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Comparison of the primary structures, cytotoxicities, and affinities to phospholipids of five kinds of cytotoxins from the venom of Indian cobra, *Naja naja*

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ABSTRACT

The molecular mechanism underlying the hemolytic and cytolytic processes of cobra cytotoxins (CTXs) is not yet fully elucidated. To examine this, we analyzed the amino acid sequences, hemolytic and cytotoxic activities, and affinities to phospholipids of the five major CTXs purified from the venom of Indian cobra, *Naja naja*. CTX2, CTX7, and CTX8 belonged to S-type, and CTX9 and CTX10 to P-type. Comparisons of CTX7 with CTX8 and CTX9 with CTX10 revealed similar primary structures and hemolytic and cytolytic activities. CTX2, whose primary structure was rather different from the others, showed several times weaker hemolytic and cytolytic biological activities than the others. The comparison of CTX2 with CTX7 suggested the importance of Lys30 in loop II for the strong hemolytic and cytolytic activities of S-type CTXs. Cloning of 12 CTX cDNAs from the *Naja naja* venom cDNA library revealed that 18 out of 23 substitutions found in CTX cDNAs were nonsynonymous. This clearly indicated the accelerated evolution of CTX genes. Multiple sequence alignment of 51 kinds of CTX cDNAs and calculations of nonsynonymous and synonymous substitutions indicated that the codons coding the three loops' regions, which may interact with the hydrophobic tails of phospholipids, have undergone an accelerated evolution. In contrast, the codons coding for amino acid residues considered to participate in the recognition and binding of the hydrophilic head groups of phospholipids, eight Cys residues, and those likely stabilizing β core structure, were all conserved.

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1. Introduction

Cobra cytotoxins (CTXs), the major components of cobra venom, cause systolic cardiac arrest, severe tissue necrosis, and other symptoms (Harvey et al., 1982; Dufton and Hider, 1991). In vitro assays have shown that they have general cytotoxicities to various types of cells (Chen et al., 1984; Hinman et al., 1990; Chien et al., 1994; Stevens-Truss and Hinman, 1997; Feofanov et al., 2004; Feovanov et al., 2005). CTXs consist of around 60 amino acids; they characteristically form five β strands and three loops that are crosslinked by four disulfide bridges (Dufton and Hider, 1991). Several isoforms of CTXs are found in the venom of a single cobra (Joubert and Taljaard, 1978; Suzuki et al., 2010). These isoforms may have resulted from gene duplication and the subsequent accelerated evolution (Ohno et al., 1998). It is unclear whether isoforms of CTXs are orthologous or paralogous to each other since their amino acid sequences are very similar (Chien et al., 1994).

CTXs have been found to bind several membrane components, namely, phospholipids (Vincent et al., 1976), heparin and heparan sulfates (Patel et al., 1997; Lee et al., 2005), potassium channel interacting proteins (KChIPs) of membrane proteins (Lin et al., 2004), and sulfatide (Wang et al., 2005; Tjong et al., 2007). Each binding of these components may participate in expressing cytotoxicity. Even though many kinds of molecules participate in hemolytic and cytolytic processes, the CTX-phospholipids interaction may play an important role in these processes since phospholipids are common components of all cell membranes. Furthermore, the fact that CTXs induce membrane leakage and the fusion of liposomes, which are composed of pure phospholipids (Chien et al., 1991; Huang et al., 2003; Chen et al., 2007), supports this hypothesis.

Abbreviations: CTX, cytotoxin; PBS, phosphate buffered saline; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolanine; PA, phosphatidic acid; SM, sphingomyeline; PE, phosphatidylethanolamine; dN, number of nucleotide substitutions per nonsynonymous site; dS, number of nucleotide substitutions per synonymous site.

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The electrostatic interaction of the CTXs with the negatively charged polar head of phospholipids, especially phosphatidylserine (PS), has been shown by a variety of techniques (Kini and Evans, 1989; Gatineau et al., 1990). Recently, nuclear magnetic resonance (NMR) and simulation studies for *Naja kauthia* CT 4 as well as *Naja oxiana* CT I and CT II showed that there are at least three putative PS binding sites on each CTX and that several Lys residues participate in the PS binding (Konshina et al., 2010; Konshina et al., 2011). In addition to positively charged residues, the hydrophobic loops of CTXs appear to interact with the hydrocarbon part of phospholipids. CTXs were classified into two types, P-type and S-type, by comparison of the amino acid sequences and activities between 10 kinds of CTXs (Chien et al., 1994). S-type CTXs characterized by the presence of Ser28 residue in loop II had weak activities. P-type CTXs characterized by the presence of Pro30 residue in loop II had strong activities. NMR and simulation studies indicated that both P- and S-type CTXs interact with the lipid bilayer with their three loops and that P-type CTXs penetrate its hydrophobic loop II deeper than S-type CTXs (Dubovskii et al., 2003; Dubovskii et al., 2005). These interactions may cause hemolysis and cytolysis by changing the surface tension (Bougis et al., 1981; Maftouni et al., 2013), charge-induced phase separation (Désormeaux et al., 1992), membrane pore-formation (Forouhar et al., 2003), or other unknown mechanisms. However, there has been no conclusive direct evidence for the mechanisms of CTX-induced hemolysis, cytolysis, and action on liposomes. In addition, many CTXs that have been identified differ significantly from each other in terms of toxicity strength (Chien et al., 1994; Feofanov et al., 2004). The structural aspects underlying these variations in cytotoxic strength have not yet been fully clarified.

The functional domain of CTXs could be partially separated to the lipid binding domain and the lytic domain (Stevens-Truss and Hinman, 1997). Many amino acid residues of CTXs have been shown and/or predicted to participate in the interaction between CTXs and phospholipids. It remains unclear, however, whether each amino acid residue participates in binding to the membrane, acts in the lytic process, and/or has other functions. Clarifying the function of each CTX amino acid residue is important in order to achieve a better understanding of the molecular mechanism of lipid membrane lysis by CTXs.

Mutational analysis is a powerful method for revealing the function of individual amino acid residues of toxins, as shown for neurotoxins which basically have the same stereospecific structure as CTXs (Antil-Delbeke et al., 2000). Methods to obtain a sufficient amount of mutant CTXs for interaction analysis have not yet been established, probably because of the toxicities of the mutant CTXs against host cells. A unique reporter assay was, however, developed using chimera mutants (Ma et al., 2002). In this respect, the comparison of a series of naturally occurring CTXs is feasible to estimate the function of each amino acid residue of CTXs. CTX genes are considered to have evolved conservatively in some parts and at accelerated rates in others. Both the conserved and diversified amino acid residues are believed to play important roles in CTX functions (Ohno et al., 1998; Sunagar et al., 2013). Estimation of the evolutionary rate of each codon is therefore also helpful to understand the function of the amino acid residues corresponding to each codon.

Previously we purified 10 kinds of CTXs from the venom of Indian cobra, *Naja naja*, designated CTX1 to CTX10, and determined the whole amino acid sequence of CTX7 (Suzuki et al., 2010). In this study, we determined the whole amino acid sequences of CTX2, CTX8, CTX9, and CTX10, and examined the hemolytic and cytotoxic activities, and the affinities to phospholipids of these five cobra CTXs to gain insight into the hemolytic mechanism. Further, we cloned 12 kinds of CTX cDNAs and analyzed the relationship between the substitution rate and the function of coded amino acid residues.

2. Materials and methods

2.1. Cytotoxins

Five kinds of CTXs, CTX2, CTX7, CTX8, CTX9, and CTX10, were purified from the venom of *N. naja* as described previously (Suzuki et al., 2010).

2.2. Amino acid sequence determination

Amino acid sequences of CTXs were determined according to the previously described method (Suzuki et al., 2010). Briefly, samples were reduced and pyridynoethylated, then digested with trypsin, AspN, or LysC in 0.1 M Tris/HCl buffer, pH 8.0 at 37 °C overnight. Then each digest was acidified with 10% trifluoroacetic acid (TFA) to give a final concentration of 0.2% and subjected to reversed-phase high-performance liquid chromatography (HPLC) with an ODS column (CAPCELL PAK C-18, 4.6 × 250 mm, Shiseido, Japan). The peptides were eluted by a linear gradient of acetonitrile and the purified peptides were manually collected. Amino acid sequences of the intact proteins and isolated peptide fragments were determined using an automatic protein sequencer (model 471; Perkin Elmer ABI Biosystems, Foster City, CA).

2.3. Hemolytic assay

Fresh human blood was mixed with 1/10 vol. of 3.8% sodium citrate, centrifuged at 3000 ×*g* for 10 min, and washed with phosphate buffered saline (PBS). The washed erythrocytes were resuspended in 80 fold (v/v) of Tyrode(-) and used for hemolytic assay. One hundred and ninety microliter aliquots of the erythrocyte suspension were mixed with 10 μL of twofold serial dilutions, starting off with stock solutions that were 30 μM CTX2, and 15 μM CTX7 to CTX10 to 2^{-10} dilutions. After incubation for 6 h with gentle agitation at 37 °C, the erythrocyte suspension was centrifuged and the amount of hemoglobin released in the supernatant was measured at a wavelength of 550 nm to determine the hemolysis. The fifty percent effective concentration (EC50) with 95% confidence intervals was calculated by Probit analysis (Finney, 1971) using XLSTAT software (Addinsoft, Paris, France).

2.4. Cell cultures

The mouse fibroblast cell line, NIH/3T3 (ATCC CRL-1658), was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere with 5% CO_2. A mouse lymphocytic leukemia cell line, L1210 (ATCC CCL-219),

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                10         20         30         40         50         60
CTX2   LQCNKLVPIA SKTCPPGKNL CYKMFMVSDL TIPVKRGCID VCPKNSLLVK YECCNTDRCN
CTX7   LKCNKLIPLA YKTCPAGKDL CYKMYMVSNK TVPVKRGCID VCPKNSLLVK YECCNTDRCN
CTX8   LKCNKLIPLA YKTCPAGKDL CYKMYMVSDK TVPVKRGCID VCPKNSLLVK YECCNTDRCN
CTX9   LKCNKLVPLF YKTCPAGKNL CYKMYMVATP KVPVKRGCID VCPKSSLLVK YVCCNTDRCN
CTX10  LKCNKLVPLF YKTCPAGKDL CYKMYMVATP KVPVKRGCID VCPKSSLLVK YVCCNTDRCN
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Fig. 1. Amino acid sequence alignment for the major five cytotoxins of *N. naja*. Consensus amino acid residues among more than three sequences are gray-shaded characters. The sequences of CTX2, CTX8, CTX9, and CTX10 were determined by an automated Edman degradation in this study. The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession numbers P86538, P86540, C0HJU1, P86541.

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