretreatment with either PE^{Asp553} or PE^{Glu57,Asp553}, cells were incubated with various concentrations of PE for 15 min. Cells were then washed and incubated for a further 4 h. Incorporation of [^{3} H]leucine was then used to measure protein synthesis. Results are shown in Fig. 6 and indicate that PE^{Asp553} at 1, 10, and 25 μ g/ml competed for PE activity, but PE^{Glu57,Asp553} showed no competition at 1 and 10 μ g/ml and only a small amount of competition at 25 μ g/ml. These data show that the change from lysine 57 to glutamate 57 reduced the ability of PE to compete for PE binding sites.

Animal Toxicity Studies—Because conversion of PE to PE^{Glu57} diminished the cytotoxic effect of PE by 50–100-fold, we expected it to be considerably less toxic to mice. Previously, it was shown that the LD₅₀ of PE for a 20-g mouse is ~0.2 μ g. This is confirmed in Table III. To examine the toxicity of the various toxins, 0.2–10 μ g of purified toxin was injected intraperitoneally into mice. Unexpectedly, all mice died within 2 days after the injection of 1 μ g or more of PE^{Glu57}. As an additional control, mice were injected with PE^{Glu234,240}. This mutant protein was also very cytotoxic. On examination, mice injected with a lethal dose of PE^{Glu57} showed liver necrosis indistinguishable from mice injected with native PE. Since PE40, which has a complete deletion of domain I (amino acids 4–252), only produced liver toxicity at very high doses (~50 μ g), it appeared that a second cell recognition region, present

TABLE II
 ID_{50} of various mutant toxins on Swiss 3T3 cells (ng/ml)

Cell extracts (50 μ l) (Experiment 1) or purified proteins (Experiments 2, 3, and 4) were added to Swiss 3T3 cells. Cells were plated at 1×10^5 cells/well in 24-well dishes. After an overnight incubation, 5 μ Ci of [^{3} H]leucine was added and the cells were incubated for 1 h more. In Experiment 1, the ID_{50} of the mutants was calculated based on a 1 ng/ml ID_{50} for native PE. ND, indicates not determined.

Toxin	Expt. 1	Expt. 2	Expt. 3	Expt. 4
PE	1	0.4	0.9	0.5
PE ^{Glu57}	94	20	50	ND
PE ^{Glu57,83,109,144,223,234,240}	235	ND	ND	ND
PE ^{Glu57,83}	74	ND	ND	ND
PE ^{Glu109,144,223,235,240}	0.6	ND	ND	0.4

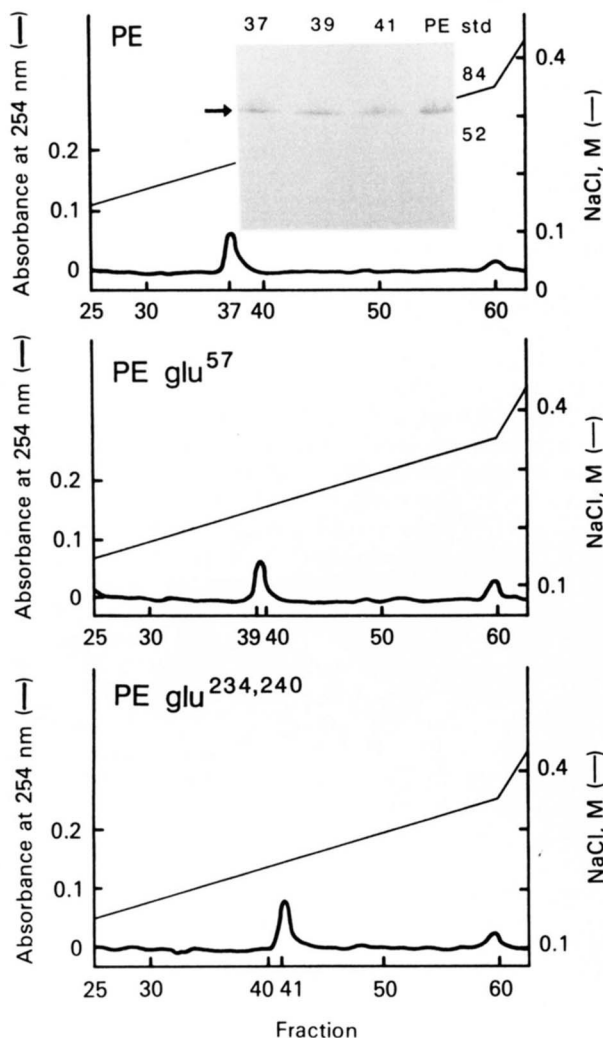


FIG. 4. Purification of PE. Upper panel, PE; middle panel, PE^{Glu57}, and lower panel, PE^{Glu234,240}. The inset in the upper panel shows a Coomassie Blue-stained sodium dodecyl sulfate-polyacrylamide gel of purified recombinant PE; from left, fraction 37 of PE, fraction 39 of PE^{Glu57}, fraction 41 of PE^{Glu234,240}, and native PE from Swiss Serum and Vaccine Institute. std, molecular weight standards.

in domain I of PE, interacted with the liver. This interaction was not effected in a major way when lysine 57 was changed to glutamate 57.

Another Function in Domain I—To confirm the presence of a second function in domain I, a mutant in which amino acids 4–225 were deleted was constructed. Domain I ends at amino acid 252. This deletion removes 10 of the 12 lysine residues present in domain I, including Lys-57. The protein produced has a M_r of 43,000 and is termed PE43. PE43 was

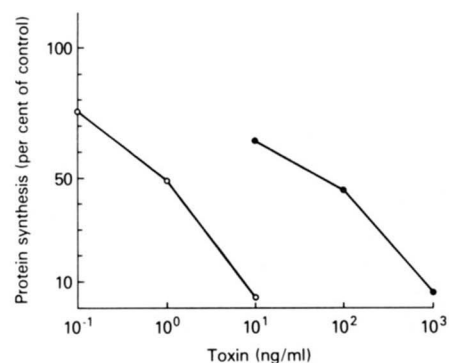


FIG. 5. Protein synthesis inhibition on Swiss 3T3 cells by PE^{Glu57}. Protein synthesis was examined using purified toxins. The procedure is described in Fig. 2. ○, PE; ●, PE^{Glu57}.

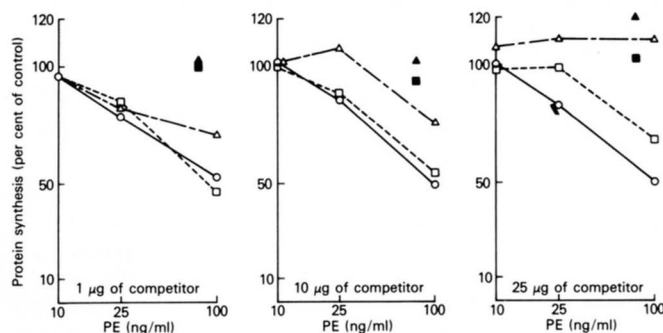


FIG. 6. Competition of PE binding to Swiss 3T3 cells. Cells were pretreated with 1, 10, or 25 μ g/ml of PE^{Asp553} or PE^{Glu57,Asp553} for 5 min. Then, native PE was added for 15 min, and cells were washed and reincubated for a further 4 h. [^{3} H]Leucine was then added for 1 h to determine relative levels of protein synthesis. ○, PE; △, PE + PE^{Asp553}; □, PE + PE^{Glu57,Asp553}; ▲, PE^{Asp553}; ■, PE^{Glu57,Asp553}.

TABLE III
 Toxicity of PE mutants in mice

Protein	Amount injected μ g	Number of deaths
PE	1	2/2
	0.4	2/2
	0.2	2/2
PE ^{Glu57}	0.1	0/2
	1	2/2
	0.8	0/2
PE ^{Glu234,240}	0.4	0/2
	1	2/2

purified as described under "Materials and Methods" and tested for inhibition of protein synthesis on Swiss 3T3 cells. The ID_{50} of PE43 is 100 ng/ml, which is the same as PE^{Glu57}

TABLE IV

Comparison of the activity of PE, PE^{Glu57}, PE43, and PE40ID₅₀, amount needed to inhibit protein synthesis by 50% in Swiss 3T3 cells.

Protein	ID ₅₀	Mouse lethal dose
		μg
PE	1.0 ng/ml	0.2
PE ^{Glu57}	100 ng/ml	1.0
PE43	100 ng/ml	1.0
PE40	>2000 ng/ml	50.0

(Table IV). In contrast, the ID₅₀ of PE40 is >2000 ng/ml. Thus, the removal of amino acids 4–225 of domain I had the same effect as mutating Lys-57 to Glu-57.

When PE43 was injected into mice it also had the same toxicity as PE^{Glu57} with a lethal dose of about 1 μg . Removal of an additional 22 amino acids generates PE40. The lethal dose of PE40 is about 50 μg (Table IV and Ref. 2). Mice injected with 1 μg of PE43 died on day 2 just as mice injected with 1 μg of PE^{Glu57} or 0.2 μg of PE, and liver necrosis was also evident. These results indicate that sequences present between amino acids 225 and 253 are very important for the toxicity of PE against 3T3 cells and against mice. Thus, there are two regions of domain I of PE that are responsible for its toxicity.

DISCUSSION

The first step in the cytotoxic action of PE is binding to a cell surface receptor (12). To determine the amino acids involved in the binding of domain I of PE to the cell, we have used site-directed mutagenesis to change all of the 12 lysine residues to glutamate. It seemed likely that a lysine was important for cell binding because treatment of PE with iminothiolane or other reagents that react with primary amines greatly decrease the toxicity of PE without affecting its ADP ribosylation activity (4). There are 12 lysine residues in domain I of PE, none in the translocation domain (II), and 3 in domain III. Glutamate was chosen as the mutant amino acid because we wished to keep the size of the amino acid about the same. Furthermore, the change in charge of an amino acid which was probably located on the cell surface should not have a large effect on the three-dimensional structure of the protein.

The data clearly show that lysine 57 is very important for the cytotoxic activity of PE on Swiss 3T3 cells. Changes in the other 11 lysine residues had very little effect on the activity of PE. In the structure published by Allured *et al.* (1), lysine 57 is located on the surface of the molecule where it might interact with a cellular receptor. Unfortunately, the nature of the cellular receptor is not yet known. To test the binding activity of the mutation at Glu-57, experiments were designed to test whether high concentrations of PE with either lysine or glutamate at residue 57 could compete for the binding of native PE. A mutant form of PE with greatly diminished ADP-ribosylating activity was used for these experiments so that the presence of excess PE would not kill the cells. It had been shown previously that conversion of glutamic acid to

aspartic acid at position 553 reduced ADP-ribosylating activity to very low or undetectable levels (9). The results clearly indicate that when lysine occupies position 57, PE^{Asp553} can bind and compete, but when glutamic acid is present there is little or no competition. Therefore, when lysine 57 is changed to glutamic acid 57, the resulting loss in cell-killing activity is due to reduced cell binding.

The reduction in cell-killing activity was consistently 50–100-fold compared with native PE. When *in vivo* toxicity in mice was similarly compared, there was, at most, a 5-fold difference. Mice die from liver toxicity when lethal amounts of native PE are given. The injection of 1 μg of PE^{Glu57} also caused death due to liver toxicity. As with native PE, death occurred 24–48 h after administration of the lethal dose. In contrast, about 50-fold more of a molecule which is devoid of domain I (PE40) was required to cause death in mice. These data indicate that the interaction of PE with the liver probably does not involve lysine 57 in an important way and that the amino acid residues which might interact with the putative second receptor reside elsewhere in domain I.

To narrow down the liver toxic region, amino acids 4–225 were deleted. The liver toxicity remained. Since deletion of amino acids 4–252 greatly decreased liver toxicity, the liver toxic region probably lies between amino acids 225 and 252. In the three-dimensional structure of PE, residues 57 and 225–252 are widely separated (1). Studies to precisely localize the liver toxic residues are now in progress.

In summary, domain I of PE has two regions that are required for toxicity. Lysine 57 is important for binding to Swiss 3T3 cells but has a relatively small effect on liver toxicity, whereas amino acids lying between residues 225 and 252 have a more important role in liver toxicity. Separation of these two functions may help produce more effective immunotoxins which can be given in large amounts without unacceptable toxicity (13).

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