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Detection of Rap1A as a yessotoxin binding protein from blood cell membranes

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ABSTRACT

As is the case with other ladder-shaped polyether compounds, yessotoxin is produced by marine dinoflagellate, and possesses various biological activities beside potent toxicity. To gain a better understanding of the molecular mechanism for high affinity between these polyethers and their binding proteins, which accounts for their powerful biological activities, we searched for its binding proteins from human blood cells by using the biotin-conjugate of desulfated YTX as a ligand. By a protein pull-down protocol with use of streptavidin beads, a band of specifically binding proteins was detected in SDS–PAGE. HPLC-tandem mass spectrometry (MS/MS) indicated that Rap 1A, one of Ras superfamily proteins, binds to the YTX-linked resins. Western blotting and surface plasmon resonance experiments further confirmed that Rap1A specifically binds to YTX with the K_D value around 4 μM.

Ladder-shaped polyether (LSP) compounds[1–3] such as brevetoxins,[4,5] ciguatoxin,[6,7] yessotoxin[8,9] (YTX; Fig. 1) and prymnesin[10] are a group of unique metabolites produced by dinoflagellates and haptophytes. LSPs commonly possess a molecular stem comprising continuous trans-fused polycyclic ethers. More than 50 naturally occurring LSPs have been reported to date, some of which are known to possess powerful biological activities and be associated with food poisoning and massive fish kill.

YTX was first isolated from the scallops *Patinopecten yessoensis*, which were contaminated with toxins of diarrhetic shellfish poisoning.[8] Afterward, YTX turned out to have weak acute toxicity to human, thus being ruled out from diarrhetic shellfish toxins. YTX and its homologs are produced by the dinoflagellates *Protoceratium reticulatum*[11–13] and *Lingulodinium polyedrum*,[13] and exhibit multiple biological activities; besides the intraperitoneal toxicity against mice,[14] YTX shows in vitro induction of apoptosis,[15–18] modulation of cellular calcium levels of human lymphocytes,[19] and enhancement of phosphodiesterase (PDE) enzymatic activity.[20] In particular, the toxin has relatively high affinity to PDE, which led to extensive studies on their interaction largely by biosensor techniques.[21–23]

Among other naturally occurring LSPs, brevetoxins and ciguatoxins are a few examples that target proteins have been identified; these toxins are known to bind to voltage-sensitive Na^+ channels[24,25] with nanomolar/subnanomolar K_D values,[26] which are thought to account for their extremely potent toxicity. More recently, another LSP, gambierol, was reported to inhibit K^+ channels.[27–29] These channel proteins largely consist of membrane integral α helices, implying that LSP skeletons generally possess high affinity to the helix motif.[30] However, the structure-based mechanism of these interactions remains elusive because their large molecular weights coupled with membrane integral nature have hampered X-ray crystallographic approaches. To address this problem, we have searched for smaller membrane proteins that specifically interact with LSPs.[31] In the previous study, we utilized YTX as a LSP model and disclosed that YTX binds to the membrane integral domain of glycophorin A.[31,32] In this study, we aimed not to identify the binding target of YTX that accounted for its biological activities, but to obtain a protein that recognized an LPS structure with high affinity. Eventually, we found a YTX-binding protein in the lysates of blood cell membranes, and examined its affinity to YTX by surface plasmon resonance techniques.

For affinity purification of target proteins of YTX, we prepared biotinylated desulfated yessotoxin, dsYTX (Figs. 1 and 3, synthetic details are provide in Supplementary data),[33] which was then immobilized to streptavidin-bearing beads. After incubation with the lysate prepared from human blood cell membranes, YTX-binding proteins trapped on the beads were eluted and subjected

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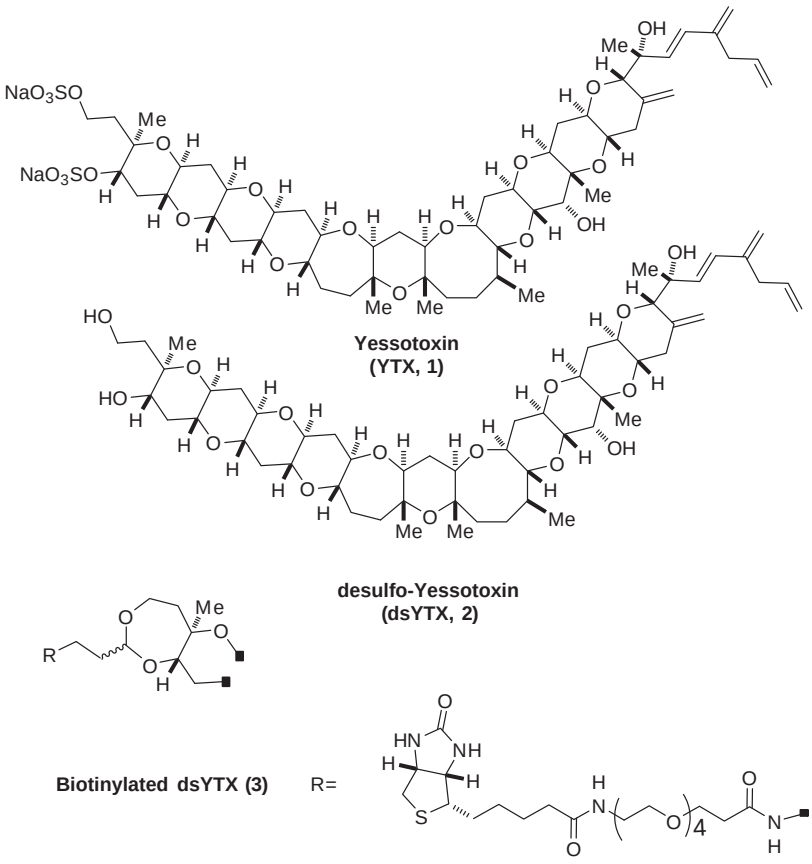

Figure 1. Structures of yessotoxin (YTX, **1**), dsYTX (**2**) and biotinylated dsYTX (**3**).

to SDS–PAGE. As depicted in Figure 2, a 21 kDa band appeared to be a candidate for YTX-binding proteins since the protein band disappeared in the presence of an excess amount of free YTX upon incubation (Fig. 2A) and increased when the beads were eluted with free YTX (Fig. 2B).

To identify the protein, in-gel tryptic digestion was carried for subsequent nano-LC–MS/MS analysis. As a result, several peptide sequences were identified, some of which corresponded to the digested peptides of Ras superfamily proteins, Rap 1A, Rap 1B, Rho A, Rho C, Rac 1, Rac 2, and K-Ras (Table 1). The identification of Rap1A, from which 11 peptides were detected by the LC–MS/MS analysis, was further carried out by Western blotting (Fig. 3).

As the next step, we evaluated the interaction between Rap 1A and YTX using surface plasmon resonance (SPR) techniques. Recombinant Rap 1A was immobilized onto the CM5 sensor chip, over which a YTX or dsYTX solution was passed.[33] Dose-dependent SPR responses were recorded for a series of concentrations of YTX and dsYTX as shown in Figure 4, which clearly indicated their direct interactions with Rap 1A. We then estimated the

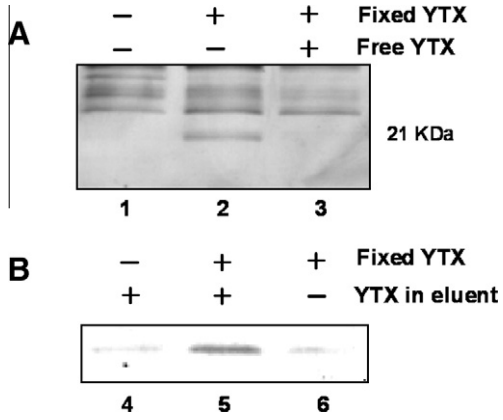

Figure 2. SDS–PAGE for the 21 kDa YTX-binding protein. (A) Human red blood cell (RBC) proteins that were trapped with control beads (lane 1) and with the ligand (**3**) fixed beads (lanes 2 and 3) were visualized by silver staining. In lane 3, free YTX (100 μM) was added to the cell lysate during incubation. (B) Human RBC proteins that were trapped with control beads (lane 4) and with the ligand (**3**) fixed beads (lanes 5 and 6) were visualized by silver staining. Elution was carried out with a buffer containing 200 μM (lanes 4 and 5) or 0 μM (lane 6) YTX.

Table 1
YTX-binding proteins detected by LC–MS/MS analysis

Protein	Number of peptide sequence[a]
Ras-related protein Rap 1A	11
Ras-related protein Rap 1B	9
Transforming protein Rho A	9
Rho-related GTP-binding protein Rho C	9
Ras-related C3 botulinum toxin substrate 1 Rac 1	7
Ras-related C3 botulinum toxin substrate 2 Rac 2	7
GTPase K-Ras K-Ras	6

[a] Number of peptides detected by nano-LC–MS/MS that correspond to the fragments of protein in the database. Details of the identified peptides are provided in Supplementary data.[33]

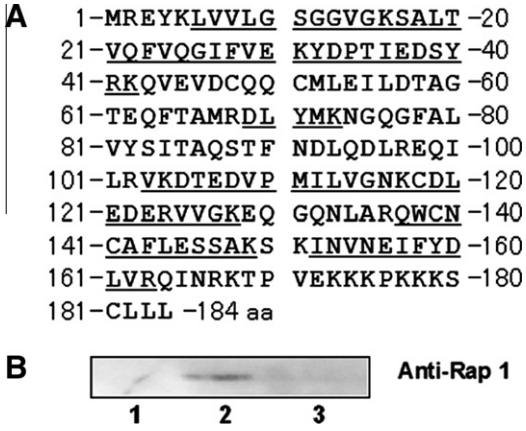

Figure 3. Peptide sequences detected by nano-LC–MS/MS and Western analysis for YTX-binding proteins. (A) The amino acid sequence of Rap 1A and the corresponding peptide sequences found in the tryptic digests (underlined). (B) Western blot analysis of YTX-binding protein. Human RBC proteins that were trapped with control beads (lane1) and with the ligand (**3**) fixed beads (lanes 2 and 3) were subjected to SDS–PAGE and visualized with a Western blot protocol using anti-Rap1A/1B antibodies. In lane 3, free YTX (100 μM) was added to the lysate during incubation.

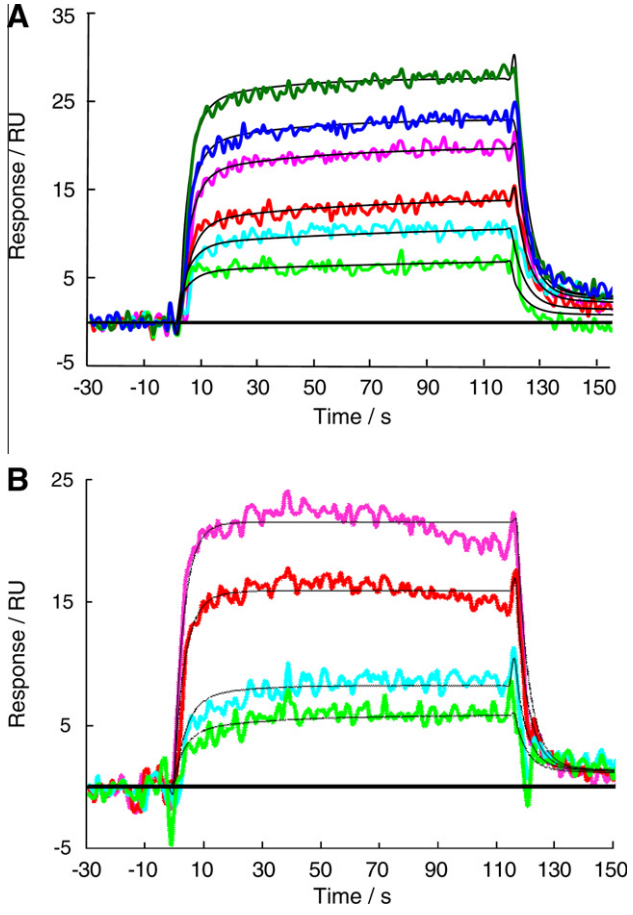

Figure 4. SPR sensor grams of YTX (A) and dsYTX (B) for Rap 1A immobilized on the surface of a CM5 sensor chip. (A) YTX (**1**) was injected in the range 0–50 μM. Color traces correspond to concentration of YTX from 5 μM (bottom) to 50 μM (top) in HBS-EP+ solution; 5 (light green), 10 (light blue), 20 (red), 30 (magenta), 40 (blue), and 50 (green) μM. (B) dsYTX (**2**) was injected in the range 0 to 30 μM. Color traces correspond to concentration of YTX in HBS-EP+ solution; 5 (light green), 10 (light blue), 20 (red), 30 (magenta) μM. Theoretical curves obtained from the heterogeneous ligand-parallel reaction model are shown as solid and black lines for sensor gram for YTX (A) and dsYTX (B), respectively.

equilibrium dissociation constant (K_D) of YTX using BIAevaluation software for a heterogeneous ligand-parallel reaction model, which gave rise to two binding affinities, K_{D1} 143.0 μM and K_{D2} 4.2 μM. The low affinity K_{D1} implies that this interaction is due to non-specific binding while the higher affinity of the K_{D2} is probably due to the specific binding of YTX to immobilized Rap 1A. The same model fitting revealed the K_D of dsYTX with Rap1A to be 0.9 μM. The higher affinity of dsYTX may be accounted for by its higher hydrophobicity. The dissociation constants of YTX and dsYTX for weakly interacting proteins such as streptavidin and membrane integral protein APH1B were comparable with that for the non-specific binding (Supplementary data).[33] We then carried out the SPR experiments with the inverted configuration, where Rap1A was passed on a dsYTX-immobilized chip, and obtained a comparable K_D value of 0.5 μM.[33]

In the present study, we screened proteins associated with blood cell membranes for YTX-binding affinity, and successfully identified one of Ras superfamily members as a binding protein. The objective of this study is to obtain a protein that recognizes an LSP structure with high affinity, so we chose blood cells rather than biologically relevant targets such as tissue cells because of their availability and well established protein profile. Thus, the present results do not necessarily imply that any of Ras superfamily proteins are involved in the toxicity and other biological activities of YTX. The seven proteins listed in Table 1, which gave rise to the sufficient number of peptide fragments in the LC–MS/MS experiments, belong to Ras superfamily proteins. This family belongs to a class of small GTPase that plays important roles in cellular signal transduction.[34–36]

We previously disclosed that various LSPs show high affinity to the membrane α-helix motif,[30,37] where hydrophobic matching plays an essential role, by using the synthetic LSP model compounds and trans-membrane peptides of glycophorin A. As is the case with phosphodiesterases (PDEs),[21,23] Rap 1A possesses the buried α-helix motifs.[37] Thus, the LSP stem of YTX may possibly bind to the hydrophobic helices as proposed for the ion-channel binding LSPs such as brevetoxins, ciguatoxins and gambierol. Apparent sequence homology to Ras proteins is observed neither in glycophorins nor PDEs, implying the relatively broad specificity of YTX to hydrophobic helices. Considering relatively high affinity of YTX to Rap1A (Fig. 4), detailed examination of their interaction may provide a clue to understanding the molecular-based mechanism of how LSPs generally recognize target proteins.

We initially expected that some of PDEs should be detected by affinity purification using YTX. Yet, no corresponding bands were recognized in the SDS–PAGE albeit the presence of small quantities of PDEs reported for erythrocytes.[38] The reason may be that quantities of PDEs are extremely small in the lysate, and/or that the linker part hampers binding of the YTX moiety to the binding pocket of PDEs.

By elucidating the structure basis of the bimolecular interactions, it would become possible to design and synthesize the small molecules that specifically recognize the trans-membrane domain of integral proteins. Further studies on the molecular recognition of Ras superfamily proteins by LSPs are now in progress in our laboratory.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.080.

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