Brief Definitive Report

A COMMON ANTIGENIC DETERMINANT FOUND IN TWO FUNCTIONALLY UNRELATED TOXINS

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A number of small molecular weight proteins (<5,000) can be grouped together on the basis of their high sulfhydryl content. In general, the cysteines of these proteins are involved in intramolecular disulfide linkages that yield thermostable molecules having a constrained tertiary structure. For example, the *Escherichia coli* heat-stable enterotoxins ST Ia and ST Ib (1, 2), neurotoxins (4–5) and geographutoxins (6), bee venom apamin (7), and the antiinflammatory agent, peptide 401 (8), possess a sulfhydryl content that exceeds 20% of their amino acid composition (see Fig. 1). Because of their small size, these toxins are amenable to analysis of the role of the disulfide network in structure and functional design. One may speculate that their primary sequence encodes two major levels of structure. On one level, the tertiary structure determines the proper linkage of the disulfide bridges, yielding a relatively rigid peptide backbone. A second level of structural complexity, dependent on the establishment of the preceding level, encodes for properly positioned side chains involved in their toxicity.

In this report, we demonstrate that the two structural levels can be partly dissociated. We have observed that two functionally unrelated toxins of less than 15 amino acids possess a homologous region that specifies a common antigenic determinant, indicative of similar tertiary structures.

Materials and Methods

Synthetic Peptides. ST Ib (6-19) and Ser^{11}ST Ib (10-15) were synthesized by stepwise solid phase peptide synthesis on a Beckman 990B peptide synthesizer (Beckman Instruments, Inc., Fullerton, CA). The amino acid protection strategy and the peptide cleavage approach were similar to previously described methodology (9). Aqueous solutions of the crude peptides (10^{-5} M) were adjusted to pH 9 with ammonium hydroxide and were left to oxidize for 5 d at room temperature. The pH of these solutions was then lowered to 6 with acetic acid and the peptides were recovered by lyophilization. These analogs were injected on a preparative LiChroprep RP-18 column (Merck & Co., Inc., Rahway, NJ) (2 × 85 cm) operating at a flow rate of 150 ml/h. The column was initially washed with 0.1% trifluoroacetic acid (TFA)/water and the peptides were eluted by developing the

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column with 0.1% TFA/15% acetonitrile/85% water for Ser[11]ST Ib (10-15) or 0.1% TFA/25% acetonitrile/75% water in the case of ST Ib (6-19). The purified peptides yielded a single peak when injected on an analytical LiChrosorb RP-18 high pressure liquid chromatography column. Peptide composition and concentration were verified by amino acid analysis.

Synthetic conotoxin GI was purchased from Peninsula Laboratories, Inc., Belmont, CA. The peptide was shown to be pure by thin-layer chromatography and the sequence composition was proven correct by amino acid analysis.

Guanylate Cyclase and Suckling Mouse Assays. The methodology used for both assays has been described (10). Only ST Ib (6-19) caused diarrhea in mice (5 ng minimal dose) and resulted in an increase in cyclic GMP levels in brush border preparations (1 ng minimal dose).

Neurotoxicity Test. The synthetic peptides were serially diluted and injected intramuscularly in suckling mice. Neurotoxicity was confirmed by the resulting flaccid paralysis or death of the animals 20 min after the injection.

Preparation of Peptide Conjugates. Aqueous solutions of synthetic peptide, carrier protein, and 1-ethyl-3(-3-dimethylaminopropyl)carbodiimide (EDAC) were prepared separately and their respective pH adjusted to 4.5–6 with 1 M HCl. These solutions were then mixed to yield a ratio of 1:1:2 (peptide/EDAC/carrier; wt/wt). After an overnight reaction period at room temperature, the peptide conjugates were desalted on a Sephadex G-25 column (2.5 × 18 cm) equilibrated in 0.1 M ammonium bicarbonate.

Immunization Scheme. Two New Zealand white female rabbits weighing 5–6 lbs were immunized with each conjugate. The animals initially received, by subscapular and intramuscular injection, 1 mg of the peptide–bovine serum albumin (BSA) conjugate emulsified in complete Freund's adjuvant. 8 wk later, a 0.5 mg dose of peptide–bovine gamma globulin (BGG) in incomplete Freund's adjuvant was administered. The rabbits were bled 9 d later.

Immunological Assays. Enzyme-linked immunosorbent assays (ELISA) were performed in triplicate in wells coated overnight with thyroglobulin or peptide-thyroglobulin conjugates (10–15 μg per well). After a 2 h incubation period with four concentrations of IgG (0.001, 0.004, 0.010, and 0.040 mg/ml) prepared from peptide antiserum, the wells were washed and then treated with goat anti-rabbit IgG alkaline phosphatase conjugate for an additional 2 h period. Color development was recorded at 405 nm, 20 min after the addition of the substrate solution. The absorbance of wells treated with IgG prepared from preimmune serum was <0.05 for all IgG concentrations tested.

Results and Discussion

The heat-stable enterotoxins ST Ia and ST Ib elaborated by enterotoxigenic *E. coli* strains cause diarrhea in man and other mammals (11) (Fig. 1). Their mechanism of action is thought to involve the stimulation of intestinal brush border guanylate cyclase (12–14). These toxins share a partial sequence homology with a family of short sea snail neurotoxins collectively known as α-conotoxins (6, 7) (Fig. 1). The intraperitoneal injection of α-conotoxins in mice results in paralysis of skeletal muscle thought to occur by inhibition of acetylcholine binding to its receptor at motor endplates (5).

To test whether the homologous segment of the two classes of toxin had a comparable tertiary structure, we prepared a 14-amino acid synthetic analog, termed ST Ib (6-19), and a six-residue peptide representing the region common to both toxins, termed Ser[11]ST Ib (10-15) (Fig. 1). Synthetic conotoxin GI was used in this study. The enterotoxic activity of ST Ib (6-19) was confirmed by both guanylate cyclase assay and the suckling mouse bioassay. Conotoxin GI was shown to induce flaccid paralysis in mice (results not shown). ST Ib (6-19) did not cause flaccid paralysis and conotoxin was not enterotoxic. Each analog was

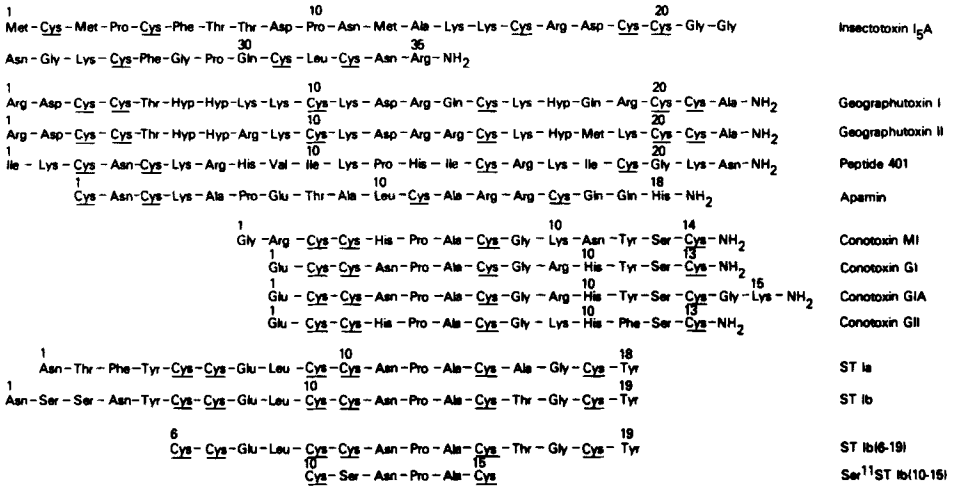

FIGURE 1. Primary sequence of several short, cysteine-rich peptides. ST Ib (6–19) and Ser[11]ST Ib (10–15) are synthetic analogs used in the present study. Note the six-residue sequence homology between conotoxins and ST I toxins. NH_2 represents a C-terminal amide group. Literature references to each primary sequence are listed in the text.

then coupled with carbodiimide to BSA, BGG, and bovine thyroglobulin. Antibodies to the peptide-BSA and peptide-BGG conjugates were raised in rabbits. For each peptide antiserum, a purified IgG fraction was obtained by ammonium sulfate precipitation and chromatography on DEAE Sephacel (16).

The results obtained from ELISAs using the peptide-thyroglobulin conjugates as the solid phase antigens are depicted in Fig. 2. Both toxin conjugates were bound by IgG prepared from antiserum to the homologous region Ser[11]ST Ib (10-15) (Fig. 2*A*), indicating that it exhibits the structural features of a common antigenic determinant. Moreover, anti-ST Ib (6-19) serum cross-reacted well with conotoxin GI (Fig. 2*B*). Since conotoxin GI does not induce diarrhea in suckling mice, nor does the intraperitoneal injection of ST Ib (6-19) in mice result in flaccid paralysis, one must conclude that the shared domain represents a structurally conserved region of both toxins that does not encode all of the sequence information needed for toxicity. Part of the homologous segment, however, has been implicated in the enterotoxic activity of ST Ia, since the substitution of Asn-12 by a tyrosine in ST Ia yielded a peptide that was 30–40 times less active than native ST Ia (17).

The conserved region appears to be normally immunorecessive, since it is not bound by antibody to ST Ib (6-19) (Fig. 2*B*) or to conotoxin GI (Fig. 2*C*). These findings indicate that the intact toxins share an element of secondary or tertiary structure that is absent in the homologous peptide segment. Alternatively, the missing element of tertiary structure required for recognition by antitoxin antibodies might be the disulfide-linked cysteine that is replaced by serine in the six-residue analog.

The cross-reactivity of the antitoxin antibodies was not symmetrical. ST Ib (6-19) was less antigenic than conotoxin GI for antibody to the heterologous toxin conjugate (Fig. 2, *B* and *C*). Note, however, that only two types of conotoxin GI–carrier conjugate can result from carbodiimide coupling that involve either

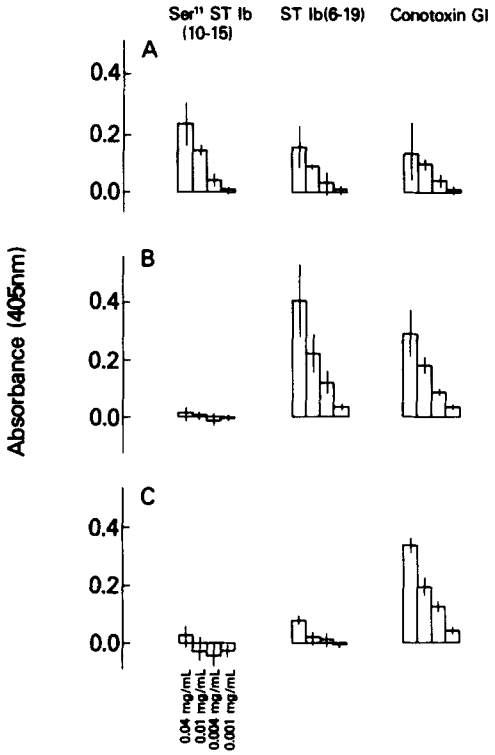

FIGURE 2. The antigenic relatedness of Ser[11]ST Ib (10-15), ST Ib (6-19), and conotoxin GI. Histograms were constructed from the ELISA results. Cross-reactivity of (*A*) anti-Ser[11]ST Ib (10-15) IgG; (*B*) of anti-ST Ib (6-19) IgG; (*C*) and of anti-conotoxin GI IgG, with thyroglobulin conjugates of each of the three peptides. The ELISA assay is described in Materials and Methods.

the amino terminal or the carboxylic group of Glu-1 (Fig. 1). These two groups are closely positioned in the sequence. The C-terminal end of conotoxin GI is an amide derivative and will not react with carbodiimide. In contrast, ST Ib (6-19) can be linked through either its amino or carboxyl terminal in addition to the carboxylic acid side chain of Glu-3. This results in an IgG response to two structurally similar conotoxin GI–carrier conjugates, as opposed to an antibody population directed against three different presentations of ST Ib (6-19) on its carriers.

The functional and structural significance of the intrachain disulfide bonds in these toxins was not directly addressed. The correct pairing of the cysteine residues in the oxidized state is probably essential for activity and may be promoted by features of the secondary structure (18). In this regard, the homologous tetrapeptide Asn-Pro-Ala-Cys represents a strong turn-forming region (19), which might promote the disulfide association between Cys-2 and Cys-7 of conotoxin GI and possibly Cys-10 and Cys-15 of ST Ib (20).

The intramolecular disulfide bonds characteristic of these toxins (Fig. 1) result in a compact, thermostable structure, relatively resistant to enzymatic digestion. This feature would promote the integrity of ST Ib in intestinal secretions.

However, these toxins may be related by their ability, once contact with their cell surface receptor occurs, to undergo a mixed disulfide reaction with a disulfide bond or a free sulfhydryl group near the receptor-binding site. For instance, the acetylcholine receptor possesses a critical disulfide bridge near the acetylcholine-binding site which can be reduced or oxidized with concomitant inhibition and restoration of the response to acetylcholine (21). This region of the acetylcholine receptor may well represent a possible binding site for conotoxin GI (15). Similarly, cystamine reversibly inhibits both the binding of ST Ia to brush border membranes and ST Ia–induced activation of guanylate cyclase (22), suggesting the presence of at least one accessible sulfhydryl group on the ST Ia receptor molecule.

Summary

The heat-stable enterotoxin ST Ib produced by enterotoxigenic *E. coli* strains shares a sequence homology with the sea snail neurotoxin, conotoxin GI. Rabbit antisera were raised against synthetic analogs of these toxins and to a six-residue peptide representing the region common to both toxins. Results from enzyme-linked immunosorbent assays indicate that the homologous region of both toxins represents part of their antigenic site. The lack of cross-reactivity exhibited by the six-residue common domain with serum directed against either toxin suggests that this region probably retains a similar conformation in the intact toxins but not in the isolated fragment.

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