Common Features in the Functional Surface of Scorpion \(\beta\)-Toxins and Elements That Confer Specificity for Insect and Mammalian Voltage-gated Sodium Channels*

Lior Cohen‡, Izhak Karbat‡, Nicolas Gilles‡, Nitza Ilan‡, Morris Benveniste‡, Dalia Gordon‡†, and Michael Gurevitz‡‡☆

From the Departments of §Plant Sciences, George S. Wise Faculty of Life Sciences and ¶Physiology and Pharmacology, Sackler School of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Tel-Aviv, Israel and §CEA, Department d’Ingenierie et d’Etudes des Proteines, C. E. Saclay, F-91191 Gif Sur Yvette Cedex, France

Scorpion \(\beta\)-toxins that affect the activation of mammalian voltage-gated sodium channels (Na\(_s\)) have been studied extensively, but little is known about their functional surface and mode of interaction with the channel receptor. To enable a molecular approach to this question, we have established a successful expression system for the anti-mammalian scorpion \(\beta\)-toxin, Css4, whose effects on rat brain Na\(_s\) have been well characterized. A recombinant toxin, His-Css4, was obtained when fused to a His tag and a thrombin cleavage site and had similar binding affinity for and effect on Na currents of rat brain sodium channels as those of the native toxin isolated from the scorpion venom. Molecular dissection of His-Css4 elucidated a functional surface of 1245 Å\(^2\) composed of the following: 1) a cluster of residues associated with the \(\alpha\)-helix, which includes a putative “hot spot” (this cluster is conserved among scorpion \(\beta\)-toxins and contains their “pharmacophore”); 2) a hydrophobic cluster associated mainly with the \(\beta_2\) and \(\beta_3\) strands, which is likely to confer the specificity for mammalian Na\(_s\); 3) a single bioactive residue (Trp-56) in the C-tail; and 4) a negatively charged residue (Glu-15) involved in voltage sensor trapping as inferred from our ability to uncouple toxin binding from activity upon its substitution. This study expands our understanding about the mode of action of scorpion \(\beta\)-toxins and illuminates differences in the functional surfaces that may dictate their specificities for mammalian versus insect sodium channels.

Scorpion “long-chain” toxins that modify the gating of voltage-gated sodium channels (Na\(_s\)) are useful probes for studying the mode of action and structural elements involved in channel function (1–3). These channels are membrane proteins composed of \(\alpha\) and \(\beta\) subunits. The 260-kDa pore-forming \(\alpha\)-subunit is organized in four repeat domains (D1–D4). Each domain contains six trans-membrane \(\alpha\)-helical segments (S1–S6) that are connected by intra- and extra-cellular loops. The S4 segments, which contain 4–8 positively charged residues at a 3-residue interval, function as a voltage sensor. Upon membrane depolarization, the sensors move outward relative to the membrane electric field, thereby imposing a conformational change leading to channel activation. Channel inactivation is mediated by the short intra-cellular loop connecting domains 3 and 4 (2).

Although the receptors for long-chain scorpion toxins have been confined to two major binding sites (site 3 for \(\alpha\)-toxins and site 4 for \(\beta\)-toxins), only partial information is available on channel residues that constitute these sites. Therefore, elucidation of the functional surface of the toxins and their channel receptors may clarify the molecular basis of toxin-channel-specific interactions.

Scorpion \(\beta\)-toxins are 61–76-residue-long polypeptides cross-linked by four disulfide bridges (4, 5). Upon binding to receptor site 4, they shift the voltage dependence of channel activation in the hyperpolarizing direction (2). Recent work has identified in D2S3–S4 of the rat brain sodium channel Na\(_{1.2}\) residues that are involved in the high binding affinity of the anti-mammalian \(\beta\)-toxin,Css4, from the Mexican scorpion Centruroides suffusus suffusus (6). In these experiments, toxin binding was voltage-independent, and a conditioning depolarizing pre-pulse was required to observe the negative shift in voltage dependence of activation. On the basis of these results, a voltage sensor trapping model was proposed to explain \(\beta\)-toxin action. According to this model, the toxin binds to the channel at resting membrane potential. Upon strong depolarization, the D2S4 segment moves outward, associates with the pre-bound toxin, and is trapped in an outward, activated position. Trapping of the voltage sensor accounts for the negative shift in the voltage dependence of activation, which typifies scorpion \(\beta\)-toxin effect. This model gained support from experiments in which the two outermost Arg residues in D2S4 of Na\(_{1.2}\) were neutralized. This led to an enhancedCss4 effect, which was independent of a depolarizing pre-pulse (7).

Because no \(\beta\)-toxin active on mammals was available thus far for molecular dissection, we have analyzed by extensive mutagenesis the functional surface of the excitatory anti-insect selective \(\beta\)-toxin, Bj-xtrIT, from Bathothus judaicus (8–11). The functional surface of Bj-xtrIT is composed of two distinct domains: one includes a putative hot spot; and the other, associated with the C-tail, supposedly confers specificity for insects (10). However, Bj-xtrIT differs prominently from anti-mammalian \(\beta\)-toxins (e.g. Cn2 from Centruroides noxius, Css2, and Css4) in specificity (5, 9, 12–14) and structure (8, 15, 16). It shares only 27% sequence identity withCss4, and the spatial arrangement of the fourth disulfide bridge and C-tail configuration vary greatly (Fig. 1) (8, 15).
With the objective of analyzing the functional surface of Css4 and identifying residues involved in its mode of action and selectivity to mammals, we expressed it in a functional form that was as active as the native toxin isolated from the scorpion venom. Thorough mutagenesis suggests that Css4 binds to receptor site 4 on mammalian Na\textsubscript{v}s via two interacting domains: the first is common to all anti-insect and anti-mammalian \(\beta\)-toxins ("pharmacophore"), and the second seems to confer the specificity for mammalian Na\textsubscript{v}s. In addition, we have found that Glu-15 is important for Css4 action, but not for binding to the receptor site, which suggests that it is involved in voltage sensor trapping (6, 7, 11).

**MATERIALS AND METHODS**

**Bacterial Strains**—Escherichia coli DH5\textalpha was used for plasmid constructions, and the BL21 strain was used for toxin expression using the pET-14b vector in a protocol similar to that described elsewhere (9).

**DNA Manipulations**—A synthetic Css4 gene was constructed using eight overlapping oligonucleotide primers designed with a codon usage based on the nucleotide sequences of long-chain scorpion toxin genes that have been successfully expressed in E. coli (Table I) (9, 17, 18). A mixture of 1 \(\mu\)M of each of primers 1–8 (Table I) heated to 95 °C was cooled stepwise to 4 °C for annealing. The mixture was diluted 1:40 with ligation buffer, and 40 units of T4 ligase (New England Biolabs) were added for overnight reaction at 15 °C. Next, 1 \(\mu\)M of the ligation mixture was used for PCR amplification from both ends of the Css4 gene using primers 9 and 10 (Table I). Primer 9 contained an NdeI restriction site, and primer 10 contained a BamHI restriction site. The PCR product was cloned into the Smal site of pBluescript (Stratagene), and the sequence was verified. An NdeI-BamHI fragment was cleaved out and subcloned into the corresponding sites of pET-14b (Novagen). Expression in E. coli BL21 cells and in vitro folding of the recombinant His-Css4 (fusion of His tag and a thrombin cleavage sequence to the N-terminal side of Css4) was performed following established protocols (18). The average yield of His-Css4 was 2 mg from 1 liter of E. coli culture. Mutations in the toxin were introduced via PCR using complementary oligonucleotide primers and the constructed Css4 gene as DNA template. All toxin mutants were produced similarly to the unmodified toxin. Sequences were verified before expression. Quantification of purified recombinant toxins was performed by amino acid analysis using an ABL system 420A/150A (Applied Biosystems Inc.) after hydrolysis by 6 \(\mu\)HCl under vacuum (18 h at 110 °C).

**Toxicity Assays**—To determine toxicity to mammals, groups of five female mice (ICR, Levenstein Farm, Israel; 20 ± 3 g) received subcutaneous injection with each toxin concentration in three independent experiments. LD\textsubscript{50} values for mice were calculated according to the sampling and estimation method of Reed and Muench (19).

**Expression of Mammalian Na\textsubscript{v}s in CHO Cells and Whole-cell Patch Clamp Recording**—CHO cells were maintained in P12 medium, supplemented with 10% fetal calf serum, in a 5% CO\textsubscript{2} incubator. Transient transfection was achieved using FuGENE 6 (Roche Molecular Biochemicals) with a 0.3 ratio of the pcMV expression vector containing NBL12a and with a vector encoding the CD8 antigen (23). Individual transfected cells were visualized with Dynabeads (Dynal ASA) binding to CD8. Currents were recorded 2–3 days after transfection. Whole-cell voltage clamp experiments were conducted using an Axopatch 200B amplifier (Axon Instrument) at room temperature. Data were acquired with a Macintosh G4 computer equipped with an ITC-16 analog-to-digital converter (Intrustechnic Corp.) using Synapse software (Synergismix). Currents were low-pass filtered at 5 kHz and sampled at a rate of 10 kHz. Cell and electrode capacitance and series resistance were compensated with an internal voltage clamp circuit. Residual linear leak and capacitance were removed by subtracting scaled control traces using P6 protocol (24). The patch pipette contained 35 mM NaCl, 105 mM CsF, 10 mM EGTA, and 10 mM Hepes (adjusted to pH 7.4 with CsOH). The bath solution contained 140 mM NaCl, 5 mM CaCl\textsubscript{2}, 1.5 mM MgCl\textsubscript{2}, 1 mM MgATP, 2 mM Na\textsubscript{2}ATP, and 10 mM Hepes (adjusted to pH 7.4 with NaOH). Toxins were dissolved in the bath solution containing 1% bovine serum albumin, and perfusion of the cells was conducted with a flow pipe glass barrel (~400 \(\mu\)m, outer diameter) positioned ~100 \(\mu\)m from the cell.

**CD Spectroscopy**—CD spectra were recorded at 25 °C using a model 202 circular dichroism spectrophotometer ( Aviv Instruments). Toxins (150 \(\mu\)g) were dissolved in 5 mM sodium phosphate buffer (pH 7) and their spectrum (190–260 nm) was the average of three measurements using a quartz cell of 0.1 mm light path. Blank spectrum of the buffer was run under identical conditions and subtracted from each of the toxin spectra.

**Three-dimensional Models and CSU Analysis**—Three-dimensional models of Css4 and LqhIT2 were constructed using the SWISS-MODEL structure homology-modelling server (www.expasy.org/swissmod/...
RESULTS

Expression and Characterization of Recombinant Css4—Protocols that were previously shown to be useful for the production and extensive mutagenesis of a number of long-chain scorpion toxins (shaded in light gray) are numbered and indicated by solid lines. The unique disulfide bond in excitatory toxins (shaded in dark gray) is indicated by a dashed line. Dashes indicate gaps. Numbering and secondary structure motifs (B, β-strand; H, α-helix) in Css4 follow the published structure of Cn2 (15). Aah, Androctonus australis hector; Bj, B. judaicus (Hettentotta judaica); Cn, C. noxius; Css, C. suffusus suffusus; Lqh, Leirus quinquestriatus hebraeus; Lqq, Leirus quinquestriatus quinquestriatus; Ts, Tityus serrulatus. Toxin sequences are from Possani et al. (5) and Gordon et al. (28). B, the Cα structure of Bj-xtrIT is derived from Protein Data Bank accession number 1BCG (8). The model includes C-terminal residues 74–76 and is taken from Gurevitz et al. (16). The Css4 model is based on the NMR structure of Cn2 (Protein Data Bank accession no 1CN2, Ref. 15; see “Materials and Methods”) and is spatially aligned with that of Bj-xtrIT. The disulfide bonds are numbered as described in A.

Commonality and Specificity in Scorpion β-Toxins. A, sequences were aligned according to the conserved cysteine residues. The disulfide bonds formed between cysteine pairs in all long-chain scorpion toxins (shaded in light gray) are numbered and indicated by solid lines. The unique disulfide bond in excitatory toxins (shaded in dark gray) is indicated by a dashed line. Dashes indicate gaps. Numbering and secondary structure motifs (B, β-strand; H, α-helix) in Css4 follow the published structure of Cn2 (15). Aah, Androctonus australis hector; Bj, B. judaicus (Hettentotta judaica); Cn, C. noxius; Css, C. suffusus suffusus; Lqh, Leirus quinquestriatus hebraeus; Lqq, Leirus quinquestriatus quinquestriatus; Ts, Tityus serrulatus. Toxin sequences are from Possani et al. (5) and Gordon et al. (28). B, the Cα structure of Bj-xtrIT is derived from Protein Data Bank accession number 1BCG (8). The model includes C-terminal residues 74–76 and is taken from Gurevitz et al. (16). The Css4 model is based on the NMR structure of Cn2 (Protein Data Bank accession no 1CN2, Ref. 15; see “Materials and Methods”) and is spatially aligned with that of Bj-xtrIT. The disulfide bonds are numbered as described in A.

was compatible with the deduced sequence of the construct made of Css4 with an N-terminal addition containing a His tag and a thrombin cleavage site (9905 Da). Activity of His-Css4 was then determined using toxicity, binding, and electrophysiological assays.

Toxicity to Mice—His-Css4 was toxic to mice (LD50 = 0.25 nmol/g by subcutaneous injection) and almost inactive on blowfly larvae (ED50 > 20 nmol/g larvae). This activity is comparable with that reported for the anti-mammalian scorpion β-toxins Css2 and Css4 (Fig. 1A) (14).

Binding of His-Css4 to Rat Brain Synaptosomes—125I-His-Css4 was used in competition binding assays to determine its binding properties to rat brain synaptosomes. Scatchard analysis of the binding curves indicated that His-Css4 bound to a single class of non-interacting binding sites (Fig. 2, inset). The
The Ki value of 1.04 ± 0.04 nM (n = 4), whereas the α-toxin Lqh2 did not inhibit the binding of 125I-His-Css4. The inset provides a Scatchard plot of the equilibrium binding curve obtained with increasing concentrations of unlabeled His-Css4. B/F, bound over free ligand. Nonspecific binding, determined in the presence of 1 μM His-Css4 (corresponding to 20% of total binding), was subtracted. The binding parameters obtained are as follows: Kd = 0.73 ± 0.11 nM (n = 4) and Bmax = 0.82 ± 0.08 pmol/mg (n = 4). A representative experiment is shown.

apparent equilibrium dissociation constant (Kd = 0.73 ± 0.11 nM; n = 4) was independent of membrane potential and was not affected by pH changes between 6 and 8 (data not shown). These results are in concert with those published previously for native Css4 and Css2, in which the apparent affinity for rat brain synaptosomes was in the range between 0.56 and 0.79 nM (14, 27). Cleavage of the N-terminal addition off Css4 by thrombin followed by HPLC purification yielded a recombinant Css4 product, rCss4, with only 4 additional residues at the N-terminal side. rCss4 competed with 125I-His-Css4 on binding to rat brain synaptosomes with a Kd value of 1.04 ± 0.04 nM (n = 3), which was practically identical to that obtained for His-Css4 (Fig. 2; Table II). This result indicates that the additional sequence at the N-terminal side of Css4 does not affect the binding affinity of Css4 for rat brain Navs.

Moreover, we compared the ability of the β-toxin Lqhβ1 (28) and the α-toxin Lqh2 (29) to compete for 125I-His-Css4 binding to rat brain synaptosomes. Low Lqhβ1 concentrations inhibited the binding of 125I-His-Css4 (Kd = 0.51 ± 0.27 nM; n = 3), indicating that the two toxins competed for binding to receptor site 4 (Fig. 2), which is recognized by all scorpion β-toxins (4, 30). In contrast, Lqh2, which binds to receptor site 3 on Naαs (20), did not inhibit 125I-His-Css4 binding to rat brain synaptosomes (Fig. 2). Receptor site capacity for His-Css4 (Bmax = 0.82 ± 0.08 pmol/mg protein; n = 4; Fig. 2) was in the same range as that obtained previously for other scorpion α- and β-toxins to rat brain synaptosomes prepared in a similar fashion (0.6–2.4 pmol/mg) (12, 13, 20, 31, 32), suggesting that they all bound to a similar population of sodium channels.

Modulation of Naα, 1.2a by His-Css4 and rCss4—The activity of His-Css4 and rCss4 was examined on Naα,1.2a channels expressed in CHO cells using a whole-cell voltage clamp configuration (Fig. 3). Similarly to the native toxin (6), His-Css4 and rCss4 required a depolarizing pre-pulse to induce a negative shift in the voltage dependence of activation, and therefore, a prepulse was included in all our electrophysiological protocols (see the Fig. 3 legend). Under control conditions, no Na current were obtained following test step depolarization from a −100 mV holding potential to voltages varying from −70 to −40 mV (Fig. 3A, top trace). However, in the presence of 2.5 μM His-Css4 (or rCss4), an inward Na current was obtained already at −60 mV, which gradually increased at −50 and −40 mV (Fig. 3A, middle trace). Current-voltage relationships at membrane potentials between −90 and +40 mV revealed a shift in the voltage dependence of activation, which typifies α-toxins (Fig. 3B) (3). Moreover, 2.5 μM His-Css4 induced an effect that was similar to that obtained with 2.5 μM rCss4. This
effect increased proportionally when the His-Css4 concentration was doubled (Fig. 3B). Because the binding affinity and electrophysiological effect of His-Css4 were very similar to those of rCss4, in the mutagenesis analysis we used His-Css4, which was easier to produce. It should be noted that observable electrophysiological effects were obtained with concentrations that were 3 orders of magnitude higher than the apparent binding affinity of His-Css4. A similar unexplained discrepancy was reported for native Css4 as well as other scorpion /H9252/toxins (e.g., Ts1, Cn2, and Css2) (7, 13, 33–38).

Molecular Dissection of His-Css4—We constructed a three-dimensional model of Css4 using the reported NMR structure of the highly homologous /H9252/toxin Cn2 (92% similarity) (Fig. 1) (15). Forty residues, which, according to the structural model, are exposed to the solvent, were scanned by substitution (mostly to Ala). Each mutant toxin, prepared following the protocol for the unmodified toxin, was analyzed in competition binding studies and by CD spectroscopy to discern between effects resulting from structural perturbation and those reflecting changes in the putative interaction with the channel receptor (Figs. 4 and 5; Table II). Of 48 mutations, 26 had little effect on the binding affinity (ΔΔG < 1.3; Table II). Residues whose substitution substantially decreased the binding affinity (ΔΔG > 1.3 kcal/mol) with no alteration of the CD spectrum were considered significant for activity and assigned to the putative functional surface (Fig. 6). To assess whether the N-terminal addition in the His-Css4 fusion peptide had an effect on function of the mutant toxins, we compared the binding affinity of mutants E15Q, E15R, E28A, E28R, N22A, and Y42A in the context of His-Css4 and rCss4 toxin forms (the latter after cleavage by thrombin) and found identical Ki values. Residues whose substitution with Ala reduced the binding affinity but also altered the CD spectrum (Y4A, T10A, F14A, W47A, and K63A) (Fig. 5; Table II) were excluded from the putative functional surface. In most instances, the CD alter-

**FIG. 3.** Effects of His-Css4, rCss4, and mutant E15R on currents of rNa,1.2a expressed in CHO cells. All voltage protocols were initiated with a depolarizing pre-pulse to 0 mV for 50 ms (data not shown), followed by 60 ms at −100 mV holding potential and a 50-ms test pulse. A, representative currents induced by test voltage pulses in the range between −70 and −40 mV, under control conditions (top traces), in the presence of 2.5 μM His-Css4 (middle traces; n = 6), and after a wash, in the presence of 10 μM His-Css4-E15R (bottom traces; n = 4). B, current-voltage relationship obtained from the same cell shown in A under control conditions, in the presence of 2.5 μM rCss4, 2.5 μM His-Css4, or 5 μM His-Css4. The observed differences between rCss4 and His-Css4 were repeated in three cells.
ations could result from perturbation of intra-molecular interactions inferred from CSU analysis of the CsS4 model (using the program CSU) (25).

Role of Amino Acids in the α-Helix and Its Vicinity—In a previous study of the anti-insect selective excitatory α-toxin, Bj-xtrIT, we have suggested that Glu-30, which is conserved in the α-helix of scorpion β-toxins, constitutes a hot spot in the interacting surface with the channel (10). We examined the role of the equivalent region in CsS4 in order to assess the commonality of such a hot spot in β-toxins. Ala scan of the residues encompassing the α-helix and the loop connecting it to 2 (residues 24–38) has shown that Tyr-24, Arg-27, Glu-28, and Gln-32 are involved in function and that Tyr-24 and Glu-28 are specifically important for activity. A stronger effect was obtained upon charge inversion of Glu-28 (E28R) as the binding affinity decreased 3 orders of magnitude (Fig. 4; Table II). Charge-neutralizing substitutions (E28A/Q/L) also markedly decreased the binding affinity (Table II), indicating that this residue is crucial for CsS4 binding interaction.

The side chains of Tyr-24 and Gln-32 project to the solvent and flank Glu-28 (Fig. 6). Substitutions Y24A and Q32A decreased the binding affinity (Table II). Increasing the side chain volume by substitution of Gln-32 with Trp (Q32W) further decreased the binding affinity, whereas a similar substitution of Tyr-24 (Y24W) had only a minor effect (Table II). The latter suggests that an aromatic moiety is important at po-
The importance of a positive charge at position 27 for Css4 function was demonstrated by its neutralization while maintaining the side chain size (R27I/Q) (Table II). These results imply that Glu-28 flanked by Tyr-24, Arg-27, and Gln-32 may be part of the contact surface of the toxin with its receptor site. This toxin region seems to be stabilized through interactions of Cys-25, Glu-28, and Gln-32 with the side chain of Phe-14, which may explain the reduced binding affinity and altered CD spectrum of mutant F14A (Fig. 5; Table II).

Mutagenesis of residues in the loop preceding the $\alpha$-helix (positions 17–23) highlighted the role of Phe-17, Leu-19, and Asn-22. Their substitution with Ala substantially decreased the binding affinity, but when substituted with amino acids with similar chemical nature and volume (F17W and L19I), only a small effect was observed (Fig. 4; Table II). From these results, we conclude that the solvent-exposed region preceding and including the $\alpha$-helix is part of the interacting surface of Css4 with its channel receptor site.

**Bioactive Role of the C-terminal Region and $\beta 2$-$\beta 3$ Strands—** Substitutions at the C-terminal region (positions 50–66) had little effect on toxin activity ($\Delta \Delta G < 1.3$ kcal/mol), except for Trp-58, whose substitution by Ala markedly decreased the binding affinity (Fig. 4; Table II). In contrast, Ala scan of residues in $\beta 2$ to $\beta 3$ (positions 39–49) highlighted a group of aromatic amino acids crucial for toxin function. Mutations Y40A, Y42A, and F44A decreased the binding affinity 3 orders of magnitude (Fig. 4; Table II). These results suggest that the aromatic nature of these residues is important for Css4 function.

**Glu-15 Has a Unique Role in Toxin Function—** We have shown previously that charge inversion at Glu-15 (E15R) of the excitatory $\beta$-toxin Bj-xtrIT abolished toxicity to insects but had little effect on binding, thus converting the toxin mutant into a potent competitive antagonist (11). Because Glu-15 is spatially conserved in the x-ray structure of Bj-xtrIT and the Cn2-based model of Css4 (Fig. 6), we challenged its role in Css4 by substitution with Gln (charge neutralization) and Arg (charge inversion). Interestingly, both mutations had a minor effect on the binding affinity (Fig. 4; Table II) but abolished toxin effect on rNav1.2a expressed in CHO cells. Whereas 2.5 $\mu M$ His-Css4 shifted the voltage dependence of activation to more negative membrane potentials, 10 $\mu M$ of mutant E15R had no effect (Fig. 3A, bottom trace).
Commonality and Specificity in Scorpion \( \beta \)-Toxins

Elucidation of structural elements that determine the interaction and specificity of scorpion \( \beta \)-toxins for mammalian Na\(_s\)s demands a suitable system for toxin modification and analysis. Here we show that the difficulty in expressing a functional Css4 could be circumvented by extension of its N terminus (His-Css4). The binding affinity for rat brain synaptosomes, as well as the effect on the rat brain sodium channel, Na\(_{1,2a}\), indicated that His-Css4 was very similar to the native Css4 isolated from scorpion venom.

The Pharmacophore of \( \beta \)-Toxins—Complete mutagenesis of His-Css4 illuminated a discontinuous functional surface (~1245 Å\(^2\)) composed mainly of two amino acid clusters (Fig. 6A). A similar cluster to that formed by residues of the \( \alpha \)-helix and its preceding loop appears in the equivalent region of the anti-insect excitative \( \beta \)-toxin, Bj-xtrIT. Thus, Tyr-24, Arg-27, Glu-28, and Gln-32 of Css4 resemble in their chemical nature and function Tyr-26, His-25, Glu-30, and Val-34 of Bj-xtrIT (Fig. 6B). It is likely that Glu-28, which is spatially conserved in all known \( \beta \)-toxins, forms a hot spot that interacts electrostatically with a putative positive charge on the receptor site, as was proposed for Glu-30 in Bj-xtrIT (10). The non-polar Tyr-24 and Gln-32, which flank Glu-28 in Css4, may function as a seal to occlude bulk solvent from the high-energy point of interaction, similar to the role proposed for Tyr-26 and Val-34 in Bj-xtrIT (10).

Further similarity between Css4 and Bj-xtrIT at the \( \alpha \)-helix region is in a functionally important positive charge, Arg-27 in Css4 and His-25 in Bj-xtrIT, which projects outward of the molecule surface in the same orientation (Fig. 6B). However, because a positively charged residue does not appear next to the conserved hot spot in all \( \beta \)-toxins (Fig. 1A), it cannot be considered part of the common binding domain of these toxins with their receptor site. Another region important for activity in both Bj-xtrIT and Css4 is the loop preceding the \( \alpha \)-helix (10) (Table II). However, despite the structural resemblance and similar spatial arrangement of a number of residues in this toxin pair, this loop differs in length and amino acid composition among various \( \beta \)-toxins (Fig. 1). Three-dimensional structure comparison among Css4 (anti-mammalian; modeled according to Cn2; Ref. 15), Bj-xtrIT (anti-insect; Ref. 8), Ts1 (Ref. 39; affects both insects and mammals; Refs. 33, 40, and 41), and a model of the depressant toxin LqhIT2 (16) indicates that a residue of hydrophobic nature (Phe-17 in Css4) is spatially conserved in this loop. Other residues of this loop that have been found to be important for activity in Css4 (Leu-19 and Asn-22; Table II) or Bj-xtrIT (10) do not match equivalent or any other residues in Ts1 or LqhIT2 (Figs. 1 and 6B).

Despite the differences, this analysis raises a similar functional region on the \( \alpha \)-helix and its preceding loop of Css4 and Bj-xtrIT and presumably in other \( \beta \)-toxins that includes the negatively charged hot spot. This common region, referred to as the pharmacophore of scorpion \( \beta \)-toxins, may explain their ability to compete to various extents on binding to receptor site 4 of different Na\(_s\)s. Indeed, Cn2 competes with Bj-xtrIT on binding to insect Na\(_s\)s (10), and Ts1 and Lqh\( \beta \) compete with both excitatory and anti-mammalian \( \beta \)-toxins on binding to insect (40, 41) and rat brain (28, 33) neuronal preparations, respectively. Thus, the pharmacophore in various \( \beta \)-toxins is smaller than that predicted previously from the dissection of Bj-xtrIT (10) and consists of spatially conserved residues of similar chemical nature. In Css4, the pharmacophore is formed by Phe-17 and Glu-28 flanked by Tyr-24 and Gln-32, whereas in Bj-xtrIT, it consists of Val-19, Asn-20, and Glu-30 flanked by Tyr-26 and Val-34. By analogy, the pharmacophore of Ts1 is made of Ile-17, Pro-19, and Glu-26 flanked by Tyr-22 and Ile-29 (Fig. 6B), although this suggestion still needs to be confirmed experimentally.

Glu-15 in Css4 Is Important for Activity but Not for Binding—To explain the effect of scorpion \( \beta \)-toxins, a voltage sensor trapping model, based on Css4 activity on Na\(_{1,2a}\) and mutations in the gating charges of D2S4, was proposed. According to the model, D2S4 can be trapped in an outward, activated position by a pre-bound Css4 (6, 7). We have shown in a previous study that Glu-15 of Bj-xtrIT is most likely involved in the interception of the voltage sensor in insect Na\(_s\)s (11) and, on this basis, challenged the role of Glu-15 in Css4. The inability of Css4-E15R to shift the voltage dependence of Na\(_{1,2a}\) activation to more negative membrane potentials while retaining almost full binding affinity for rat brain synaptosomes (Figs. 3 and 4; Table II) was strikingly similar to the loss of toxic effect on blowfly larvae while maintaining binding affinity for insect Na\(_s\)s of the E15R mutant of Bj-xtrIT (11). Thus, it seems that Css4 and Bj-xtrIT have a similar mechanism of action. It is noteworthy that other \( \beta \)-toxins such as Ts1, Lqh\( \beta \), and LqhIT2 lack a negatively charged residue at the position equivalent to Glu-15 in Bj-xtrIT and Css4 (Figs. 1A and 6B). Because all \( \beta \)-toxins induce a similar effect on channel activation (28, 33, 34, 42, 43), we assume they also trap the voltage sensor, but via different interactions.

The Functional Domain in Css4 That Confers Specificity for Mammals—Although Bj-xtrIT and Css4 share a common functional domain, which includes the pharmacophore, they differ markedly in specificity for insect and mammalian Na\(_s\)s (6, 9, 14). Therefore, it is rational to assume that the second amino acid cluster in the functional surface of each toxin determines its specificity. In Bj-xtrIT, this cluster is formed by a stretch of hydrophobic residues (Val-71, Gln-72, Ile-73, and Ile-74) positioned on the C-tail (9, 10), which is unique in length and configuration in excitatory toxins (Figs. 1 and 6B) (8, 16). In Css4, the only C-tail residue important for activity is Trp-58 (Table II), and sulfenylation of its equivalent residue (Trp-54; Figs. 1A and 6B) in Ts1 reduced the activity to both insects and mammals (44). However, we identified in Css4 another functional domain that consists of Leu-19 and Asn-22 of the loop preceding the \( \alpha \)-helix and Tyr-40, Tyr-42, and Phe-44 of the \( \beta \) strand and the loop connecting \( \beta \)2 with \( \beta \)3 (Fig. 6). In Bj-xtrIT, the equivalent \( \beta \)-3 region is partially masked by the C-tail (8), and mutagenesis of the solvent-exposed Trp-44 (equivalent to Tyr-42 of Css4) had no effect on toxin function (10). For these reasons, we suggest that this 508-Å\(^2\) domain confers the specificity of Css4 for rat brain Na\(_s\)s.

Interestingly, although the residues involved in specificity of Bj-xtrIT and Css4 appear in an entirely different region of the sequence, in both toxins they form a hydrophobic entity facing the solvent in a spatially similar orientation relative to the \( \alpha \)-/\( \beta \)-core of the toxin (Fig. 6B). However, a salient difference between the functional surfaces in both toxins is the distance between the hot spot (Glu-28 in Css4 and Glu-30 in Bj-xtrIT), centered in the pharmacophore, and the hydrophobic domain that supposedly confers specificity (average distance, ~12.3 ± 1.4 Å in Css4 and ~16.8 ± 0.8 Å in Bj-xtrIT; Fig. 6B). The hydrophobic domain of Css4 resembles that of Ts1 only in part. Tyr-36 and Trp-39, whose sulfenylation reduced the activity of Ts1 (44), are the equivalents of Tyr-40 and Phe-44 in Css4 (Fig. 6B). However, two additional Tyr residues appear in \( \beta \)3 of Ts1 (positions 43 and 45), and Ala-38 appears in the position occupied in Css4 by Tyr-42 (Figs. 1A and 6B). Furthermore, the loop that precedes the \( \alpha \)-helix is shorter in Ts1 than in Css4, and no spatial equivalents to Leu-19 and Asn-22 of Css4 exist. These differences may be related to the preference of Css4 for mammalian Na\(_s\)s compared with the high affinity of Ts1 for both insect and mammalian Na\(_s\)s.
Commonality and Specificity in Scorpion β-Toxins

We show that the functional surface of scorpion β-toxins, which bind to Na⁺ receptor site 4, consists mainly of two distinct domains: a pharmacophore common to various toxins that may explain their ability to compete in binding, and another hydrophobic domain that most likely determines the specificity for various Na⁺s. The high binding affinity of the toxin to the sodium channel most likely results from the interactions of both the pharmacophore and the hydrophobic domain. Such a bipartite functional surface was also shown in scorpion α-toxins, which bind to receptor site 3 (45). This proposed mode of toxin interaction with the Na⁺ receptor has been suggested previously for potassium channel pore blockers (46) and nAChR blockers from cone snails (47) and therefore is likely to be a general mechanism by which a variety of toxins interact selectively with their ion channel receptors.

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Common Features in the Functional Surface of Scorpion β-Toxins and Elements That Confer Specificity for Insect and Mammalian Voltage-gated Sodium Channels
Lior Cohen, Izhar Karbat, Nicolas Gilles, Nitza Ilan, Morris Benveniste, Dalia Gordon and Michael Gurevitz

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