Chapter 12

Role of Heparan Sulfates and Glycosphingolipids in the Pore Formation of Basic Polypeptides of Cobra Cardiotoxin

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Abstract

Cobra venom contains cardiotoxins (CTXs) that induce tissue necrosis and systolic heart arrest in bitten victims. CTX-induced membrane pore formation is one of the major mechanisms responsible for the venom's designated cytotoxicity. This chapter examines how glycoconjugates such as heparan sulfates (HS) and glycosphingolipids, located respectively in the extracellular matrix and lipid bilayers of the cell membranes, facilitate CTX pore formation. Evidences for HS-facilitated cell surface retention and glycosphingolipid-facilitated membrane bilayer insertion of CTX are reviewed. We suggest that similar physical steps could play a role in the mediation of other pore forming toxins (PFT). The membrane pores formed by PFT are expected to have limited lifetime on biological cell surface as a result of membrane dynamics during endocytosis and/or rearrangement of lipid rafts.

Introduction

Biological membrane consists of many glycoconjugates that promote protein-protein interaction. HS are a class of negatively charged glycosaminoglycans (GAGs) that are composed of heterogeneous disaccharide repeating units. The binding of HS with biologically active ligands, such as basic polypeptides of chemokines or cytokines,[1-4] plays a significant role in many disease and cell development.[1,5] Glycosphingolipids usually exist in the outer leaflet of membrane bilayer as a dynamic lipid domain, termed as lipid raft and are crucial for cell signaling and membrane translocation.[6-9] They are also targets for toxin bindings, as demonstrated by the interactions between the cholera toxin and GM1 glycosphingolipids, where they induce an oligomerization process that leads to pore formation. Although the significance of protein conformational changes during pore formation has been widely acknowledged,[10-12] the exact mechanisms that allow proteins to anchor and insert the cell membrane remain unclear. In this review, we will explain the significant role of other molecules distributed on cell surface, such as HS and glycosphingolipids, in membrane pore formation. Specific focus is placed on the PFT of three-fingered CTX, a basic polypeptide whose core structure is tightened by four disulfide linkages that minimize conformational change.

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Proteins: Membrane Binding and Pore Formation, edited by Gregor Anderluh and Jeremy Lakey

Amphiphilic Properties of Three-Fingered CTXs

Cobra CTXs are β-sheet polypeptides with hydrophobic residues located mainly on the tips of the three-fingered loops (Fig. 1A,B). Similar to other basic proteins like defensin, anti-microbial and cell penetrating peptides,[13,14] they contain positively charged clusters that interact with negatively charge molecules on the cell membranes. In the case of CTXs, most of their positively charged residues are flanking on the two sides of the continuous hydrophobic stretch formed by the

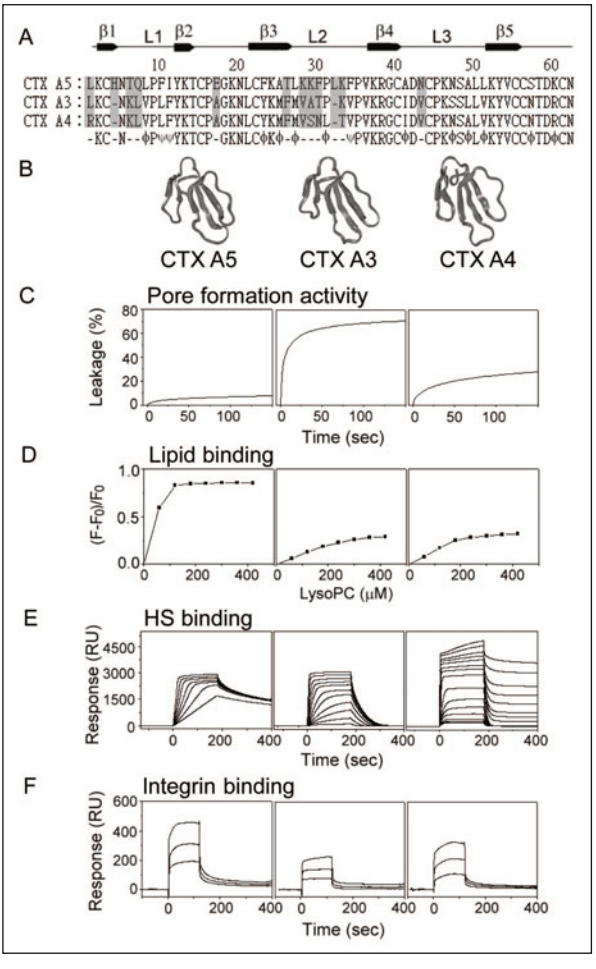

Figure 1. Structure and activity correlation of CTX homologues (A) Sequence alignment of CTX homologues indicates that amino acid residues located near the tips of loop 1 (L1) and loop 2 (L2) play an important role in target selection. The consensus sequence of CTX is also shown with symbols ϕ and ψ to indicate semi-conserved residues with similar physical property. B) Three-fingered CTXs with similar β sheet structures among all CTX homologues (CTX A5 PDB ID: 1kxi; CTX A3 PDB ID: 1i02; CTX A4 PDB ID: 1kbs) (C) Representative traces of CTXs-induced leakage of fluorescence probe in sulfatide containing vesicles. D) Representative binding isotherm as measured by the change of Tyr intrinsic fluorescence intensity after CTXs binding to Lyso-PC micelles. E) Surface plasma resonance studies on the binding of CTXs to immobilized heparin. F) Surface plasma resonance studies on the binding of CTXs to immobilized αvβ3 integrin. For the details, please see references 22,23,28.

three-fingered loops. While the CTXs share structural similarity, the amino acid groups on the loop ends differ- exhibiting different lipid binding preferences.[15,16] Most CTXs, although water-soluble, can bind to membrane lipid bilayers and induce leakage of membrane vesicles through pore formation activities. Interestingly, the lipid binding ability is not directly related to the toxin's pore forming property, suggesting that toxin insertion and oligomerization could be more significant in the pore formation processes. This is illustrated in Figure 1C,D, in which noncytotoxic CTX A5 with the highest membrane binding has the least pore forming activity.

Diverse Targets of CTX Homologues

In the crude venom, there are seven to ten CTX homologues with distinct biological activities.[17-21] CTXs exhibit binding specificity toward not only HS and lipid, but also membrane proteins such as integrin (Fig. 1E,F). Recent X-ray structural determination of a CTX A3-heparin hexasaccharide complex revealed a structural basis responsible for the differences in the binding strengths among CTX homologues toward HS.[22] The structure also suggests a molecular mechanism for toxin retention near the membrane surface, in which heparin-induced conformational changes of CTX A3 lead to citrate-mediated dimerization. Citrate is a major component of snake, bee, scorpion and ant venom and serves as a counter ion for the basic polypeptides of many types of venoms.

Despite of the overall structural similarity, not all CTX homologues are PFT (Fig.2A). A similar scenario should be considered for other basic polypeptides with pore forming activity. For instance, diverse activities of defensin homologues have been reported. While some defensins bring about membrane pore formation in bacteria, others could function as chemokines in order to promote cell-signaling process. Similarly the CTX A3 homologue A5 can bind with $\alpha_v\beta_3$ integrin to perturb the wound-healing processes of the bitten victim.[23] The synergistic action between the toxin-induced pore forming activity and toxin-induced cell signaling process is expected to be crucial for the overall cell cytotoxicity.

CTX A3 Pores in Sulfatide Containing Membranes

Although CTX A3 has been shown to induce membrane leakage in model membranes,[15] the formation of specific membrane pores in biological membrane has only recently been demonstrated. First, whole cell recording of H9C2 cardiomyocytes by electrophysiological methods has demonstrated that CTX A3 could induce extra conductance formation in a voltage- and dose-dependent manner.[24] This is due to the single channel-like events as observed by the outside-out patch clamp experiments. However, CTX A3-induced conductance is sensitive to pretreatment of the cells with sulfatase, anti-sulfatide IgG or anti-sulfatide IgM, which indicates that sulfatide, a sulfated-galactose glycosphingolipid, is involved with the process. The dose-response curve of the sulfatide dependent CTX A3-induced conducting pathway corresponded roughly to the square of the CTX A3 concentration.[24] These results indicate that a bimolecular interaction, such as sulfatide-induced dimerization of CTX A3 molecules might be the rate-limiting step for the observed effect.

Pore Formations also Trigger Endocytosis

After CTX A3 form pore in H9C2 or rat cardiomyocytes, it becomes internalized. The internalization is due to endocytosis of the cell membrane as part of the membrane repair mechanism,[29] which also regulates the lifetime of the CTX A3 pore. This process involves the 3′-sulfated galactose headgroup of sulfatide, because either the removal of the preexisting sulfate moiety by sulfatase or blocking of the negatively charged sulfate through the pre-incubation of anti-sulfatide antibody can block the internalization of CTX A3. The extragenously added sulfatide-enhanced CTX A3 internalization further confirmed sulfatide as a membrane target of CTX A3 on the membrane surface. In fact, cocrystallization of sulfatide with CTX A3 in the presence of $C_{10}E_6$ detergent (an artificial membrane environment) shows that the sulfatide lipid headgroup is buried within the pocket formed by the CTX A3 dimer (PDB ID: 2bhi).

HS Facilitate Cell Surface Retention of CTXs

HS have been suggested to be responsible for inducing gradient of biologically active ligands, such as growth factors, near the cell membranes and mediate cell development. CTX contains positively charged cluster domains that are attracted to the anionic pockets of HS.[25] This electrostatic attraction leads to a local CTX enrichment because the multiple binding sites allows for cross-linking with HS in the extracellular matrix. By immobilizing Chinese hamster ovary cells in micro-capillary tubes and heparin on sensor ships, we showed that HS-mediated cell retention of CTX A3 near membrane surface is citrate dependent.[22] This observation- together with crystal structure of CTX A3/HS complex (PDB ID: 1xt3), provides a structural basis of cell retention through the interaction of the molecules (Fig 2B). Not all CTX homologues behave similarly in the HS-induced cell surface retention; different CTX homologues target different cell type depending on their HS binding specificity.[26]

HS Stabilizes Membrane Bound Form of CTX

Understanding HS-induced conformational change of GAG binding protein is essential for the study of its function. The X-ray structure of the FGF, FGF receptor and heparin ternary complex provides a basis for investigating the role of HS in FGF signaling.[27] Under the same token, proton NMR study on the binding of heparin-derived hexasaccharide to CTX A3 at the β-sheet region induces a *local* conformational change of CTX A3 near its membrane binding loops and promotes the binding activity of CTX toward phospholipid micelles.[28] The detected change is due to the structural coupling between the connecting loop and its β-strains without involving a *global* conformational change. This explains how the association of hydrophilic carbohydrate molecule of HS with amphiphilic proteins of CTX could initiate protein-lipid interaction without involving extensive structural alteration. A similar mechanism that favors the lipid-protein interaction through the HS binding may be operative at the membrane surface.

From HS to Membrane Sulfatides

Many toxins hijack GAGs in the extracellular matrix for specific targeting. The negatively charged HS may, therefore, serve as a high capacity region for concentrating basic toxins that can be specifically transferred to a glycosphingolipid domain in the outer leaflet of the membrane bilayer. Although CTX A3 fails to bind to gangliosides at the lipid rafts, it shows specificity in binding with sulfatide as a dimer.[24] Similar to cell signaling molecules, the location of sulfatide, with respect to proteoglycans that contain specific HS sequences, affect CTX pore forming activity significantly. Taken together, we suggest that animal toxins use a complex strategy to find their targets by using sequential events of binding to molecules that contain a similar motif within a protein molecule (Fig. 2).

Peripheral Binding Modes

The peripheral binding structural model of CTX A3/sulfatide complex in sulfatide containing phosphatidylcholine micelles has been determined by NMR and molecular docking methods.[23] The intermolecular NMR nuclear Overhauser effect has been observed in order to allow the computer docking of sulfatide headgroup against the available CTX A3 structure. The three-fingered hydrophobic loops of CTX A3 can be seen to penetrate into the fatty acyl region of the lipid bilayers (Fig. 3). Such a binding mode may allow a deep CTX A3 penetration into lipid bilayers without major conformational change of the structure. This is in sharp contrast to the sulfatide-CTX A3 complex structure determined by X ray method, which shows that the three-fingered hydrophobic loops are in opposite direction to the fatty acyl chain of sulfatide molecule.

Lipid Headgroup Conformational Change to Facilitate CTX Insertion

The relative orientation of sulfatide against CTX A3 in lipid bilayers is likely to change during the pore forming process- if the hydrophobic interaction between CTX A3 and sulfatide is to be maximized. In fact, in order to form a stable CTX A3 dimer with sulfatide chelated in between,

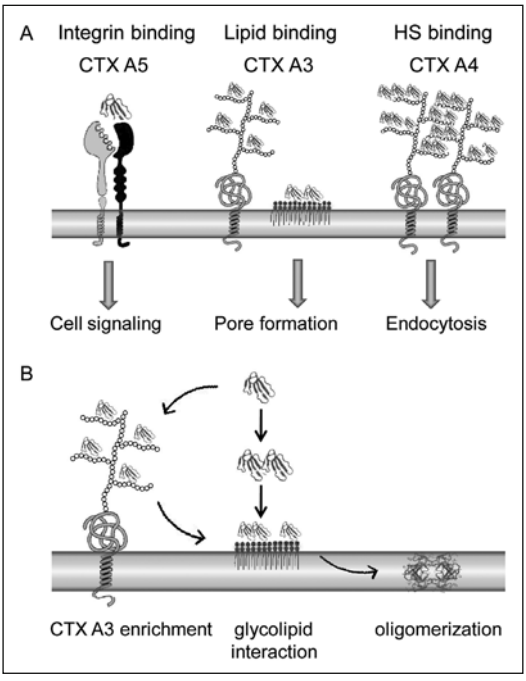

Figure 2. Schematic diagrams show how CTX homologues can interact with diverse targets on the cell membrane to trigger different cell response (A) and how HS in proteoglycan could facilitate cell surface retention of CTXs and its pore formation (B).

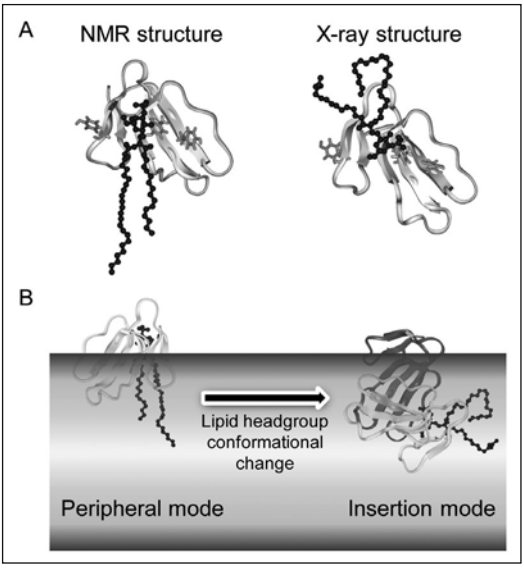

Figure 3. Available NMR and X-ray structures of sulfatide-CTX A3 complex (A) suggest that lipid headgroup conformational change play a role in the insertion and dimerization of CTX A3 (B) to account for the pore forming activity.

both the Gal-3S headgroup and the fatty acyl chain will have to experience *global* reorientation relative to the CTX A3 molecule. The sulfatide headgroup conformation will undergo sequential change from CTX free form of *-sc/ap/sc/ap* to the peripheral mode of *sc/ap/sc/ap* and then to the insertion mode of *sc/ap/ap/ap*.[29] Such a conformational change is likely to be soft and dynamic. Our results suggest a scenario for CTX A3 pore that is closer to the recent theoretical simulation for the distorted toroidal model,[30,31] with lipid as an essential element[32] to participate as an agent to stabilize the CTX dimer and a facilitator to promote CTX insertion and oligomerization during the pore formation process.

Pore Dynamics

The lifetime of CTX A3 pore formation in sulfatide containing vesicles has been studied by the dequenching process of lipid vesicles using entrapped fluorescence probe. Assuming that the pore size of the vesicle fall in the range of 35 to 40 Å, the open lifetime of the pore can be determined to be longer than 1-3 msec.[29] The estimated lifetime of the CTX pore formed in vesicles is thus comparable to that of the lifetime of the sulfatide dependent single conductance in the biological membrane, as determined by the patch-clamp experiment. It should be noted that pore formation dynamics in the biological membrane also depend on the endocytosis process triggered by calcium influx.[33] The apparent consistency of the pore lifetime in the model and biological membranes should therefore be considered with caution. Nevertheless, we can investigate whether dynamics of nano-size lipid raft would affect the stability of the pore because glycosphingolipid functions as part of the pore.

Conclusion

The acidic glycoconjugates on membrane surface are shown to be able to interact with basic polypeptides of cobra CTX and induce pore formation. HS retain CTX at the membrane surface and induce the formation of membrane bound form; while the sulfatide lipid domain facilitates CTX dimerization and promote membrane insertion through lipid headgroup conformational change. Since many PFT are also basic polypeptides, the physical process established for cobra CTX might be applicable to the pore formation mechanism of PFT. It remains to be seen how the dynamics of lipid raft, the rate of calcium dependent endocytosis and the flip-flop process within lipid bilayer in the cell membrane, could mediate the dynamics of PFT pore formation.

Acknowledgements

This work was supported by grants from National Science Council, Taiwan.

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