

Primary Structure of Diphtheria Toxin Fragment B: Structural Similarities with Lipid-binding Domains

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ABSTRACT Two different lipid-associating domains have been identified in the B fragment of diphtheria toxin using automated Edman degradation of its cyanogen bromide peptides, secondary structure prediction analysis, and comparisons with known phospholipid-interacting proteins.

The first domain is located in the highly hydrophilic (polarity index [PI] = 61.0%) 9,000-dalton N-terminal region of fragment B. This region shows primary and predicted secondary structures dramatically similar to those found for the phospholipid headgroup-binding domains of human apolipoprotein A1 (surface lipid-associating domain).

The second domain is located in the highly hydrophobic (PI = 32.4%) middle region of fragment B. Its structure resembles that found for the membranous domain of intrinsic membrane proteins (transverse lipid-associating domain).

In contrast, the hydrophilic C-terminal 8,000-dalton region of fragment B (PI = 53.8%) does not show structural similarity with lipid-associating domains.

Diphtheria toxin (62,000 daltons) is a typical example of the group of toxic proteins that use receptor-mediated internalization to reach their cytoplasmic targets (20). The proteolytically activated molecule consists of two functionally distinct fragments linked together by a disulfide bridge. The C-terminal fragment B (40,700 daltons) binds to specific eukaryotic cell-membrane receptors and mediates the entry of the enzymically active N-terminal fragment A (21,150 daltons) into the cytoplasm where A catalytically ADP-ribosylates elongation factor 2 and, thereby, inhibits protein synthesis of the cell (9).

The primary structure of fragment A and most of its catalytic properties are known (11, 14, 17, 18). Fragment B has been shown to contain two functional regions, (a) the 17,000-dalton C-terminal region, which is involved in the binding to the specific cell receptors (25), and (b) the 23,000-dalton N-terminus, which participates to the interaction of fragment B with the cell membrane bilayer (1, 2). However, neither the molecular mechanism of this interaction nor the type of interaction is known.

Finding answers to these questions would be helped by a knowledge of the amino acid sequence of fragment B. In a previous work (12), we isolated and characterized the cyanogen bromide peptides of fragment B; their proposed molecular weights and alignment were: CB4 (4,700 daltons), CB2 (14,000 daltons), CB5 (2,500 daltons), CB1 (12,000 daltons), and CB3 (8,000 daltons) (Fig. 4).

This work reports, for the first time, a partial amino acid sequence of fragment B which includes the N-terminal amino acid sequence of peptides CB2, CB1, and CB3, and the complete primary structure of peptides CB4 and CB5.

Comparison of these sequences with those of known lipid-interacting proteins and conformational predictions allowed us to identify two distinct lipid-associating domains; a hydrophilic surface lipid-associating domain, as found in serum apolipoproteins, located at the N-terminus of fragment B, and a hydrophobic transverse lipid-associating domain, as found in intrinsic membrane proteins, located in the middle region of fragment B.

MATERIALS AND METHODS

Materials

Partially purified diphtheria toxin was purchased from Connaught Laboratories, Toronto, Canada, and purified to homogeneity, according to Michel and Dirkx (17), in the presence of 20 mM phenylmethylsulfonyl fluoride. Polyamide thin-layer sheets (F 1,700) were obtained from Schleicher and Schüll, GmbH, D-3354 Dassel, W. Germany. Reagents and solvents for automated amino acid sequence analysis, all sequanal grade, were obtained from E. Merck D-1600 Darmstadt, W. Germany. All other chemicals were analytical grade.

Methods

The cyanogen bromide peptides of diphtheria toxin fragment B were prepared according to Falmagne et al. (12). Automated Edman degradation was performed

with an updated 890 B Beckman sequencer (Beckman Instruments, Inc., Fullerton, Calif.) using the 0.1 M Quadrol program of Brauer et al. (3). All peptides, except the largest CNBr peptide CB2, were degraded in the presence of 3 mg of Polybrene Aldrich-Europe, B-2340 Beerse, Belgium (15).

The phenylthiohydantoin derivatives were quantitatively identified by gas-liquid chromatography on Chromosorb WHP coated with 10% SP-400 (Supel Co., Inc., Bellefonte, Pa.) (21), by reverse-phase high pressure liquid chromatography on Partisil-10 ODS-2 (Whatman, Inc., Clifton, N. J.) in a methanol gradient (23–56%, vol/vol) in 0.005 M sodium acetate (26), and qualitatively by thin-layer chromatography on 5.0 × 5.0 cm polyamide sheets (16).

Conformational predictive analysis was applied according to the method of Chou and Fasman (6), using their improved sets of α -helical, β -pleated sheet, and β -reverse turn conformational parameters (7, 8).

Polarity indexes of the polypeptide segments were calculated according to Capaldi and Vanderkooi (5) as the mole percent of the total hydrophilic residues of a considered segment. The hydrophobic index of the apolar face of an amphipathic helix was computed according to Segrest and Feldmann (22).

RESULTS

Amino Acid Sequence Analysis

All amino acid sequences were determined by automated Edman degradation of 50–200 nmol of peptides. Fig. 1 shows the N-terminal sequences of the large CNBr peptides CB1 (12,000 daltons), CB2 (14,100 daltons), and CB3 (8,000 daltons) and the complete sequences of the smaller CNBr peptides CB4 (41 residues) and CB5 (25 residues).

The sequence of CB5 was completed by C-terminal degradation with carboxypeptidases A and B (10). To obtain its complete sequence, CB4 was further cleaved at its arginyl residue with trypsin after citraconylation of the lysines and at its tryptophanyl residue with cyanogen bromide in the presence of heptafluorobutyric acid (19).

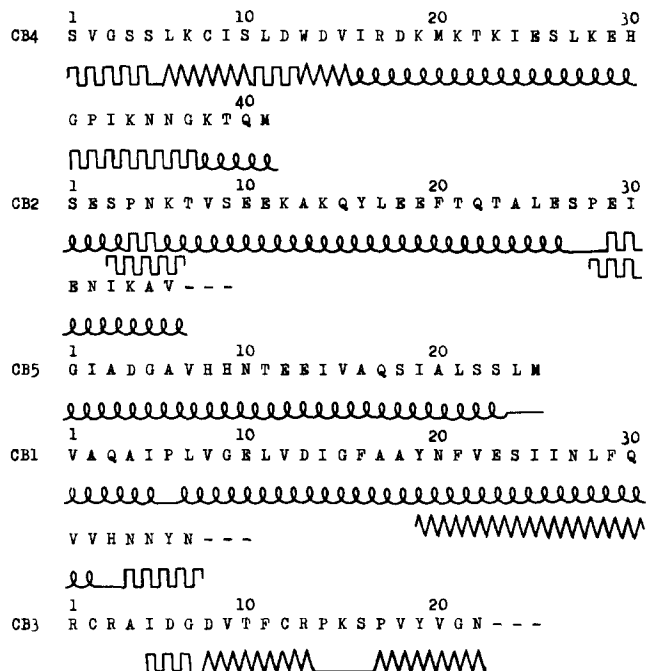


FIGURE 1 Complete amino acid sequences of CNBr peptides CB4 and CB5 and N-terminal amino acid sequences of CNBr peptides CB1, CB2, and CB3. One letter amino acid abbreviations used are: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophane; Y, tyrosine. Predicted secondary structures are presented using the following symbols: α -helix (Ω), β -pleated sheet (\wedge), β -turns or loops (Ω), and nondefined (—) conformations. In case of ambiguity, both conformations are presented.

According to the alignment proposed by Falmagne et al. (12) (Fig. 4), peptide CB4 and the N-terminal 36-residues segment of the adjacent peptide CB2 cover the 77-residues N-terminal sequence of fragment B; peptide CB5 and the N-terminal sequence of CB1 are in the middle region of B; the N-terminal sequence of CB3 is located at ~8,000 daltons from the C-terminus.

Because fragment B behaves like a membrane protein (1, 2), we first calculated the polarity indices of the known sequences.

The N-terminal 77-residues segment of B exhibits a polarity index of 61.0%, which is much higher than the value of $47 \pm 6\%$ proposed by Capaldi and Vanderkooi (5) for water-soluble proteins and demonstrates the extremely hydrophilic character of the N-terminal region of fragment B.

In contrast, the N-terminal sequence of CB1, located in the middle region of B, has a polarity index of 32.4%, which is similar to the polarity indices of the hydrophobic segments of membrane proteins, such as cytochrome b_5 (30.0%), cytochrome b_5 reductase (35.0%), Semliki Forest virus glycoprotein (28.0%), and glycoporphin (29.0%) (4).

Polarity indices of CB5 (44.0%) and of the N-terminal 22-residues segment of CB3 (40.9%) are close to the mean value of most water-soluble proteins.

The difference between the highly hydrophilic N-terminal and the highly hydrophobic middle segments of fragment B is stressed by the fact that both are enclosed in that limited portion of fragment B known to interact with membrane lipids, i.e., the 23,000-dalton N-terminus. We, therefore, compared the structure of these two strikingly different segments with the structure of known lipid-interacting proteins.

At the present time, two such structures have been fully studied and described. They are (a) the surface lipid-associating domains of plasma lipoproteins, specifically designed to associate with the polar headgroups of the phospholipids and (b) the transverse lipid-associating domains of intrinsic membrane proteins that interact specifically with the hydrocarbon core of the phospholipid bilayer (23).

Structural Properties of the N-terminal Hydrophilic Segment of Fragment B

Comparison of the 77-residues N-terminal hydrophilic sequence of fragment B with the sequence of human apolipoprotein A1 (Apo A1) revealed a significant degree of similarity (the probability that the similarity could be caused by chance equals 2×10^{-7}). Indeed, when residue 155 of Apo A1 is aligned on residue 1 of fragment B (Fig. 2), identical or homologous residues appear in 34% of the positions. Most homologies are clustered between residues 14–31 and 48–70 of fragment B, corresponding in Apo A1 to residues 168–185 (50% homology) and 202–223 (49% homology), respectively. Comparison of the conformational analyses of the homologous segments in the two proteins showed that α -helix is predicted for all of them.

To visualize the distribution of polar and apolar residues on the predicted helical segments, their helical nets were drawn. The projection of helix 16–30 of fragment B (Fig. 3A) clearly shows that this helix is amphipathic; it has a narrow hydrophobic face (residues Ile 16, Met 20, Ile 24, and Leu 27) surrounded by a large hydrophilic face (Fig. 3A, 2). On this face (Fig. 3A, 1), positively charged residues are at the periphery (residues Arg 17, Lys 19, 21, 23, and 28, and His 30) and negatively charged residues at the center (residues Asp 18, Glu 25 and 29), forming ion pairs or triplets. The helical segment 47–67 of

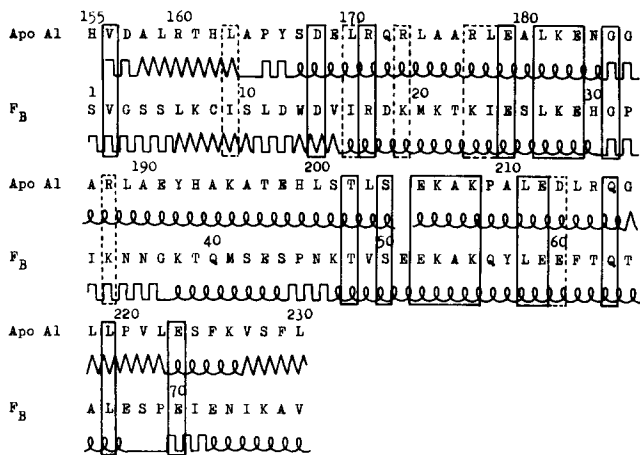


FIGURE 2 Comparison of the amino acid sequences and predicted secondary structures of segment 1-77 of diphtheria toxin fragment B and segment 155-230 of human apolipoprotein A1. One letter annotations are used as in Fig. 1. Straight lines surround identical residues and dashed lines surround homologous residues.

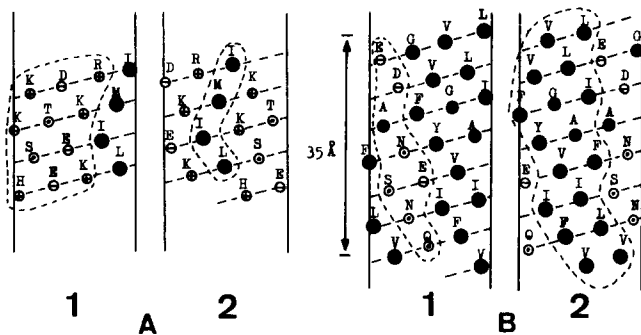


FIGURE 3 α -Helical nets of the hydrophilic amphipathic helix 16-30 of peptide CB4 (A1, and 2) and the hydrophobic helix 7-32 of peptide CB1 (B1, and 2). Symbols \bullet , \circ , \oplus , and \ominus represent, respectively, the hydrophobic, neutral, positively, and negatively charged residues. Amino acid residues are named using the one letter code as in Fig. 1.

fragment B shows a similar distribution of its residues and is also amphipathic (not shown).

Such α -helical amphipathic domains with a narrow hydrophobic face and a large hydrophilic face containing ion pairs or triplets have been shown to be characteristic of serum lipoproteins and are responsible for their binding to phospholipid headgroups of phosphatidylcholine molecules (24), leading to reversible associations between proteins and surfaces of bilayers and to the formation of soluble lipid-protein complexes.

No statistically significant similarities were found between Apo A1 and the apolar middle region of fragment B nor its C-terminal region.

Structural Properties of the Hydrophobic Middle Region of Fragment B

The clustering of apolar residues in the N-terminal amino acid sequence of peptide CB1 is responsible for the high hydrophobicity of the middle region of fragment B. Conformational analysis of this sequence predicts helicity at residues 1-5 and 7-32, although β -pleated sheet structure is also probable at residues 19-30 (Fig. 1).

Like the helical segments of the N-terminal region of fragment B, helix 7-32 of CB1 is amphipathic, as shown by the distribution of its residues on the drawn helical net (Fig. 3B). However, the projection clearly shows that, for CB1, the hydrophobic face of the helix is the largest, with numerous apolar and bulky residues (Fig. 3B, 2); it surrounds a narrow polar face, containing few polar and neutral residues, which runs like a groove parallel to the axis of the helix (Fig. 3B, 1). This helix resembles the transverse lipid-associating domains of intrinsic membrane proteins, responsible for their anchoring in the membrane bilayer. As with them, its polarity is very low (polarity index = 26.9%) and the hydrophobic index of its large hydrophobic face is very high (hydrophobic index = 3.9), close to the value found for the apolar face of the membranous segment of glycoporphin (23).

Structural Properties of the C-Terminal Polar Region of Fragment B

The N-terminal sequence of peptide CB3, located at the C-terminal end of fragment B, shows no structural similarities with both types of lipid-binding domains. It contains the disulfide bridge of fragment B (cys 2 and 12).

DISCUSSION

Our findings suggest that two different kinds of lipid-associating domains are present in that region of diphtheria toxin fragment B endowed with lipid-binding properties.

The first one, located in the 9,000-dalton N-terminal region of B (Fig. 4), shows an apolipoprotein-like lipid-associating structure. This highly hydrophilic region of B could, thus, function like apolipoproteins when interacting with a biological membrane; the large polar face bearing ion pairs or triplets interacts specifically with the charged headgroups of phospholipids, and the molecule is stabilized parallel to the membrane by hydrophobic interactions of its narrow apolar face with the membrane lipid core. This is the general model proposed for the surface lipid-associating domain of plasma lipoproteins (23).

The second lipid-associating domain of fragment B, located in its middle region, is structurally related to the membranous segments of integral membrane proteins and designed to interact by its large apolar face with the hydrocarbon core of the phospholipids. This highly hydrophobic region of B could, thus, be a transverse domain involved in a process of membrane penetration. The 7-33 α -helix of CB1 would be ~ 35 Å long, which is approximately the thickness of the aqueous discontinuity of hydrated phospholipid bilayers.

The few negatively charged residues on the narrow polar face of this helix suggest stabilization of B in the membrane lipid core by interaction either with itself after formation of a multimeric complex or with preexisting integral membrane proteins, perhaps involved as carriers in the diphtheria toxin-receptor complex (13).

The C-terminal 8,000-dalton region of fragment B belongs to a receptor binding site of the diphtheria toxin molecule (25). It shows no structural similarities with the above lipid-interacting structures.

Here we suggest that the combination in diphtheria toxin fragment B molecule of a surface lipid-associating domain, as found in serum apolipoprotein, and a transverse lipid-associating domain, as found in intrinsic membrane proteins, can

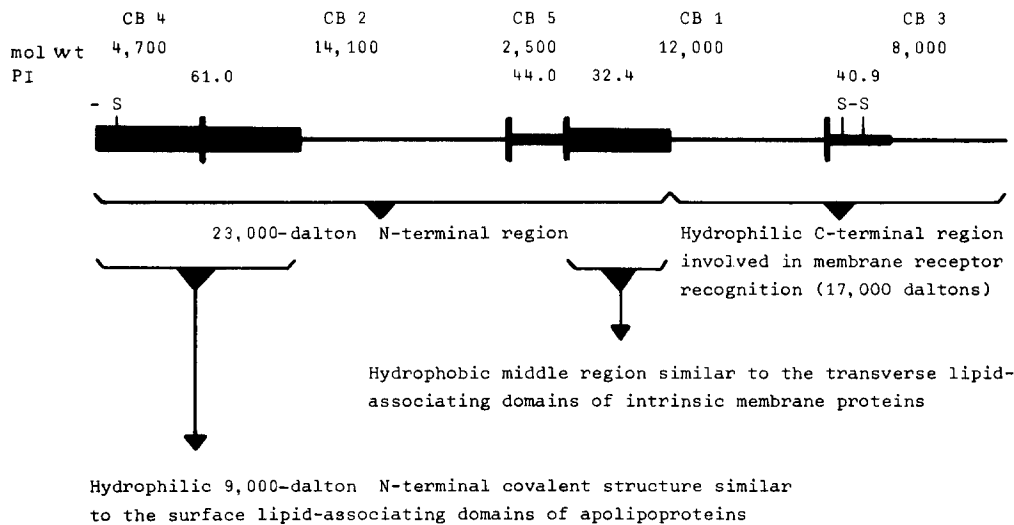


FIGURE 4 Topographic properties of diphtheria toxin fragment B: the CNBr peptides are aligned according to the proposal of Falmagne et al. (12). Molecular weights (mol wt) are expressed in daltons (d), and polarity indexes (PI) in percent (%). Vertical lines indicate positions of methionine residues.

confer upon this molecule the dynamic properties leading to its association and anchoring in a cytoplasmic membrane.

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