Cytotoxic potency of cardiotoxin from *Naja sputatrix*: development of a new cytolytic assay

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The possible involvement of specific regions/loops of cardiotoxin from *Naja sputatrix* venom in mediating its cytolytic activity is evaluated using a new cytolytic assay. In this assay, the amount of chloramphenicol acetyltransferase (CAT) that is released upon lysis of the cellular membranes by the cytotoxin has been measured as an index of cytolysis. This newly developed CAT system is more sensitive than the traditional haemolysis method utilizing red blood cells or the lactate dehydrogenase assay for cytolysis. Series of chimaeric toxin molecules have been constructed by swapping the loops between highly hydrophilic neurotoxin and highly hydrophobic cardiotoxin molecules from *Naja sputatrix*, which are known to exhibit structural similarity (three-finger conformation) but to have different functional

properties. Comparison of the cytolytic activities of the recombinant chimaeric toxins demonstrated the possible involvement of all three loops of cardiotoxin in its cytolytic potency. However, the first two loops of the protein appear to make the major contribution to its lytic activity. cDNAs encoding cardiotoxin and the chimaeric toxins, when expressed in transfected cultured Chinese hamster ovary cells, resulted in cell lysis, indicating that these cDNAs can be developed as useful cytolytic agents.

Key words: chimaeric toxins, chloramphenicol acetyltransferase, homology modelling, sequential PCR mutagenesis, three-finger toxins.

INTRODUCTION

The cardiotoxins (CTXs) and short-chain neurotoxins (NTXs) found in snake venom are highly similar proteins, each comprising 60–65 amino acid residues. They adopt a similar structural scaffold, the three-finger loop structure, but differ significantly in their biological functions [1–3]. α -NTXs are hydrophilic proteins that specifically block neurotransmission across the neuromuscular junction [4–7]. On the other hand, snake venom CTXs are highly hydrophobic and basic polypeptides. Interestingly, CTXs can exert a wide variety of biological activities, such as depolarization and contraction of muscular cells, lysis of various types of cells such as red blood cells, epithelial cells and fetal lung cells, and also selective killing of certain types of tumour cells [8–11]. These highly hydrophobic proteins are found exclusively in the venom of cobras and ringhals. More than 95 CTXs from various sources have been reported, and their sequences show almost 90% similarity [12,13].

The three-dimensional structures of the three-fingered toxins indicate that the backbone of this family is composed of a globular core with four disulphide bridges and three fingers emerging from the core. Structural and evolutionary comparisons suggest that the globular core maintains the overall structure of these toxins [14,15], whereas major structural plasticity is present at the tips of the loops [16]. Recent studies have also documented that the minor changes in these loop regions might account for the functional differences between the toxins [17]. Among the biological functions of CTXs, their cytolytic and/or haemolytic properties are the most commonly studied. The lytic activity of CTXs is associated with their binding to cell membranes and resultant damage to the membranes. Chien et al. [18] divided

CTXs into two distinct types, P-type and S-type, based on Pro-31 and Ser-29 residues within a putative phospholipid binding site in the loop II region. The P-type CTXs have been found to possess greater haemolytic activity, whereas S-type CTXs show greater muscle cell depolarization activity. The S-type molecule has also been proposed to interact with the membrane through only one of its three hydrophobic loops [19], presumably loop II [20], whereas the membrane binding motif of the P-type CTXs has been proposed to involve all the three hydrophobic loops [21]. Although the chemical modification of specific amino acids has shed some light on the structure–activity relationships of CTXs, the amino acids exclusively responsible for the specific action of CTXs still need to be elucidated. However, the lysine residues flanking the tip of loops I–III have been suggested to represent an important functional motif in the binding of CTXs to the lipid bilayer [22]. Our previous study on six *Naja sputatrix* CTX isoforms demonstrated that they exhibited different degrees of cytolytic potency [13]. Primary sequence comparisons between these CTXs showed that most of the conserved amino acid residues participate in structural roles, forming the three-finger loop conformations, thus maintaining the structural scaffold, while the variable residues around the tips of the loops participate in their biological activities [23]. Loop II has been proposed to be the most important cytolytic domain for these CTXs [13].

To further examine the structure–function relationships of *N. sputatrix* CTX loops, CTX-4b, the P-type CTX that shows the greatest cytolytic activity, and NTX-2, with no such activity [7,13], have been chosen as templates for the construction of a series of chimaeric molecules by site-directed mutagenesis. In this study, a new cytolytic monitoring system has also been developed

Abbreviations used: CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; CTX, cardiotoxin; GST, glutathione S-transferase; LDH, lactate dehydrogenase; MEM, minimal essential medium; NTX, neurotoxin; prefixes in chimaeras denote the source of loops I–III, e.g. CNC-CTX contains loops I and III of CTX-4b (denoted by the Cs in CNC) and loop II of NTX-2 (denoted by the N in CNC).

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to assay for cytotoxicity. Our results indicate that all three loops of CTX work co-operatively to promote high cytotoxic activity, with the first two loops (I and II) of CTX being the major contributors. We have also shown for the first time that mammalian cells, such as Chinese hamster ovary (CHO) cells, undergo cytotoxicity when transfected with CTX cDNAs.

MATERIALS AND METHODS

Cell culture and DNA transfection

The CHO (A.T.C.C. CCL-61) cell line was maintained in α -minimal essential medium (α -MEM) supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μ g/ml streptomycin. Transfections were carried out with 6 μ g of reporter plasmids by the calcium phosphate method [24]. Stable transfectants were selected in G418 (Sigma)-supplemented growth media.

Construction of chloramphenicol acetyltransferase (CAT) reporter plasmid

Plasmid pMAMneoCAT (Clontech) was digested with *Nhe*I and *Nde*I to remove the MMTV (mouse mammary tumour virus) promoter. The whole CTX-2 gene promoter was amplified by PCR from a full-length CTX-2 promoter template [25]. After digestion with *Nhe*I and *Nde*I, the fragment was ligated into pMAMneoCAT promoter-less reporter vector DNA.

CAT assay

The transfected cells were treated with TEN buffer (40 mM Tris/HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl) for 5 min. The cells were then harvested by scraping with a rubber policeman and resuspended in 100 μ l of 0.25 M Tris/HCl, pH 8.0. The cells were ruptured by three rounds of freeze/thawing cycles (37 °C/solid CO₂ in ethanol). The extract was spun in a Microfuge for 5 min to remove cellular debris, and the resultant supernatant was retained. Protein estimation was carried out by the Bradford method using a Bio-Rad kit. The extract was heated at 65 °C for 10 min to inactivate deacetylases.

The assay mixture contained 5 μ l of n-butyryl-CoA (5 mg/ml; Sigma), 20 μ l of cell extract and 6 μ l of [¹⁴C]chloramphenicol (specific radioactivity 0.025 mCi/ml; Amersham Biosciences), and the mixture was made up with 0.25 M Tris/HCl to a final volume of 125 μ l. After incubation at 37 °C for 2 h, the reaction products were extracted with 500 μ l of ethyl acetate and the organic phase was evaporated to dryness in a Savant SpeedVac. The resultant residue was resuspended in 30 μ l of ethyl acetate and spotted on to a silica gel TLC plate (Merck). The plate was placed in a tank pre-saturated with chloroform/methanol (97:3, v/v) until the solvent front had travelled half-way up the plate. Radioactive chloramphenicol was visualized by autoradiography using Kodak XAR-5 film, for qualitative analysis.

Quantitative assessment of CAT enzymic activity was also carried out using liquid scintillation counting assays, as described by Seed and Sheen [26]. After the reaction, 300 μ l of mixed xylenes was used to extract the sample for 30 s. The sample was spun at 10000 g in a microcentrifuge for 3 min to achieve good phase separation. The mixed xylene phase was transferred into a fresh tube. After extraction with 100 μ l of 0.25 M Tris/HCl (pH 8.0) twice, 200 μ l of the upper xylene phase was transferred into a scintillation vial. Then an appropriate scintillation fluid (e.g. Ready-Safe™ scintillant) was added, and the radioactivity in the samples was measured in a liquid scintillation counter.

The radioactivity (c.p.m.) measured in each sample represents the butyrylated chloramphenicol products. Nuclear extracts from CHO cells were used as negative controls.

Homology modelling and three-dimensional protein structure of three-finger toxins

Homology modelling was carried out by submitting the amino acid sequences of toxins to the Swiss-Model web site for automatic homology modelling (first approach mode; <http://www.expasy.ch/swissmod>). The Swiss PDB viewer was used for three-dimensional structural comparisons and manipulation [27].

CD spectral analysis

CD spectra were recorded on a Jasco spectropolarimeter, model J715, equipped with an interfaced personal computer. The instrument was calibrated with ammonium *d*-10-camphorsulphonate. CD spectral data averaged from at least five recordings were considered acceptable. Results were expressed as mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = \theta/60lc$, where θ is the observed ellipticity in degrees, c is the concentration in dmol/l, l is the length of the light path in cm and the number 60 is the number of amino acid residues in the protein [28]. All CD spectra were measured at appropriate concentrations using 0.1 cm path-length cells.

The β -sheet content of all recombinant proteins was calculated based on the CD spectral values (200–240 nm) using the k2d analysis program at <http://www.embl-heidelberg.de/~andrade/k2d/> [29,30].

Construction of CTX/NTX chimaeras

CTX-4b and NTX-2 DNA fragments were cloned previously by reverse transcription-PCR from *N. sputatrix* snake venom mRNA [7,13]. In the present study, the DNA fragments encoding chimaeric toxins were constructed by sequential PCR. To construct the CNC-CTX chimaera, which contains loops I and III of CTX-4b (denoted by the Cs in CNC) and loop II of NTX-2 (denoted by the N in CNC), three rounds of PCR were carried out. In the first round of PCR, the region of DNA containing NTX loop II was amplified by using the NTX-2 template and primers CNC-F and CNC-R. The primers contained a short stretch of CTX-4b sequence at their 5'-ends (Table 1). The amplified DNA fragment was purified by gel electrophoresis and subjected to two separate amplifications (rounds 1 and 2). CTX-4b templates and the primer CTX-his-F or CTX-R were added separately to the purified first-round-PCR products. After denaturation at 95 °C for 3 min, primer CNC-F or CNC-R and PCR reagents were added. After 30 cycles, the truncated recombinant products were purified by agarose-gel electrophoresis. Finally, these products were mixed in equimolar amounts with primer set CTX-his-F and CTX-R for the third round of amplification. At the end of this amplification, the full-length CNC-CTX chimaera DNA was obtained. It was then subcloned into a pQE30 vector. To construct the other two chimaeric molecules, i.e. CCN-CTX (loops I and II of CTX with loop III of NTX) and NCC-CTX (loop I of NTX with loops II and III of CTX), similar PCR amplification strategies were used. For the construction of NCC-CTX, the NTX-2 template and primers NCC-R and NTX-GST-F were used (GST denotes glutathione S-transferase). After purification of the PCR product, it was mixed with diluted CTX-4b template and another round of PCR was carried out by using primer set NTX-GST-F and CTX-R. The CCN-CTX chimaera

Table 1 Primers used for construction of the recombinant chimaeric toxins

The complete NTX-2 and CTX-4b nucleotide sequences can be retrieved from GenBank (accession nos. AF097000 and U86595 respectively).

Primer	Descriptions	Sequence
CNC-F	Loops I and III of CTX and loop II of NTX; forward primer	5' AAGAACTTATGCTATAAAAGAG 3'
CNC-R	As above; reverse primer	5' GAGACTGCTTTTAGGGCAACCATC 3'
NCC-R	Loops II and III of CTX and loop I of NTX; reverse primer	5' AGTTCTTCCTGCTGAACAACCTGT 3'
NTX-GST-F	Neurotoxin GST fusion; forward primer	5' CCGCGTGGATCCCTGGAATGCCAC 3'
CCN-F	Loops I and II of CTX and loop III of NTX; forward primer	5' GGGCAACCATCCCTTTTGACAG 3'
NTX-R	NTX; reverse primer	5' AGAGCCAAGCTTATCAGTTGTTGCAGC 3'
NCN-F	Loops I and III of NTX and loop II of CTX; forward primer	5' GGGGAGACCAATTGCTATAAAATGTA 3'
NCN-R	As above; reverse primer	5' GGGCAACCATCCCTTTTGACAG 3'
NNC-F	Loops I and II of NTX and loop III of CTX; forward primer	5' TGTGTTTGCCCTAAAGCAGT 3'
CTX-R	CTX; reverse primer	5' GGAGGTAAGCTTATCAGTTGCATCTG 3'
CNN-R	Loops II and III of NTX and loop I of CTX; reverse primer	5' CTCCTGCTGGACAAGTC 3'
CTX-GST-F	CTX GST fusion; forward primer	5' GCGCGTGGATCCTTAAATGT 3'
CTX-his-F	CTX His-tagged fusion; forward primer	5' GGGATCCCTGGTTCGCGTGGTTCCCTAAAA 3'
NTX-his-F	NTX His-tagged fusion; forward primer	5' CGGGATCCCTGGTTCGCGTGGTTCCCTGGAA 3'
CTX-F	CTX forward primer for eukaryotic expression	5' GAATTCGCCGCCACCATGTTTAAATGTAAC 3'
NTX-F	NTX forward primer for eukaryotic expression	5' GAATTCGCCGCCACCATGCTGGAATGCCAC 3'

was constructed in a similar manner by using primers CCN-R, CTX-GST-F and NTX-R (Table 1). The primers used for the preparation of other constructs, each containing a single loop from CTX (CNN, loop I; NCN, loop II; NNC, loop III), are listed in Table 1.

All PCR amplifications were carried out on a Perkin Elmer Cetus Thermal Cycler Model 480 for 30 cycles, with each cycle consisting of a denaturing step at 94 °C for 1 min, an annealing step at 50 °C for 1 min and an elongation step at 72 °C for 2 min, followed by a final extension step of 10 min at 72 °C. The reaction mixture contained 250 mmol of each dNTP, 10 mmol each of the sense and antisense primers, 50 µl of reaction buffer (50 mM KCl, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl₂) and 1 unit of *Taq* polymerase.

To clone the corresponding recombinant toxin genes into a eukaryotic expression system, primers CTX-4b-F, NTX-F, CTX-R and NTX-R were used for PCR and then the amplified fragments were digested by *Eco*RI and *Xho*I. The restricted fragments were ligated into *Eco*RI/*Xho*I-treated pcDNA4/TO/myc-His-A vector. The reading frame and orientation of each of the constructs were confirmed by dideoxy DNA sequencing on an automated DNA sequencer (Model 373A; Applied Biosystems).

Expression and purification of recombinant chimaeric toxins

DNA fragments encoding recombinant CTX-4b, NTX-2 and all other chimaeric toxins were cloned into the bacterial expression vector pQE30 or pGEX-KG at *Bam*HI and *Hind*III restriction digestion sites and expressed as GST or His-tagged fusion proteins. The orientation and in-frame fusion of each of the recombinant genes with the GST gene or to the histidine tag in the vector were confirmed by DNA sequencing.

To facilitate the removal of GST and the His tag, thrombin-cleavage sites were incorporated just in front of the toxin proteins. The GST and His tags were removed by thrombin protease digestion. Hence only two extra amino acids, Gly and Ser, will remain attached to the toxin after digestion with thrombin.

The expression of cloned genes was induced by isopropyl β-D-thiogalactoside (0.1 mM) for 3–5 h and the expressed products were analysed by Tris/Tricine SDS/PAGE [31]. The GST fusion products were purified on a glutathione-agarose resin according to the manufacturer's protocol (Pharmacia Biotech) and cleaved

with thrombin to release the recombinant CTXs. For His-tagged fusion proteins, the recombinant proteins were purified under denaturing conditions. After dialysis to remove urea, the recombinant fusion proteins were cleaved by thrombin protease according to the protocol described by the manufacturer (Pharmacia Biotech).

Refolding of recombinant chimaeras

Before being refolded, the thrombin-digested recombinant proteins were first fully denatured by dilution into 5 vol. of 8 M urea in 50 mM Tris/HCl (pH 8.0)/2 mM EDTA containing 50 mM β-mercaptoethanol and incubated in the buffer for 2 h at room temperature. *In vitro* renaturation of unfolded proteins was performed overnight at room temperature by dialysis in PBS, pH 8, containing 4 mM GSH and 2 mM GSSG. Refolded proteins were finally purified using reverse-phase HPLC as previously described [13]. Briefly, the refolded chimaeras or the native proteins were purified by reverse-phase HPLC on a C18 column (Jupiter C18; 5 µm; 4.6 mm × 250 mm). The column was equilibrated with 0.1 % trifluoroacetic acid in water and the protein was eluted using a linear gradient of 0–60 % solvent B (0.1 % trifluoroacetic acid in acetonitrile). MS analyses were carried out on a Q-TOF mass spectrometer (Micromass UK Ltd). The processing of the mass spectra was performed using Micromass MassLynx software according to the manufacturer's protocol.

The refolding of these proteins to form secondary structures was monitored by CD spectra at a protein concentration of 20 µM on a Jasco spectropolarimeter (J715).

Cytotoxicity assay

The CHO-CAT cell line, which expresses the *CAT* gene endogenously, was inoculated into 24-well plates. When the cells had reached 80–90 % confluency, they were subjected to cytotoxicity tests. The medium in each well was aspirated, the cells were washed with 1 × PBS, and an appropriate amount of toxin dissolved in 200 µl of PBS was then added and incubated at 37 °C in a CO₂ incubator for 1 h. After treatment, the assay buffer was removed and stored at –70 °C for measurement of CAT activity. Percentage cytolytic activity was calculated as:

$$\text{Cytotoxicity (\%)} = ([\text{CAT}]_t - [\text{CAT}]_c) / ([\text{CAT}]_s - [\text{CAT}]_c)$$

where $[CAT]_c$, $[CAT]_t$ and $[CAT]_s$ represent the CAT activities in the media of untreated control cells, CTX-treated cells and cells that had been completely lysed by 40 μ g of *N. sputatrix* crude venom respectively.

For *in vivo* cytotoxicity assays, 5×10^5 CHO-CAT cells were inoculated on to 6-well plates 1 day before transfection. After co-transfection of CHO-CAT cells with pcDNA6/TR or pcDNA/TO/myc-His-A containing different recombinant toxin DNAs, the cells were incubated in complete α -MEM for 24 h. Then the transfected cells were induced by 1 ml of α -MEM containing 1 μ g/ml tetracycline for another 24 h. Approx. 500 μ l of medium was removed and heated at 65 °C for 10 min before proceeding to the CAT assay. Toxin protein expression was confirmed by Western blot analysis using anti-His-HRP (horseradish peroxidase) antibody. To standardize the results for potential cell death during the transfection procedure, medium removed from pcDNA6/TR-transfected cells was used as a negative control. The results were corrected for the c.p.m. readings from the negative control, and all transfections were carried out in triplicate.

Haemolysis assay

Freshly collected human blood in sodium citrate buffer was washed in isotonic NaCl solution. The cell suspension was centrifuged to obtain a clear supernatant. The washed cells were resuspended in the same buffer for haemolysis. Aliquots of the washed cells were then added and diluted to the desired CTX concentration in a final volume of 1 ml at 37 °C. After a 1 h incubation, the intact cells were spun down in a microcentrifuge. The absorbance of the supernatant was measured at 540 nm for quantification of the lysis of red blood cells. As a positive control, red blood cells were ruptured in water. Haemolytic potency was expressed as percentage haemolysis, assuming lysis in water to be equal to 100 % within a given incubation time.

RESULTS AND DISCUSSION

Establishment of a new cytolytic toxin monitoring system and assessment of the autolysis of CHO-CAT cells

To generate stably transfected cell lines that can constitutively express the *CAT* gene, a eukaryotic expression vector containing the *CAT* gene was constructed as described in the Materials and methods section. About 1 week after transfection of CHO cells with a *CAT* expression vector and selection by G418-containing medium, cells were serially diluted in G418/ α -MEM and inoculated into 96-well plates for cloning. After 6 weeks of selection and purification, the stably transfected CHO cells were expanded and established as the desired cell line. A total of 25 G418-resistant transfectants were harvested, lysed and tested for endogenous CAT activity. Figure 1(A) shows the TLC results of CAT activity for 13 of the tested clones. Seven clones exhibited strong endogenous CAT activity. One of these, clone 18, was chosen for further analysis and stored frozen under liquid nitrogen. This clone was designated CHO-CAT.

The ability of CHO-CAT cells to remain as intact cells and also to prevent the CAT enzyme from being spontaneously released into the buffer (in the absence of a cytolytic agent) is important, to increase the signal-to-noise ratio of the cytotoxicity assay. To test this property, the growth medium was removed and PBS was added to 80 % confluent CHO-CAT cells. PBS was sampled at different time points. Figure 1(B) shows that, even after 8 h in PBS, the cells were still intact, with no detectable enzymic activity in the incubation buffer. Most of the enzyme

activity was confined to the interior of the cells. A relatively high level of CAT activity was observed when cell extracts (after lysis) were assayed for the enzyme (Figure 1B).

Comparison with the red blood cell cytolytic analysis system and the lactate dehydrogenase (LDH) assay

Red blood cells are commonly used to determine cytolytic activity [32]. However, the accuracy, sensitivity and linearity of such an assay are extremely poor. When a red blood cell system was used for the comparison of cytotoxicity elicited by different CTX isoforms, no significant differences were observed.

Although a number of CTXs exhibit haemolytic activity, some CTXs do not. However, they may lyse other cell types, especially some tumour cells [33]. In Figure 1(C), the sensitivity and accuracy of these two cytolytic assay systems were compared based on the cytolytic potency of the protein in relation to its concentration. In the new CHO-CAT cell cytolytic assay, 50 % lysis was observed at 100 nM protein, while the haemolytic assay showed only < 5 % lysis at 100 nM toxin. On the other hand, 50 % lysis was observed at 700 nM toxin in the conventional LDH assay for cytotoxicity determined previously [13] for the same CTX-4b. Thus the CHO-CAT system is at least 10 times more sensitive than the haemolytic assay and 7 times more sensitive than the LDH assay. Significant (~10 %) lysis has been observed even at 60 nM toxin concentration with the CHO-CAT system, while almost no lysis was observed at this concentration in the haemolytic assay. The high sensitivity of the CHO-CAT system might be due to the following. First, it is well known that transformed cells, such as CHO cells, are more sensitive to cytotoxins [34]. The morphology and characteristics of this cell line are very similar to those of tumour cells, such as high growth rate, high nucleus/cytoplasm ratio, etc. [35]. Secondly, CAT is extremely stable, so that the CAT produced can accumulate during the incubation period. Thirdly, if the enzymic reaction time was prolonged, very low levels of endogenous CAT enzyme could be detected. Besides these advantages, CAT is a prokaryotic enzyme and is absent from mammalian cells. These features make it the most suitable enzyme for the assay. Several commercially available CAT ELISA kits, e.g. from Boehringer Mannheim or Promega, can also be used by those investigators who prefer to use non-radioactive substrates.

Homology modelling, construction and purification of chimaeric three-finger toxins

Post-synaptic NTXs and CTXs belong to the family of three-fingered toxins. Even though they have a similar three-dimensional superimposable scaffold, no functional similarities have been reported. It is well known that protein function is due to its specific spatial folding. Ricciardi et al. [17] demonstrated that subtle structural deviations could affect specific functional topographies. Through loop-swapping between different toxins, these authors [17] have shown that structurally different regions not only can confer their specific functions to the host molecule, but also can keep their native conformation similar to that of the donor molecule and even retain the conformation of adjacent residues. Structural and functional comparisons have revealed that the three loops of NTX/CTX may be the most important regions for their neurotoxicity or cytotoxicity. By a comparison of primary sequences, Jeyaseelan et al. [13] suggested that the most important domain for the cytolytic activity of CTX is loop II of the protein. Hence, to explore the cytolytic activity of CTX, the P-type CTX (highly cytolytic) CTX-4b and the highly neurotoxic NTX-2 of *N. sputatrix* were selected as initial templates.

Figure 1 Development of a CHO-CAT cytolytic assay

(A) TLC for the assessment of CAT activity in CHO cell lines stably transfected with the *CAT* gene. All clones (clones 6–18 are shown) were G418-resistant and expanded from single cells. Clone 18 (CHO-CAT) was chosen for further analysis. Cm, chloramphenicol; bCm, butyrylated chloramphenicol. (B) Assessment of CAT expression. CHO-CAT cells were inoculated into 24-well plates at 3×10^5 cells/well. After overnight growth, the medium was replaced by 500 μ l of PBS. Samples of 50 μ l of PBS were removed every 2 h from each well. After 8 h, 40 μ g of crude venom was added to lyse all the cells and CAT activity was analysed by liquid scintillation counting as described in the Materials and methods section. ▲, CAT activity of whole-cell extracts; ■, CAT activity of supernatant. (C) Comparison of cytotoxicity of red blood cells with the CHO-CAT system. CHO-CAT cells and red blood cells were incubated with different concentration of CTXs for 1 h at 37 °C, and cytotoxic and haemolytic assays were carried out as described in the Materials and methods section.

Homology modelling of CTX-4b and NTX-2 was carried out in order to verify structural deviation between the two proteins. The modelled structures of CTX-4b and NTX-2 have been superimposed in order to compare the overall structural differences between them (Figure 2A). The major structural deviations have been found to be located within loops I and II. Initially, single-loop transfers were carried out to test whether the cytotoxic properties of CTX-4b could be transferred to NTX-2. As a co-operative interaction between loops has been demonstrated in previous reports [36,37] to be required for CTX to display its cytotoxicity, we also transferred two loops at a time from the CTX molecule to its NTX counterpart by sequential PCR. Compared with other mutagenesis protocols, sequential PCR represents an extremely simple and successful method for the shuffling of DNA fragments. The basic three-finger structure was kept undisturbed by maintaining the conserved residues as well as the eight cysteine residues. The detailed experimental procedures have been outlined in the Materials and methods section, and the exchanged areas in the chimaeric toxins are shown in Figure 2(C).

Two expression strategies were employed, based on the yield and solubility of recombinant proteins. The chimaeric proteins CNN-NTX, NCN-NTX, NNC-NTX, CCN-CTX and NCC-CTX were expressed as GST fusion proteins. Other proteins,

including CTX, CNC-CTX and NTX, were expressed by a His-tag expression system. After digestion by thrombin, all recombinant proteins were refolded in refolding buffer containing 4 mM GSH and 2 mM GSSG. Figure 3 shows that the thrombin-digested recombinant toxins purified by reverse-phase HPLC were homogeneous. The secondary structures were examined by far-UV CD. A negative trough at around 211 nm and a large positive trough at around 195 nm which is typical of β -sheet organization, were observed in all the chimaeric constructs, as well as in native CTX (Figure 3C). This strongly suggests that the dominant β -sheet structure that characterizes the three-finger-like fold has been preserved in the refolded recombinant chimaeric toxins (Table 2). However, close inspection of the CD spectra suggests that CCN-CTX, NCN-NTX and CNC-CTX might exhibit a tightly packed structure like that of the native toxins, but that NCC-CTX might exhibit a slightly different (possibly improperly folded) structure.

Cytolytic activity of recombinant chimaeric toxins

CTX-4b, a P-type CTX from *N. sputatrix*, was selected as a model for cytotoxic assays based on the previous studies of Jeyaseelan et al. [13] and Chien et al. [18]. To evaluate the

Figure 2 Homology models of CTX-4b, NTX-2 and CCN-CTX

(A) Superimposed molecular models of CTX-4b and NTX-2. CTX-4b and NTX-2 were modelled based on a primary structure homology model and superimposed to identify the variant regions. (B) Model of CTX-4b superimposed on that of CCN-CTX. The β -strands are indicated as yellow lines. The highly variable region is shown in red on the CTX-4b molecule. The corresponding amino acids within the regions are labelled. (C) Sequence alignment of recombinant toxins. The loops exchanged are underlined. The regions of the three loops are also indicated.

cytolytic activities of the different chimaeric toxins, CHO-CAT cells were incubated with various concentrations of the toxins. Each experiment was carried out in triplicate. The results demonstrated that *in vitro* refolded recombinant CTX-4b retained the cytolytic function of native CTX-4b (Figure 4A). NTX, NCC-CTX and the single-loop-transferred chimaeric constructs NNC-, CNN- and NCN-NTX did not exhibit any cytolytic activity. As expected from the homology modelling data (Figure 2B), where the first two loops in the CCN-CTX model superimposed well with those of native CTX-4b, the cytolytic potency of CCN-CTX was found to be much higher than that of CNC-CTX. Based on the CD spectral data, the loss of cytotoxic activity in NCC-CTX could be due to possible perturbation of its tertiary structure caused by exchange of the loops (Figure 2C). Transfer of individual loops of CTX on to the NTX backbone did not result in chimaeric proteins with cytolytic activity, even though the spatial structure, based on CD spectral analysis, was observed to be well preserved (Figure 3C). Hence it is clear that all three

of the finger-loops work in a co-operative manner. However, the first two loops are thought to be the major functional domains with regard to cytotoxicity, as observed for the cytolytic activity of CCN-CTX. Although the chimaeric toxin CNC-CTX exhibited a tightly packed structure, like the native toxins, the cytolytic activity of this toxin was still markedly lower (Figure 4A) than that of the other constructs, indicating that functional topology differs from the structural scaffold, and that loops I and II play a crucial role in the cytotoxic action of the protein. *In vitro*, CTXs execute their cytotoxic activity by binding to zwitterionic phospholipids through hydrophobic interactions [38,39] and to anionic glycosaminoglycans via specific electrostatic interactions [40]. The continuous hydrophobic patch formed in the CTX molecule might be one of the major reasons for its detergent-like cell lysis activity. The hydrophobicity of CCN-CTX (Figure 5) is higher than that of CNC-CTX in the loop II region. This difference could possibly explain the high and low cytotoxicity observed for CCN-CTX and CNC-CTX respectively. However,

Figure 3 Purification and properties of recombinant toxins

(A) SDS/PAGE of HPLC-purified recombinant proteins. LMW, low-molecular-mass markers (Amersham Pharmacia; CFA 755). (B) Reverse-phase HPLC and mass spectra results. The recombinant and native proteins were purified to homogeneity on HPLC using a C18 column (Jupiter C18; 4.6 mm $\times$ 250 mm). Protein was eluted using 0.1% trifluoroacetic acid in acetonitrile (0–60%). The inset in panel (III) shows the mass spectra results for CCN-CTX on Q-TOF. (C) CD spectra showing the molecular ellipticity of native CTX and the recombinant chimaeric toxins.

Table 2 Prediction of protein secondary structure from CD spectra

		CTX					
	CTX-4b	refolded	CCN-CTX	CNC-CTX	NCC-CTX	NCN-NTX	NTX-2
α -Helix	> 0.18	> 0.18	> 0.18	> 0.19	> 0.31	> 0.23	> 0.27
β -Sheet	> 0.33	> 0.30	> 0.30	> 0.28	> 0.11	> 0.29	> 0.35
Random coil	> 0.48	> 0.51	> 0.51	> 0.53	> 0.58	> 0.48	> 0.38

the role of the hydrophobic nature of the N-terminal and loop I region of the cytotoxin is yet to be defined. Sivaraman et al. [41] proposed that the N- and C-terminal ends of CTX III in *Naja atra* aid in the compaction of the CTX molecule. The CD-spectral data also showed that replacing loop I of CTX with loop I of short-chain NTX in NCC-CTX disturbs the spatial structure of the three-finger scaffold (Figure 3C). Interestingly CCN-CTX exhibited a tightly packed structure. The β -sheet content of CCN-CTX is similar to that of native CTX-4b and recombinant CTX-4b (Table 2).

Stevens-Truss and Hinman [42] reported that the absence of an aromatic residue at position 11 in loop I resulted in lower cytolytic activity of CTX. Interchanging amino acid sequences in loop III selectively impaired target-specific binding and cytotoxicity, while loss of a positively charged residue at the tip of loop

II significantly reduced cytotoxicity. The functionally important residues that are located at the tips of three loops are believed to be flexible, and this flexibility is postulated to be crucial in the binding of CTXs to their respective acceptor or receptor [43]. On the other hand, Dufourcq et al. [19] reported that the hydrophobic patch located at the tip of loop I could constitute the erythrocyte-interaction site of CTXs, and that the loop I residues are more flexible than the residues in loop II and III. Chien et al. [18], however, postulated that the S-type CTXs penetrate the membrane via only one loop, presumably loop II. However, in the P-type CTXs (membrane-active toxins), all three loops were demonstrated to be involved in the interaction with the lipid bilayer [21]. In our present study, the involvement of loops I and II was observed to be significant for the cytotoxic activity of CTX. The chimaeric toxin CCN-CTX was found to be the most active cytotoxic protein, followed by CNC-CTX and (least active) NCC-CTX (Figure 4A). The CD spectra and the predicted β -sheet and α -helix structural content of the refolded proteins (CCN-CTX and CNC-CTX; Figure 3C and Table 2), as well as the hydrophobicity profiles (Figure 5), were found to correlate well with those of the native proteins, except for the NCC-CTX chimaera, which exhibited a slightly different CD spectrum.

Transfection of cDNAs into mammalian cells and cytotoxic assays

To rule out the possible misfolding of proteins within the bacterial expression system, we co-transfected pcDNA6/TR with

Figure 4 Cytolytic activity of chimaeric toxins

(A, B) Cytolytic activity determined for native and recombinant toxins at different concentrations of toxin (A) and at 400 nM toxin (B). CTX-n, native CTX; CTX-r, recombinant CTX. (C) *In vivo* expression of toxin cDNAs and cytolytic activity of the proteins. The cytolytic activities of different chimaeric toxins in the eukaryotic system are compared. The plasmid pcDNA4/TO/myc-His-A containing the toxin gene was transfected into CHO-CAT cells. After 48 h, the transfected cells were incubated in the same medium or subjected to 1 μ g/ml tetracycline (Tet) treatment for another 24 h. The cells were collected and boiled with SDS loading buffer for Western blot analysis by using anti-His-HRP antibody. All tests were carried out in triplicate, and the results are means $\pm$ S.D. of three independent experiments.

recombinant pcDNA4/TO/myc-His-A tetracycline-inducible vector containing different recombinant toxin genes, including the chimaeric constructs, into CHO-CAT cells and examined their cytotoxic effects.

The pcDNA4/TO/myc-His vector contains two tetracycline operator 2 (TetO2) binding sites within the human cytomegalovirus promoter for the tetracycline-regulated expression of the target gene of interest [44], and allows the high-level expression of toxin genes. Since gene expression can be stringently controlled by the Tet repressor, this system has been successfully used for the investigation of other highly cytotoxic proteins in eukaryotic cells [44–47]. The DNA fragments encoding the corresponding toxins were subcloned into pcDNA4/TO/myc-His-A vector and transiently co-transfected with pcDNA6/TR vector into

Figure 5 Hydropathy profiles of toxins

The hydropathy of native CTX-4b and NTX-2 (upper panel) and the profiles of chimaeras CCN-CTX, CNC-CTX, NCC-CTX and NCN-NTX (lower panel) are shown.

CHO-CAT cells. After 24 h of growth in complete medium, protein expression was induced by α -MEM containing 1 μ g/ml tetracycline. The expression of toxin proteins in CHO-CAT cells was confirmed by Western blotting using anti-His-HRP labelled antibodies (Figure 4C, inset). Figure 4(C) shows the cytotoxic effects of the different constructs. A pattern similar to that of *in vitro* assays was observed, whereby CCN-CTX showed the highest lytic activity and NCC-CTX was the least active construct. These data not only further supported the results of the *in vitro* cytolytic assays, but also demonstrated that CTXs are capable of lysing cells from inside the cells as well. Recently Jayaraman et al. [48] reported that the DNA binding ability of CTXs could account for their cytolytic properties. Therefore CTXs can bring about cytotoxicity, and possibly cell death, when expressed within eukaryotic cells as reported. However, potential applications of chimaeric CTXs in inducing cell death in certain types of tumour cells need to be investigated further.

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REFERENCES

- Kumar, T. K., Jayaraman, G., Lee, C. S., Arunkumar, A. I., Sivaraman, T., Samuel, D. and Yu, C. (1997) Snake venom cardiotoxins – structure, dynamics, function and folding. *J. Biomol. Struct. Dyn.* **15**, 431–463
- Menez, A., Gattineau, E., Roumestand, C., Harvey, A. L., Mouawad, L., Gilquin, B. and Toma, F. (1990) Do cardiotoxins possess a functional site? Structural and chemical modification studies reveal the functional site of the cardiotoxin from *Naja nigricollis*. *Biochimie* **72**, 575–588
- Tsetlin, V. (1999) Snake venom alpha-neurotoxins and other 'three-finger' proteins. *Eur. J. Biochem.* **264**, 281–286
- Endo, T. and Tamiya, N. (1991) Structure-function relationships of postsynaptic neurotoxins from snake venoms. In *Snake Toxins* (Harvey, A. L., ed.), pp. 165–222. Pergamon Press, New York
- Takacs, Z., Wilhelmsen, K. C. and Sorota, S. (2001) Snake alpha-neurotoxin binding site on the Egyptian cobra (*Naja haje*) nicotinic acetylcholine receptor is conserved. *Mol. Biol. Evol.* **18**, 1800–1809

- 6 Ackermann, E. J. and Taylor, P. (1997) Non-identity of the alpha-neurotoxin binding sites on the acetyl choline receptor by modification of alpha-neurotoxin and receptor structures. *Biochemistry* **36**, 12836–12844
- 7 Afifyan, F., Armugam, A., Gopalakrishnakone, P., Tan, N. H., Tan, C. H. and Jeyaseelan, K. (1998) Four new postsynaptic neurotoxins from *Naja naja sputatrix* venom: cDNA cloning, protein expression, and phylogenetic analysis. *Toxicon* **36**, 1871–1885
- 8 Harvey, A. L., Marshall, R. J. and Karlsson, E. (1982) Effects of purified cardiotoxins from the Thailand cobra (*Naja naja siamensis*) on isolated skeletal and cardiac muscle preparations. *Toxicon* **20**, 379–396
- 9 Chen, X. H. and Harvey, A. L. (1993) Effects of different antagonists on depolarization of cultured chick myotubes by cobra venom cardiotoxins and Pyrularia thionin from the plant *Pyrularia pubera*. *Toxicon* **31**, 1229–1236
- 10 Teng, C. M., Jy, W. and Ouyang, C. (1984) Cardiotoxin from *Naja naja atra* snake venom: a potentiator of platelet aggregation. *Toxicon* **22**, 463–470
- 11 Zaheer, A. and Braganca, B. M. (1980) Comparative study of three basic polypeptides from snake venoms in relation to their effects on the cell membrane of normal and tumor cells. *Cancer Biochem. Biophys.* **5**, 41–46
- 12 Dufton, M. J. (1984) Classification of elapid snake neurotoxins and cytotoxins according to chain length: evolutionary implications. *J. Mol. Evol.* **20**, 128–134
- 13 Jeyaseelan, K., Armugam, A., Lachumanan, R., Tan, C. H. and Tan, N. H. (1998) Six isoforms of cardiotoxin in malayan spitting cobra (*Naja naja sputatrix*) venom: cloning and characterization of cDNAs. *Biochim. Biophys. Acta* **1380**, 209–222
- 14 Brown, L. R. and Wuthrich, K. (1992) Nuclear magnetic resonance solution structure of the alpha-neurotoxin from the black mamba (*Dendroaspis polylepis polylepis*). *J. Mol. Biol.* **227**, 1118–1135
- 15 Hatanaka, H., Oka, M., Kohda, D., Tate, S., Suda, A., Tamiya, N. and Inagaki, F. (1994) Tertiary structure of erabutoxin b in aqueous solution as elucidated by two-dimensional nuclear magnetic resonance. *J. Mol. Biol.* **240**, 155–166
- 16 Albrand, J. P., Blackledge, M. J., Pascaud, F., Hollecker, M. and Marion, D. (1995) NMR and restrained molecular dynamics study of the three-dimensional solution structure of toxin FS2, a specific blocker of the L-type calcium channel, isolated from black mamba venom. *Biochemistry* **34**, 5923–5937
- 17 Ricciardi, A., le Du, M. H., Khayati, M., Dajas, F., Boulain, J. C., Menez, A. and Ducancel, F. (2000) Do structural deviations between toxins adopting the same fold reflect functional differences? *J. Biol. Chem.* **275**, 18302–18310
- 18 Chien, K. Y., Chiang, C. M., Hseu, Y. C., Vyas, A. A., Rule, G. S. and Wu, W. G. (1994) Two distinct types of cardiotoxin as revealed by the structure and activity relationship of their interaction with zwitterionic phospholipid dispersions. *J. Biol. Chem.* **269**, 14473–14483
- 19 Dufourcq, J., Faucon, J. F., Bernard, E., Pezelet, M., Tessier, M., Bougis, Z., Van Rietschotten, J., Delori, P. and Rochat, H. (1982) Structure-function relationships for cardiotoxins interacting with phospholipids. *Toxicon* **20**, 165–174
- 20 Lauterwein, J. and Wuthrich, K. (1978) A possible structural basis for the different modes of action of neurotoxins and cardiotoxins from snake venoms. *FEBS Lett.* **93**, 181–184
- 21 Dubovskii, P. V., Dementieva, D. V., Bocharov, E. V., Utkin, Y. N. and Arseniev, A. S. (2001) Membrane binding motif of the P-type cardiotoxin. *J. Mol. Biol.* **305**, 137–149
- 22 de Planque, M. R., Kruijtz, J. A., Liskamp, R. M., Marsh, D., Greathouse, D. V., Koeppe, II, R. E., de Kruijff, B. and Killian, J. A. (1999) Different membrane anchoring positions of tryptophan and lysine in synthetic transmembrane alpha-helical peptides. *J. Biol. Chem.* **274**, 20839–20846
- 23 Dufton, M. J. and Hider, R. C. (1983) Conformational properties of the neurotoxins and cytotoxins isolated from Elapid snake venoms. *CRC Crit. Rev. Biochem.* **14**, 113–171
- 24 Jordan, M., Schallhorn, A. and Wurm, F. M. (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.* **24**, 596–601
- 25 Ma, D., Armugam, A. and Jeyaseelan, K. (2001) Expression of cardiotoxin-2 gene. Cloning, characterization and deletion analysis of the promoter. *Eur. J. Biochem.* **268**, 1844–1850
- 26 Seed, B. and Sheen, J. Y. (1988) A simple phase-extraction assay for chloramphenicol acyltransferase activity. *Gene* **67**, 271–277
- 27 Guex, N. and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**, 2714–2723
- 28 Sivaraman, T., Kumar, T. K., Jayaraman, G., Han, C. C. and Yu, C. (1997) Characterization of a partially structured state in an all-beta-sheet protein. *Biochem. J.* **321**, 457–464
- 29 Andrade, M. A., Chacón, P., Merelo, J. J. and Morán, F. (1993) Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network. *Protein Eng.* **6**, 383–390
- 30 Merelo, J. J., Andrade, M. A., Prieto, A. and Morán, F. (1994) Proteinotopic feature maps. *Neurocomputing* **6**, 443–454
- 31 Schagger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379
- 32 Fletcher, J. E., Jiang, M. S., Tripolitis, L., Smith, L. A. and Beech, J. (1990) Interactions in red blood cells between fatty acids and either snake venom cardiotoxin or halothane. *Toxicon* **28**, 657–667
- 33 Costa, L. A., Miles, H., Araujo, C. E., Gonzalez, S. and Villarrubia, V. G. (1998) Tumor regression of advanced carcinomas following intra- and/or peri-tumoral inoculation with VRCTC-310 in humans: preliminary report of two cases. *Immunopharmacol. Immunotoxicol.* **20**, 15–25
- 34 Borkow, G. and Ovadia, M. (1999) Selective lysis of virus-infected cells by cobra snake cytotoxins: a sendai virus, human erythrocytes and cytotoxin model. *Biochem. Biophys. Res. Commun.* **264**, 63–68
- 35 Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D. and Darnell, J. (1999) *Molecular Cell Biology*, 4th edn, W.H. Freeman, New York
- 36 Jayaraman, G., Kumar, T. K., Tsai, C. C., Srisailam, S., Chou, S. H., Ho, C. L. and Yu, C. (2000) Elucidation of the solution structure of cardiotoxin analogue V from the Taiwan cobra (*Naja naja atra*) – identification of structural features important for the lethal action of snake venom cardiotoxins. *Protein Sci.* **9**, 637–646
- 37 Gatineau, E., Takechi, M., Bouet, F., Mansuelle, P., Rochat, H., Harvey, A. L., Monteny-Garestier, T. and Menez, A. (1990) Delineation of the functional site of a snake venom cardiotoxin: preparation, structure, and function of monoacetylated derivatives. *Biochemistry* **29**, 6480–6489
- 38 Chien, K. Y., Huang, W. N., Jean, J. H. and Wu, W. G. (1991) Fusion of sphingomyelin vesicles induced by proteins from Taiwan cobra (*Naja naja atra*) venom. Interactions of zwitterionic phospholipids with cardiotoxin analogues. *J. Biol. Chem.* **266**, 3252–3259
- 39 Chiang, C. M., Chien, K. Y., Lin, H. J., Lin, J. F., Yeh, H. C., Ho, P. L. and Wu, W. G. (1996) Conformational change and inactivation of membrane phospholipid-related activity of cardiotoxin V from Taiwan cobra venom at acidic pH. *Biochemistry* **35**, 9167–9176
- 40 Patel, H. V., Vyas, A. A., Vyas, K. A., Liu, Y. S., Chiang, C. M., Chi, L. M. and Wu, W. (1997) Heparin and heparan sulfate bind to snake cardiotoxin. Sulfated oligosaccharides as a potential target for cardiotoxin action. *J. Biol. Chem.* **272**, 1484–1492
- 41 Sivaraman, T., Kumar, T. K., Chang, D. K., Lin, W. Y. and Yu, C. (1998) Events in the kinetic folding pathway of a small, all beta-sheet protein. *J. Biol. Chem.* **273**, 10181–10189
- 42 Stevens-Truss, R. and Hinman, C. L. (1997) Activities of cobra venom cytotoxins toward heart and leukemic T-cells depend on localized amino acid differences. *Toxicon* **35**, 659–669
- 43 Lee, C. S., Kumar, T. K., Lian, L. Y., Cheng, J. W. and Yu, C. (1998) Main-chain dynamics of cardiotoxin II from Taiwan cobra (*Naja naja atra*) as studied by carbon-13 NMR at natural abundance: delineation of the role of functionally important residues. *Biochemistry* **37**, 155–164
- 44 Yao, F., Svensjo, T., Winkler, T., Lu, M., Eriksson, C. and Eriksson, E. (1998) Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Hum. Gene Ther.* **9**, 1939–1950
- 45 Pollock, R., Issner, R., Zoller, K., Natesan, S., Rivera, V. M. and Clackson, T. (2000) Delivery of a stringent dimerizer-regulated gene expression system in a single retroviral vector. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 13221–13226
- 46 Massie, B., Couture, F., Lamoureux, L., Mosser, D. D., Guilbault, C., Jolicœur, P., Belanger, F. and Langelier, Y. (1998) Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette. *J. Virol.* **72**, 2289–2296
- 47 Nakagawa, I., Nakata, M., Kawabata, S. and Hamada, S. (1999) Regulated expression of the Shiga toxin B gene induces apoptosis in mammalian fibroblastic cells. *Mol. Microbiol.* **33**, 1190–1199
- 48 Jayaraman, G., Krishnaswamy, T., Kumar, S. and Yu, C. (1999) Binding of nucleotide triphosphates to cardiotoxin analogue II from the Taiwan cobra venom (*Naja naja atra*). Elucidation of the structural interactions in the dATP-cardiotoxin analogue II complex. *J. Biol. Chem.* **274**, 17869–17875