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Towards universal approach for bacterial production of three-finger Ly6/uPAR proteins: Case study of cytotoxin I from cobra *N. oxiana*

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ABSTRACT

Cytotoxins or cardiotoxins is a group of polycationic toxins from cobra venom belonging to the 'three-finger' protein superfamily (Ly6/uPAR family) which includes small β-structural proteins (60–90 residues) with high disulfide bond content (4–5 disulfides). Due to a high cytotoxic activity for cancer cells, cytotoxins are considered as potential anticancer agents. Development of the high-throughput production methods is required for the prospective applications of cytotoxins. Here, efficient approach for bacterial production of recombinant analogue of cytotoxin I from *N. oxiana* containing additional N-terminal Met-residue (rCTX1) was developed. rCTX1 was produced in the form of *E. coli* inclusion bodies. Refolding in optimized conditions provided ~6 mg of correctly folded protein from 1 L of bacterial culture. Cytotoxicity of rCTX1 for C6 rat glioma cells was found to be similar to the activity of wild type CTX1. The milligram quantities of ^{13}C,^{15}N-labeled rCTX1 were obtained. NMR study confirmed the similarity of the spatial structures of recombinant and wild-type toxins. Additional Met residue does not perturb the overall structure of the three-finger core. The analysis of available data for different Ly6/uPAR proteins of snake and human origin revealed that efficiency of their folding *in vitro* is correlated with the number of proline residues in the third loop and the surface area of hydrophobic residues buried within the protein interior. The obtained data indicate that hydrophobic core is important for the folding of proteins with high disulfide bond content. Developed expression method opens new possibilities for structure-function studies of CTX1 and other related three-finger proteins.

1. Introduction

The venom of snakes of the *Elapidae* family is a complex mixtures of dozens polypeptides. The main lethal components of the venom are the protein toxins with characteristic three-finger fold (three-finger proteins, TFPs) [1,2]. These proteins contain three loops protruding from a small β-structural core, composed from two β-sheets and cross-linked by four invariant disulfide bonds (Fig. 1) [3]. Evolution of snake venom TFPs led to a significant functional diversification. Inhibitors of nicotinic and muscarinic acetylcholine receptors, β1-/β2-adrenergic receptors, L-type calcium channels, acetylcholinesterase and even acid sensing ion channels have been isolated from *Elapidae* venom [2,4]. The three-finger fold is adopted also by a variety of non-toxic proteins belonging to the Ly6/uPAR family which were found in insects and vertebrates including mammals [5].

Cytotoxins or cardiotoxins (CTXs) is a highly conserved group of snake three-finger toxins which share significant sequence homology (Fig. 1) [6,7]. Biological effects of CTXs include depolarization of myocytes, hemolysis and prevention of platelet aggregation [8]. Some CTXs can cause cardiac arrest of a perfused heart and for that reason they were called cardiotoxins [9]. CTXs can inhibit vital proteins such as protein kinase C and Na+, K + ATPase [10].

Abbreviations: CTX, cytotoxin; Mblgn-2, mambalgin-2 from *D. polylepis*; rCTX1, recombinant analogue of cytotoxin-1 from *Naja oxiana*; TFPs, three-finger proteins; ws-Lynx1, water soluble domain of human Lynx1; wtCTX1, wild-type cytotoxin-1 from *Naja oxiana*; WTX, weak toxin from *N. kaouthia*.

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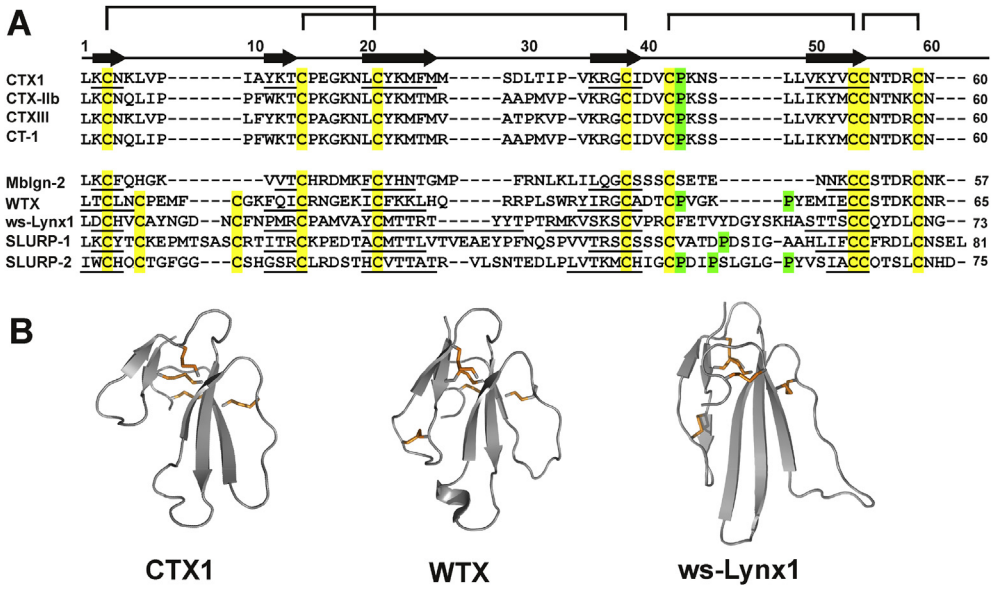

Fig. 1. (A) Amino acid sequence alignment of CTX1 from *N. oxiana* (UniProt number: P01451) with other cytotoxins and human three finger proteins: CTX-IIb from *N. mossambica* (P01467), CTXIII from *N. atra* (P60301), CT-1 from *N. pallida* (P01468), Mblgn-2, - mambalgin-2 from *D. polylepis* (P0DKS3), WTX from *N. kaouthia* (P82935), water-soluble domain of human Lynx-1 (isoform 2 of Ly-6/neurotoxin-like protein 1 Q9BZG9-2), SLURP-1 (Secreted Ly-6/uPAR-related protein 1, P55000), SLURP-2 (isoform 3 of Ly-6/neurotoxin-like protein 1 Q9BZG9-3). Cysteine residues and proline residues from the loop 3 are highlighted. The fragments corresponding to β-strands in the spatial structures are underlined. (B) Comparison of spatial structures of CTX1, ws-Lynx1 and WTX [P33A] mutant.

However, this inhibitory effect could be connected with the attenuation of elastic properties of the lipid membranes upon incorporation of CTXs into them [11]. The tips of CTXs loops, which are enriched by hydrophobic amino acid residues, form the membrane-binding motif [12,13]. CTXs bind to membranes via interaction with anionic lipids and induce membrane disintegration [9,14]. Some CTXs exhibit antibacterial activity and destabilize the bacterial cell membranes, rich in anionic phospholipids [15]. Of practical interest is the cytotoxic activity of CTXs against cancer cells [16–20]. This activity varies considerably for different CTXs, and depends on a cell type. Cytotoxicity of CTXs usually is realized in a micromolar range of concentrations [21,22] and is related to the toxin accumulation in lysosomes [22]. When a concentration of CTXs in lysosomes exceeds some threshold value, lysosomal membrane is disrupted thus initiating apoptosis or necrosis of cancer cells [18,22]. CTXs are considered as prospective agents for anticancer therapy [23]. However the rational design of CTX-based drugs with improved and directed antitumor activity requires systematic structure-function studies. In spite of high relevance, such studies are complicated by difficulties with a production of these proteins. The membrane activity of CTXs makes them toxic to the host cells, significantly hampering recombinant production.

The high content of disulfide bonds (4–5 disulfides per 60–90 residues) also complicates bacterial production of CTXs and other TFPs. Disulfide bonds do not form in a cytoplasm, thus alternative approaches of recombinant production are required. These alternative methods involve: (1) periplasmic expression (via bacterial secretion systems), (2) production in the form of hybrid constructs fused with the proteins promoting disulfide bond formation, (3) direct expression in the form of cytoplasm inclusion bodies with subsequent refolding [24]. In addition, (4) the usage of special mutant *E. coli* strains, which have more oxidizing environment inside the cell [25], sometimes permits the production of folded proteins with multiple disulfide bonds in the bacterial cytoplasm. Previously, for bacterial production of CTXs the direct expression [26–29] or expression of the fused constructs with GST [30] or beta-galactosidase [31] was explored. However, the majority of these works did not result in the production of a recombinant CTX with biological activity and spatial structure similar to the native toxin. According to our knowledge, only one from the previous works described the successful refolding of CTX (CTXIII from *N. atra*) from *E. coli* inclusion bodies [28], but the final yield of the refolded CTXIII was not reported. Limited number of examples of successful CTX production confirms high complexity of the problem.

Previously, we successfully produced a number of Ly6/uPAR proteins of different origin using direct expression with subsequent refolding. These are two snake toxins: WTX from *N. kaouthia* [32] and mambalgin-2 (Mblgn-2) from *D. polylepis* (M.A.S. & E.N.L. unpublished), and three human proteins: ws-Lynx1 (water soluble domain of Lynx1) [33], SLURP-1 [34], and SLURP-2 [35] (Fig. 1). In this work we tested the applicability of this approach to high-efficient production of CTXs on the example of CTX1 from *N. oxiana*. The developed production system provided the milligram quantities of unlabelled and $^{13}C,^{15}N$-labeled recombinant CTX1 analogue, containing additional N-terminal Met-residue (rCTX1). NMR analysis and cytotoxic assay confirmed that the rCTX1 has the spatial structure and biological activity similar to the wild-type toxin (wtCTX1). The analysis of available data for different Ly6/uPAR proteins revealed the correlation between efficiency of TFPs folding *in vitro* and their structure. Obtained data confirmed the large adaptability of the tested approach to production of various Ly6/uPAR proteins.

2. Materials and methods

2.1. Bacterial production of rCTX1

The *CTX1* gene encoding 60 amino acid residues of the CTX1 toxin from *N. oxiana* with additional ATG codon at the 5′-end was constructed from six overlapping synthetic oligonucleotides using PCR and considering the codon frequency in *E. coli* (Evrogen). The *CTX1* gene was cloned into the *pET-22b*(+) expression vector (Novagen) at the *Nde*I and *Bam*HI restriction sites. BL21 (DE3) *E. coli* cells transformed with the *pET-22b*(+)/*CTX1* vector were grown at