Functional expression of α-latrotoxin in baculovirus system

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Received 1 December 1998

Abstract To facilitate the study of the mechanism of α-latrotoxin action, it is necessary to create a biologically active recombinant toxin. Mature α-latrotoxin is naturally produced by post-translational cleavage, probably at two furin sites located at the N- and C-termini of the precursor. A recombinant baculovirus has now been constructed, which encodes the melittin signal peptide fused to the 130-kDa mature toxin between the furin sites. Insect cells, infected with this baculovirus, secreted recombinant α-latrotoxin. This was partially purified and proved indistinguishable from the natural toxin with respect to its molecular mass, immunostaining, toxicity to mice, binding to α-latrotoxin receptors (latrophilin or neurexin Iα) and electrophysiological recording in the mouse diaphragm. The successful expression of recombinant α-latrotoxin permits mutational analysis of the toxin.

Key words: α-Latrotoxin; Baculovirus; Expression; Latrophilin; Neurexin

1. Introduction

α-Latrotoxin (α-LTX) is a protein neurotoxin from the black widow spider venom that causes an exhaustive release of neurotransmitters from nerve terminals and endocrine cells in vertebrates [1–3]. This makes α-LTX an important natural tool to study exocytosis. However, due to regional and seasonal variability of the venom composition and because some peptides from the venom co-purify with the toxin [4], α-LTX effects may vary between preparations. It is possible to overcome these problems by producing a recombinant α-LTX. This would also allow for mutational analysis of the toxin, thus facilitating the study of its mechanism of action.

Production of the active, soluble α-LTX and its homologues in bacteria proved very difficult due to incorrect protein folding ([5] and data not shown). Attempts had also been made to express α-LTX and α-latroinsectotoxin precursors in a baculovirus system, but the proteins synthesised appeared non-functional and insoluble [6].

This paper presents a reliable method for functional expression and purification of the mature, active form of α-LTX, using an alternative strategy of the toxin production in baculovirus system.

2. Materials and methods

2.1. Generation of recombinant baculovirus

Overlapping plasmids pT-4N and pT-3C [7], covering the complete α-LTX cDNA, were digested respectively with *Bam*HI/*Eco*RI and *Eco*RI/*Hpa*II endonucleases and ligated into a pBlueScript vector (Stratagene) cut with *Bam*HI/*Acc*I. This construct was used as a template in a polymerase chain reaction (PCR) with primers (L01, 5′-TTGGGATCCGAAGGAGAAGATTTAACT-3′; L08R, 5′-GAACCGTCGACTTACCTCCGAAATTTTCCGCT-3′) that introduced *Bam*HI and *Sal*I restriction sites, respectively, and the resulting fragment was subcloned in frame with the melittin signal peptide of the baculovirus transfer vector pMelBacA (Invitrogen) cut with the same enzymes. Sf9 cells were cultured in complete TNM-FH medium (Invitrogen) and co-transfected with this construct and the viral DNA (Bac-N-Blue kit, Invitrogen). The medium above transfected cells was tested by plaque assay, and blue recombinant plaques were selected. Recombinant baculoviruses AcMNPv-α-LTX-M were amplified and their purity was confirmed by PCR using proprietary primers (Invitrogen). High-titre, pure viral stocks were obtained after two additional rounds of amplification.

2.2. Expression and purification of α-LTX

For large-scale expression, Hi5 cells were cultured in Express Five serum-free medium. Cells were plated at a density of 15×10^6 cells per 150-cm^2 flask and then infected with AcMNPv-α-LTX-M baculovirus at a multiplicity of infection (MOI) of 10 pfu/cell. The medium was replaced 24 h after infection; the new medium was harvested 72 h later, adjusted to pH 8.2 using 1 M Tris base, clarified by centrifugation at $5000\times g$ for 15 min and filtered through a 0.22 μm filter (Whatman). The medium (200 ml) was diluted 5 times with 25 mM Tris-HCl, pH 8.2 (buffer A), and passed through a Q 15 anion-exchange filter (Sartorius). The filter was washed with 200 ml of buffer A and eluted with 2 ml of buffer A supplemented with 1 M NaCl. The eluate was immediately diluted to 20 ml with buffer A and loaded onto a Mono-Q column (Pharmacia) equilibrated in buffer A containing 0.1 M NaCl. The column was eluted with a linear gradient of NaCl (0.1–0.5 M) in the same buffer. Fractions obtained during the chromatography were analysed by SDS-electrophoresis and Western blotting. Fractions containing the recombinant α-LTX were diafiltered and concentrated in a Centricon unit (Amicon).

2.3. Electrophysiological experiments

Intracellular recordings of spontaneous miniature endplate potentials (MEPPs) were made using conventional microelectrodes on a mouse diaphragm, which was perfused with normal saline of the following composition (in mM): NaCl, 135; KCl, 4; NaH_2PO_4, 0.9; $MgCl_2$, 1; $CaCl_2$, 3.6; $NaHCO_3$, 16.3; glucose, 11; pH 7.2–7.4. Traces were stored on magnetic tape and subsequently processed electronically.

2.4. Expression of α-LTX receptors in COS-7 cells and binding experiments

COS-7 (gift from C. Isacke) cells were grown at 5% CO_2 in standard DMEM supplemented with 10% foetal calf serum, 2 mM glutamine and antibiotics (Gibco). Latrophilin [8] or neurexin Iα [9] cDNAs were subcloned into pcDNA3.1 vector (Invitrogen) under the control of the CMV promoter. These constructs were introduced into exponentially growing COS cells using the SuperFect transfection reagent (Qiagen). The cells were harvested 24 h after transfection and washed several times with PBS by centrifugation for 10 min at $1000\times g$. The cells were then resuspended in the Hi5 medium containing secreted recombinant α-LTX (1×10^6 cells/200 μl), incubated for 10 min at room temperature and pelleted by centrifugation. The pellets were analysed by SDS-gel electrophoresis and Western blotting using a monoclonal anti-α-LTX antibody YaL-1 (gift from V. Pashkov).

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PII: S0014-5793(98)01624-X

3. Results and discussion

3.1. Construction of recombinant baculovirus

The molecular mass of α-LTX deduced from the cDNA is 156.9 kDa [7]. However, based on SDS electrophoresis [10] and MALDI mass spectrometry (I. Dulubova and T. Chait, personal communication), the molecular mass of the toxin purified from the venom is significantly smaller (about 131 kDa). It is reasonable to suggest that α-LTX is synthesised as a larger precursor, which is cleaved post-translationally. This modification may be required for the toxin activation. Indeed, expression of the full-length α-LTX in insect cells resulted in a functionally inert protein [6]. The N-terminus of mature α-LTX has been experimentally determined [11]. Surprisingly, it is preceded not by a defined signal peptide but by amino acids R-M-R-R, which constitute the recognition site (R-X-R/K-R) of furin, a subtilisin-like proteolytic enzyme involved in the processing of many protein precursors [12]. Furin has been shown to cleave and activate, among other proteins, such toxins as Shiga toxin [13], *Clostridium septicum* α-toxin [14], and diphtheria toxin [15]. It is possible that during maturation, a furin-like protease hydrolyses α-LTX, producing an active toxin. Still, the cleavage of α-LTX precursor at the N-terminus cannot account for a protein of 130 kDa. Therefore, another proteolytic site should be located in the C-terminal part of the protoxin. In fact, another potential furin site (K-F-R-R) has been found in the C-terminal region of the precursor. Proteolysis at both sites would produce a protein of exactly 131.5 kDa, which is very close to the estimated molecular mass of the natural toxin. Therefore, a fragment of α-LTX cDNA (encompassing base pairs 142–3679, which encode amino acids 1–1179 between the two putative cleavage sites) was used to construct the recombinant vector.

Construction of the transfer plasmid pMelBac-α-LTX-M is schematically shown in Fig. 1. Overlapping α-LTX cDNA clones [7] were linked together (see Section 2) and then used as a template to PCR-amplify the sequence between base pairs 142 and 3679. PCR primers L01 and L08R were designed to introduce appropriate restriction sites and a stop codon at the 3′ end. The resulting cDNA fragment was introduced into the baculovirus transfer vector pMelBacA in frame with the melittin signal peptide under the control of the polyhedrin promoter. This exogenous signal peptide was used to facilitate secretion of recombinant α-LTX that lacks its own signal sequence. After co-transfection of Sf9 cells with pMelBac-α-LTX-M and baculoviral DNA, six recombinant plaques were selected. The titre of final stocks of amplified recombinant viruses reached $1–3\times10^8$ pfu/ml.

3.2. Expression and purification of recombinant α-LTX

The cabbage looper cell line Hi5, which is known to express secreted proteins more efficiently than Sf9 or Sf21 cell lines [16], was used to produce α-LTX. First, to optimise the yield of expressed recombinant toxin, we determined an optimal combination of two parameters: expression time and MOI. Hi5 cells were infected at MOI 1, 5, 10 or 20 pfu/cell and aliquots of the media collected 24, 48, 72 and 96 h after infection. Samples were analysed by immunoblotting using antibodies against α-LTX (data not shown). It was found that the secreted recombinant toxin reached a maximum of about 1 μg/ml in the culture medium 72–96 h post infection, whilst the optimal MOI was 10 pfu/cell. For large-scale expression, the cells were infected using 10 pfu/cell. One day following infection, the medium was changed to remove serum proteins, thus simplifying α-LTX purification. This did not affect the level of the toxin production, since at this time most of the live viruses in the medium had infected the cells and secretion of α-LTX had not yet started.

Purification of recombinant toxin was carried out using a

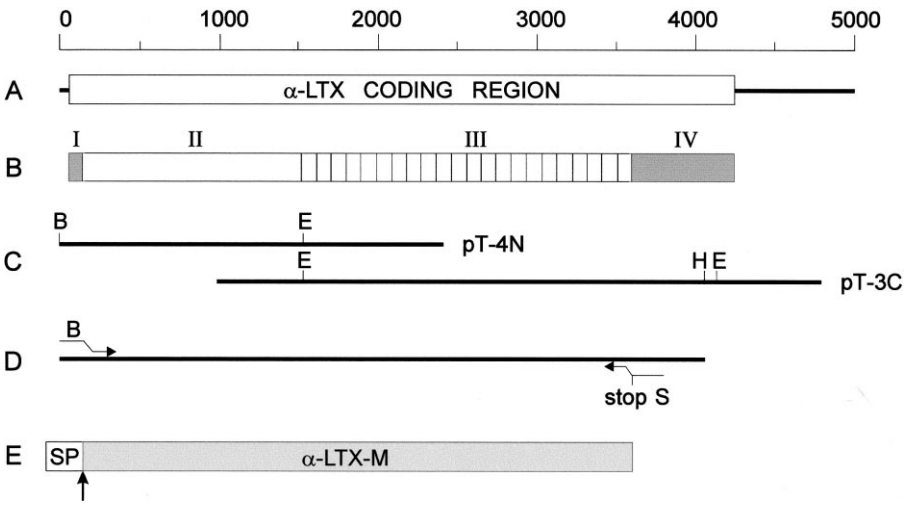

Fig. 1. The structure of α-LTX and construction of a recombinant vector for the toxin expression using baculovirus. A: The coding (open box) and untranslated regions of the α-LTX cDNA. B: Domain structure of the α-LTX precursor. I, leader sequence; II, N-terminal domain; III, central domain containing 22 ankyrin repeats; IV, C-terminal domain. Hatched domains are absent from the mature protein. C: Overlapping α-LTX cDNA clones. These were cleaved and re-ligated at the *Eco*RI restriction site to produce a continuous template for PCR. Letters above denote positions of recognition sites for restrictases *Bam*HI (B), *Eco*RI (E), *Hpa*II (H), and *Sal*I (S). D: PCR amplification of the cDNA fragment encoding mature α-LTX. The upstream and downstream primers were designed to anneal at the ends of the sequence encoding the mature protein and to introduce restriction sites and a stop codon. E: The structure of recombinant α-LTX. The PCR-amplified DNA fragment was cut with *Bam*HI and *Sal*I and ligated in frame with the melittin signal peptide (SP) of pMelBac vector cleaved with the same enzymes. The arrow marks the position of post-translational cleavage of the recombinant protein by insect cells, resulting in the mature toxin (α-LTX-M). The scale bar (in base pairs) is shown at the top.

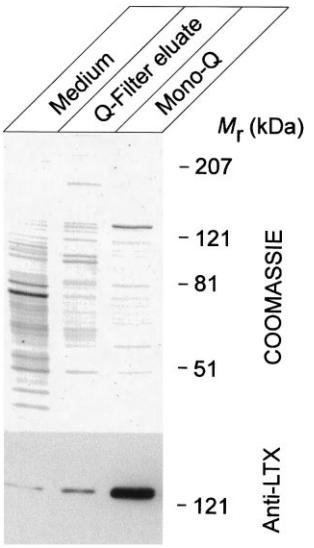

Fig. 2. Purification of α-LTX from culture medium. Cell culture medium (Medium) was concentrated on anion-exchange filter and eluted with 1 M NaCl ('Q-filter eluate'). Anion-exchange chromatography of this eluate on a Mono-Q column, eluted with a linear gradient of NaCl, produced a fraction containing recombinant α-LTX ('Mono-Q'). Samples taken after each purification step were analysed by SDS gel electrophoresis and Western blotting with an anti-α-LTX antibody using enhanced luminescence detection.

three-stage procedure. Initially, it was necessary to raise the pH of the medium; this allowed the toxin to bind to the anion exchangers used later. At the next stage, the preparation was passed through an anion-exchange filter, which adsorbed all recombinant toxin and some contaminating proteins (Fig. 2). This gave approximately 10-fold enrichment of the toxin. Finally, α-LTX was further purified by high-pressure anion-exchange liquid chromatography. Like the natural toxin, recombinant α-LTX eluted from the column at 0.3–0.4 M NaCl. The toxin-containing fractions were combined and concentrated by ultrafiltration (Fig. 2). Judging by SDS electrophoresis, the purity of the final toxin preparation was 40–50%. Since the total protein concentration in this sample was 0.7 mg/ml, the estimated concentration of α-LTX appeared to be 0.25 mg/ml. Thus, the yield of the purified toxin was 0.2–0.3 mg/l of cell culture medium.

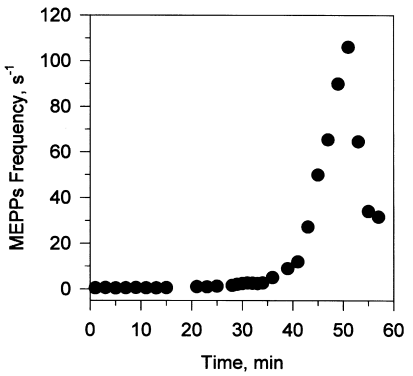

Fig. 3. Time course of the changes in MEPP frequency caused by recombinant α-LTX at a single neuromuscular junction. α-LTX (4 nM) was bath-applied to a mouse diaphragm at time 0 and MEPPs were recorded as described in Section 2. Each point represents values averaged over 2-min periods.

3.3. Biological activity of expressed α-LTX

The recombinant toxin was highly toxic to mice; its LD_{100} of less than 170 μg/kg body weight was comparable to that of the natural α-LTX [17]. One of the main features of α-LTX is its ability to cause a dramatic increase in the frequency of spontaneous quantal release from nerve terminals in vertebrates [18]. To investigate whether the recombinant toxin possesses the same functional activity, we studied its effect on the frequency of MEPPs in mouse nerve-muscle preparations. A typical experiment is shown in Fig. 3. Under our experimental conditions MEPPs occurred at a rate of about 0.5–0.7 MEPPs/s in resting nerve-muscle preparations ($n = 5$). Following bath application of 3–5 nM of recombinant α-LTX, MEPP frequency started increasing after a delay of 20–30 min. Maximal effect was achieved 40–50 min after the toxin application, reaching 80–100 MEPPs/s ($n = 5$). The MEPP frequency then gradually decreased indicating the depletion of neurotransmitter stores in terminals. These results demonstrate that recombinant α-LTX is able to stimulate neurotransmitter release comparable to that evoked by the native toxin.

α-LTX exerts its action at the nerve terminal after binding to a specific presynaptic receptor. To date, two potential toxin receptors have been found, neurexin Iα and latrophilin [8,19–21]. To elucidate whether recombinant α-LTX binds to these receptors, we expressed latrophilin and neurexin in COS-7 cells, which do not normally bind the toxin. When transfected cells were incubated with the culture medium containing the recombinant toxin and then pelleted by centrifugation, α-LTX co-precipitated only with those cells that expressed either of these receptors but not with the control cells (Fig. 4). This result indicated that the recombinant toxin bound specifically to both latrophilin and neurexin Iα.

The recombinant toxin, thus, possesses all the properties of natural α-LTX, suggesting that the latter is indeed proteolysed at the putative furin sites during maturation in the black widow spider venom glands.

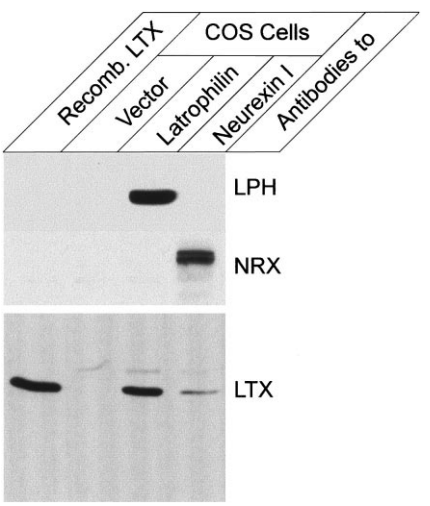

Fig. 4. Binding of recombinant α-LTX to latrophilin and neurexin Iα. COS-7 cells were transfected with vector, latrophilin or neurexin cDNA as shown. After incubation in α-LTX-containing medium (Medium), the cells were pelleted by centrifugation and analysed by SDS gel electrophoresis and immunoblotting with antibodies to latrophilin (LPH), neurexin Iα (NRX) or α-LTX (LTX). Recombinant α-LTX from the medium bound specifically only to the cells that expressed either latrophilin or neurexin Iα.

This paper describes a novel method for functional expression of α-LTX based on the use of baculovirus system[1]. The problem of producing an active, soluble form of the toxin was solved by introducing a strong signal peptide at the N-terminus of the toxin and by the removal of its C-terminus, whose presence renders the toxin inactive. This approach will allow us to carry out mutational analysis of α-LTX functional domains.

Acknowledgements: The authors are grateful to V. Pashkov for providing monoclonal anti-LTX antibodies and to C. Isacke for the gift of COS-7 cells. This work was supported by a grant from Russian Foundation for Basic Research (97-04-48552) and by a Wellcome Senior European Research Fellowship (to Y.A.U.).

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[1] When this paper was being prepared for publication, a similar approach to α-LTX expression was published [22].