Review

Cytolytic Proteins from Cnidarians – an Overview

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Dedicated to the memory of Professor Franc Gubenšek

Abstract

Cnidarians, mostly soft-bodied water organisms, produce several classes of toxins deployed in biological warfare or signalling. Cytolytic toxins, that form pores in cell membranes, form a significant part of their "weaponry". Here, we describe the physiological relevance of membrane permeabilization, and present basic data on those proteinaceous cnidarian cytolysins proven or presumed to damage cell membranes by pore formation. We describe cytolysins that have been at least partially characterized, both functionally and structurally.

Keywords: Cnidaria, cytolysin, membrane, pore-forming toxin, sea anemone, toxin

1. Introduction

Cnidarians (classes Anthozoa, Scyphozoa, Cubozoa, Hydrozoa) are, evolutionarily, an ancient group of water, mostly marine, animals. Characteristic of these sedentary or swimming organisms are specialized cells, nematocytes, that produce capsular organelles called nematocysts, together with a variety of toxins. Nematocysts are used for prey capture and stinging in attack and defence. These highly complex devices contain and allow delivery of the venom including toxins. In addition to the latter, some cnidarian groups can actively secrete a variety of offensive and defensive allomones.[1–5] Sequencing of genomes (*Hydra magnipapillata* and the sea anemone *Nematostella vectensis*) and ESTs (expressed sequence tags) has shed more light on the complexity of the cnidarians' toxins.[4,5] In Anthozoa (sea anemones) and Hydrozoa (hydrae), the most abundant and biologically effective weapons appear to be neurotoxins blocking Na^+ and K^+-channels, and cytolysins affecting the integrity of targeted cell membranes.[5–8] However, the occurrence of these types of toxins in cnidarian venoms does not explain all the effects produced *in vivo* by cnidarian stings, in particular those caused by medically important species of Scyphozoa (jellyfish), Cubozoa (Cubomedusae), and some Hydrozoans (i.e., Portuguese man-of-war).[9,10]

Cellular life is dependent on the integrity of cellular membranes that is responsible for controlling the proper transmembrane distribution of solutes. It is not surprising that membrane permeabilization induced by specifically designed peptides and proteins has evolved as a common strategy deployed in various biological organisms, all leading to cell necrotic or apoptotic death. Some of the best known executive molecules are antimicrobial peptides acting as natural antibiotics[11] or as crucial effectors of innate immune systems. They are produced almost universally, from bacteria to plants and animals.[12]

Another group of proteins that puncture cell membranes are the pore-forming proteins of the vertebrate humoral immune defence. In this system, proteins of complement lyse foreign cells, such as Gram-positive bacteria and protozoans, while perforin, together with granzymes, secreted in a controlled manner from cytotoxic lymphocytes, eliminates organism's own defective cells by triggering their apoptosis.[13]

Moreover, the intrinsic apoptotic pathway includes two nucleus-encoded proteins, Bax and Bak, that are able to pierce the mitochondrial outer membrane to mediate cell death.[14]

There is a group of intrinsic proteins, such as amyloid-β or α-synuclein, that when misfolded, are causative agents of neurodegenerative diseases (i.e. Alzheimer's and Parkinson's disease). There is increasing evidence that one consequence of the deposition of such misfolded proteins on cell membranes increases the latters' permeability, thus contributing to cell deterioration.[15,16]

Polypeptide cytolysins are an abundant and widespread group of toxins produced by bacteria, plants and animals.[17] They damage cellular membranes *via* enzymatic degradation of membrane lipids (by cytolytic phospholipases and sphingomyelinases) or integrate into the membrane and form transmembrane pores (pore-forming toxins, PFTs). PFTs are found more frequently than cytolytic enzymes, suggesting that their effectiveness in damaging host membranes is evolutionarily advantageous. The general mechanism of action of PFTs can be separated into distinct steps: (i) secretion as soluble proteins that attach to the host membrane by recognizing, more or less specifically, lipids and/or lipid domains, (ii) oligomerization in the membrane plane, and (iii) projection of specific amphiphilic α- or β-structured polypeptide segments across the lipid bilayer to make a transmembrane hydrophilic pore lined by either a bundle of α-helices (α-PFTs) or several β-hairpins (β-PFTs).[18,19]

In this overview, we focus briefly on cnidarian cytolysins, presumably pore-forming toxins, and present their structural (amino acid and/or nucleotide sequences) and functional characteristics. More comprehensive information on cnidarian cytolysins is available in a number of specific reviews published in the last decade,[3,9,20,21] and in a series of excellent reviews dealing with many aspects of cnidarian venoms and toxins (Toxicon Vol. 54, Issue 8). Furthermore, we do not attempt to classify cnidarian cytolysins, since their structural characterization in particular is far from complete. We therefore use the provisional term "cytolysin type", which is based on basic structural and functional data. However, based on structure-function and phylogenetic criteria, some cnidarian cytolysins are currently classified as "1.C. Pore-Forming Toxins (Proteins and Peptides)", and are included in the Transporter Classification Database (TCDB).[22]

2. Cnidarian Cytolysin Types

2. 1. Actinoporins (M ~20 kDa)

Actinoporins are the most studied cnidarian pore forming toxins. Although described in many different sea anemone species, most of the research in the last decade has been carried out on equinatoxin II (EqtII) from the sea anemone *Actinia equina* and sticholysins I and II (StI and StII) from *Stichodactyla helianthus*.[3] These are closely similar cysteine-less proteins. The most distant members of the family still contain more than 50% of identical residues. Studies on actinoporins have been directed mainly to elucidation of their 3D structure (Figure 1)[23–26] and molecular mechanism of pore formation,[3,27–29] while their biological role in the life of sea anemones is not yet understood. In nM concentrations these proteins lyse red blood and other cells and are potent toxins when injected into experimental animals.[3] Interestingly, similar proteins with clear physiological effects on target cells have been described in fungi, oomycetes, molluscs and even plants. It thus appears that the actinoporin structure is employed for targeting different cell types.[30–34]

Pore formation by actinoporins proceeds by several steps, involving binding to a lipid membrane containing sphingomyelin (SM), displacement of the N-terminal region to the lipid-water interface, oligomerization within the plane of the membrane, and final transmembrane pore formation.[28,29] Some of these steps are known in great detail, for example the mechanism of SM recognition has been described at the molecular level (Figure 1)[35] and its role in the functioning of actinoporins has been extensively studied.[36,37] On the other hand, the molecular details of pores are not understood completely and the number of protein molecules participating in forming pores, together with structural details of the pores, remain to be elucidated.

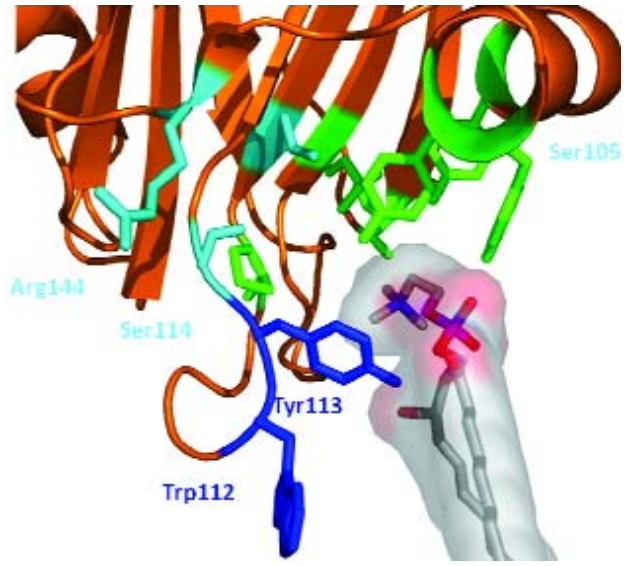

Figure 1. 3D structure of actinoporin EqtII. A) The 3D structure is composed of a β-sandwich flanked on two sides by α-helices. The N-terminal region (coloured blue) is the only part of the molecule that can undergo conformational rearrangements without disrupting the general fold of the molecule. B) An enlarged view of the membrane binding region at the bottom of the molecule and below the C-terminal helix (orange in A). Residues important for membrane and sphingomyelin (SM) interactions are designated by sticks. The bound SM structure is also shown with surface presentation. Blue: residues identified as being important for membrane interactions in cysteine-scanning mutagenesis. Green: residues participating in binding phosphocholine by StI. Dark blue: residues important for SM recognition.

2. 2. Sea Anemone Cytolysins (M ~30 kDa)

A cardiostimulatory and cytolytic 28 kDa protein, UpI, was isolated from *Urticina piscivora*.[38,39] In contrast to actinoporins, this basic protein contains several cysteine residues. A similar cytolytic protein UcI was isolated from a related species, *U. crassicornis*, and partially characterized.[40] The N-terminal amino acid sequences of these two proteins (Figure 2) are very similar, but differ from

those of the 20 kDa actinoporins and, further, from those of any other proteins in databases. UpI and UcI may be regarded as members of a new family of cytolytic proteins from sea anemones, so far found only within the genus *Urticina*.

Figure 2. Alignment of the N-terminal amino acid sequences of *U. piscivora* and *U. crassicornis* cytolysins UpI and UcI. Identical residues are shaded.

UcI has a molecular mass of around 30 kDa and lacks phospholipase A_2 activity. It is lytic to erythrocytes at nanomolar concentrations by forming pores of ~1.3 nm in diameter. It binds to lipid monolayers and effectively permeabilizes sonicated lipid vesicles composed of both SM and cholesterol (CHOL), but does not interact with either pure cholesterol or sphingomyelin dispersions. In contrast, the cytolytic activity of UpI was inhibited only by SM and not by CHOL pre-incubation.[38] The sequence similarity of these cytolysins suggests that their mechanisms of binding, insertion and pore formation are similar. The observed necessity for combined SM and CHOL as lipid acceptors for this type of cytolysins suggests that specific lipid microdomains are essential for binding and/or pore formation.

2. 3. Sea Anemone MACPF Toxins (M ~55 kDa)

In 2002, a novel type of cnidarian cytolysin, similar to the membrane-attack complex/perforin (MACPF) family of proteins, was discovered in nematocysts of the stinging sea anemone *Phyllodiscus semoni*.[41] The 60 kDa cytolysins PsTX-60A and PsTX-60B, and closely related proteins, were lethal in shrimps (LD_{50} ~800–900 μg/kg) and hemolytic (EC_{50} ~600 and ~300 ng/mL) against sheep erythrocytes. Complete cDNA sequences and deduced primary structures (501 and 488 amino acids, respectively) revealed homology with AvTX-60A toxin (Q76DT2) from the sea anemone *Actineria villosa*.[42–45] Like perforin, these cytolysins possess an EGF-like domain next to the MACPF domain, but lack the C2 domain for attachment to lipid membranes. AvTX-60A toxin was lethal in mice, exhibiting a minimum lethal dose of 250 μg/kg.[42]

A search of databases, using the sequence of PsTX-60B as a query, indicated the presence of very similar proteins in the sea anemone *Nematostella vectensis* (E-values $\leq 9 \times 10^{-37}$). The discovery and biochemical characterization of the cnidarian MACPF-cytolysins calls for comparative mechanistic studies of their membrane permeabilization. Both sea anemone species contain actinoporin type cytolysins in addition to the MACPF toxins.

2. 4. Box Jellyfish (Cubozoa) and Jellyfish (Scyphozoa) Cytolytic Toxins (M ~50 kDa)

Very potent cytolysins, of ~50 kDa and belonging to the jellyfish toxin family, have been isolated from the medically important box jellyfish species *Carybdea rastonii*, *C. alata*, *Chiropsalmus quadrigatus, and Chironex fleckeri*.[46–49] Their amino acid sequences are similar, and indicate weak structural similarities to pore-forming insecticidal δ-endotoxins Cry1Aa, Cry3Bb and Cry3A.

The *C. rastonii* nematocyst cytolysin, synthesized in the tentacles, is lethal in mice (LD_{50} ~ 20 μg/kg) and crayfish (LD_{50} ~5 μg/kg), and causes cutaneous inflammation in humans. Cytolysin CqTX-A from the deadly box jellyfish *C. quadrigatus* (CqTX-A, 44 kDa), a major nematocyst toxin, was lethal to crayfish (LD_{50} ~ 80 μg/kg) and hemolytic to sheep red blood cells (ED_{50} ~160 ng/ml).[48] This protein family includes predicted proteins from the box jellyfish *Malo kingi* that causes the »Irukandji« syndrome in humans, and from the hydrozoan *Hydra magnipapillata*. Recently, a novel 31 kDa cytolysin with structural homology to box jellyfish hemolysins was isolated from the scyphozoan *Cyanea capillata*. The protein is cytotoxic to human hepatocytes at 1.3 μg/mL.[50]

The numerous reports on the box jellyfish venoms and toxins have brought to light many of their interesting functional properties, but the use of crude venoms or partially purified venom proteins precludes firm conclusions on the detailed molecular mechanism of action of these hemolysins on cellular and artificial lipid membranes.

2. 5. Hydralysins (M ~26 kDa)

A novel group of cnidarian toxins, called hydralysins, was purified from non-cnidocystic tissues of green hydra *Chlorohydra viridissima* in 2003. Being weakly hemolytic, they were assigned functionally as being paralytic, and selectively cytotoxic to insect cells.[51] Hydralysins are distinct from other cnidarian toxins. Structurally and functionally, they can be classified as a group of β-PFTs similar to bacterial and fungal toxins such as aerolysin, ε-toxin, α-toxin, and the fungus *Laetiporus sulphureus* cytolysin LSL.[52] In particular, they exhibit high structural similarity to the 30 kDa parasporin-2 from the bacterium *Bacillus thuringiensis* (E-value 6×10^{-44}).[53]

Hydralysins bind to cell membranes and form pores with an internal diameter of ~1.2 nm. Their hemolytic activity is unaffected by pre-incubation of the proteins with cholesterol, sphingomyelin, phosphatidylcholine, galactose, mannose, or lactose, suggesting that these membrane components are not the primary acceptors responsible for their membrane binding. Rather, the cytolytic effect of hydralysins is cell type-selective, suggesting a specific receptor that is not a phospholipid or a carbohydrate. On erythrocyte membranes hydralysins were visualized by

immunofluorescence as discrete spots, suggesting that they bind to specific environments (microdomains) on the membrane. It was suggested that the paralytic and cytolytic activities of hydralysin, which are correlated, are both a consequence of receptor-mediated pore formation.[54]

2. 6. Fire Coral (Hydrozoa) Cytotoxin (M ~18 kDa)

Fire corals are known to produce severe pain and inflammation on contact. Crude nematocyst venom of fire corals was found to be highly lethal to mice, hemolytic, and dermonecrotic. Hemolysins from *Millepora dichotoma* and *M. platyphylla* have been characterized as 30 kDa proteins.[55]

An 18 kDa cytotoxin-1 (MCTx-1) was isolated from nematocysts of *M. dichotoma* var. *tenera*. The precursor form (222 amino acids), deduced from the mRNA nucleotide sequence, is presumably processed by cleavage of a signal peptide and propeptide. The mature protein, which contains three disulphides and short repeats, is similar to dermapontins, extracellular matrix proteins found in animal species from sponges to mammals. The protein is lethal in crayfish (LD_{50} of ~106 μg/kg) and highly cytotoxic for L1210 mouse leukaemia cells (EC_{50} ~79 ng/mL). It induces agglutination of erythrocytes.[56] The molecular mechanism of cytotoxicity of MCTx-1 is not known but probably differs from that of the fire coral 30 kDa hemolysins.

3. Miscellaneous Cytolysins

The diversity of pore-forming toxins from Cnidaria may be reflected in other purified cytolysins that have been not structurally characterized. However, they appear to be different from the above described proteins.

3. 1. Low-molecular Cytolysins from the Sea Anemone *Radianthus macrodactylus* (M 5 to 10 kDa)

Two low molecular weight cytolytic toxins of 5.1 and 6.1 kDa were isolated from aqueous extracts of the sea anemone *Radianthus macrodactylus*. Both proteins were non-toxic to mice and crabs. In contrast to *R. macrodactylus* 20 kDa actinoporins, their hemolytic activity was not inhibited by exogenous sphingomyelin.[57]

3. 2. A Cholesterol-inhibited Cytolysin from Sea Anemone *Metridium senile* (M ~80 kDa)

An 80 kDa cytolysin, metridiolysin, was purified from the tissues of the North Atlantic sea anemone, *Metridium senile*. It was suggested to be a heterodimeric protein. Metridiolysin is lethal to mice and cytolytic to blood platelets and fibroblasts, but not to bacterial protoplasts and spheroplasts. The optimal pH for hemolysis, which was inhibited specifically by cholesterol, was between 5 and 6.[58]

3. 3. A High Molecular Weight Hemolysin from Hydrozoan *Physalia physalis* (M ~150 kDa)

A hemolytic protein, physalitoxin, lethal to mice has been isolated from the nematocyst venom of *Physalia physalis*. The heterotrimeric and glycosylated hemolysin was inactivated by concanavalin A.[59]

4. Conclusions

The genomic and EST nucleotide sequences of *N. vectensis* and *H. magnipapillata* reveal how complex cnidarian venoms can be, even in the case of cytolytic toxins. Bioinformatics analysis and experimental evidence reveal that the occurrence of a certain type of cytolysin is not restricted to a specific cnidarian group, as thought earlier. The example of the actinoporins indicates that certain toxin folds are evolutionarily "successful", and are conserved in moderately unrelated eukaryotic organisms (animals and plants).

Despite the fact that cnidarian venoms and toxins have received much attention, the purification of their higher molecular proteins is a formidable task, and is seriously hampered by the great instability of venom and extracted protein components. This is particularly true for box jellyfish, jellyfish, and hydrozoans venoms and toxins. To this end, combined genome or EST sequencing, bioinformatics, molecular biology tools and novel biochemical and biophysical approaches are promising to intensify research into the structure and function of cnidarian cytolysins. In general, knowledge of the 3D-structures, membrane acceptors/receptors, and molecular mechanisms of action of these cytolysins at the membrane level, together with the corresponding specific responses of targeted cells, are lacking.

There is a need to intensify research on pharmacologically active proteins from cnidarians, especially those from the medically relevant species of Cubozoa and Hydrozoa. It may provide clues to improved medical treatment of cnidarian stings and, on the other hand, cnidarian cytolysins could prove useful in biotechnology. For example, actinoporins have been applied in constructing immunotoxins and, in cell biology, as research tools.[60,61] A pivotal characteristic of pore-forming toxins is their propensity for insertion into non-polar phases, found typically in lipid membranes. However, for a number of them it has been shown that they bind to the membrane surface by recognizing a specific membrane structural component. Such pro-

teins have the potential for exploitation in research on membrane structure and function in general. For example, a fluorescently labelled actinoporin, equinatoxin, has been employed as a specific marker of membrane lipid microdomains and for membrane sphingomyelin.[35–37]

It has to be recognized, however, that the biological role(s) of many cnidarian cytolysins remain unknown.

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Povzetek

Ožigalkarji so vodni organizmi večinoma brez skeleta, in ki tvorijo različne toksine. Uporabljajo jih v »kemijski vojni« in za signaliziranje. Pomemben delež v njihovi »orožarni« predstavljajo citolitični toksini, ki tvorijo pore v celičnih membranah. Kratko opišemo, kakšen je biološki pomen permeabilizacije membran in predstavljamo osnovne značilnosti ožigalkarskih beljakovinskih citolitičnih toksinov, ki so domnevno ali dokazano tvorci por v celičnih membranah. Predstavljamo citolizine z vsaj delno znanima zgradbo in načinom delovanja.