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Identification of a target protein of *Hydra* actinoporin-like toxin-1 (HALT-1) using GST affinity purification and SILAC-based quantitative proteomics

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ABSTRACT

Hydra actinoporin-like toxin-1 (HALT-1) is a 20.8 kDa pore-forming toxin isolated from *Hydra magnipapillata*. HALT-1 shares structural similarity with actinoporins, a family that is well known for its haemolytic and cytolytic activity. However, the precise pore-forming mechanism of HALT-1 remains an open question since little is known about the specific target binding for HALT-1. For this reason, a comprehensive proteomic analysis was performed using affinity purification and SILAC-based mass spectrometry to identify potential protein-protein interactions between mammalian HeLa cell surface proteins and HALT-1. A total of 4 mammalian proteins was identified, of which only folate receptor alpha was further verified by ELISA. Our preliminary results highlight an alternative-binding mode of HALT-1 to the human plasma membrane. This is the first evidence showing that HALT-1, an actinoporin-like protein, binds to a membrane protein, the folate receptor alpha. This study would advance our understanding of the molecular basis of toxicity of pore-forming toxins and provide new insights in the production of more potent inhibitors for the toxin-membrane receptor interactions.

1. Introduction

Pore-forming toxins (PFTs) are characterized by their unique ability to form pores in the cell membrane. They are produced by a variety of organisms such as bacteria (Geny and Popoff, 2006), yeast-fungi (Bussey, 1991) and human (Voskoboinik et al., 2010). In sea anemones, a group of alpha helical-pore-forming toxins (α-PFTs) was isolated and named actinoporins (Ferlan and Lebez, 1974; Kem, 1988; Turk, 1991). This group of toxins has characteristic features of PFTs, including small protein size of 18–20 kDa, an isoelectric point above 9, lack of cysteine residues and sphingomyelin dependence for membrane binding (Anderluh and Maček, 2003; Norton, 2009). The most studied members of the actinoporin family are equinatoxin II (EqtII) of *Actinia equina* and sticholysin II (StnII) of *Stichodactyla helianthus* (Šuput, 2014; Rojko et al., 2016).

After the first actinoporin was identified in *Actinia equina*, many other actinoporin-like proteins were identified in other cnidarians. For example, six *Hydra* actinoporin-like toxins (HALTs) were identified in the genome of *Hydra magnipapillata* (Glasser et al., 2014). The amino acid sequences of these toxins share 30% identity with those of actinoporins from sea anemones (Glasser et al., 2014). In addition, HALT-1 showed haemolytic and cytolytic activity towards red blood cells and HeLa cells (Glasser et al., 2014; Liew et al., 2015). HALT-1 has a conserved cluster of aromatic amino acids and an amphiphilic N-terminus, which are proposed to play important roles in membrane binding and pore formation, respectively (Liew et al., 2015). Although HALT-1 is structurally similar to other actinoporins, a recent study showed that this toxin produced a different pore size from EqtII and did not use sphingomyelin as membrane target (Glasser et al., 2014). These findings may not be surprising, since HALT-1 and EqtII share only 30% similarity in protein sequence and several amino acid residues known to be involved in sphingomyelin binding in equinatoxin and sticholysin are not conserved in HALT-1 (Liew et al., 2015).

Although numerous experiments support a critical role for sphingomyelin as the membrane attachment site for sea anemone antinoporins (Bakrač and Anderluh, 2010), some reports suggested otherwise. For example, De los Ríos et al. (1998) and Caaveiro et al.

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(2001) tested the lytic action of StnII and EqtII with model membranes having different lipid compositions. Their results showed that these toxins appeared to have the ability to bind to and permeabilize membranes in the absence of sphingomyelin. In fact, EqtII was able to permeabilize large unilamellar vesicles (LUV) containing 30% cholesterol in egg phosphatidylcholine (Caaveiro et al., 2001) as well as LUVs composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glyerol-3-phosphocholine (DPPC) and cholesterol (1:1:1) (Schön et al., 2008). A similar result was obtained with another actinoporin, sticholysin of *Stichodactyla helianthus*. This actinoporin was able to permeabilize LUVs composed only of cholesterol and phosphatidylcholine (De los Ríos et al., 1998; Anderluh and Maček, 2003; Kristan et al., 2009). Thus, at least in some *in vitro* experiments, sphingomyelin is not required for membrane permeabilization by actinoporins.

In the present study, we have searched for alternative membrane receptors, in particular membrane proteins, which could play a role in HALT-1 permeabilization of membranes. The cell membrane is a complex mixture of lipids and proteins and both are functionally important in many cellular processes including as a barrier to separate the interior of the cell from its environment, to selectively transport substances across the membrane and to act as membrane-bound receptors or mediators of signal transduction (Fernandis and Wenk, 2007). A few bacterial pore-forming toxins have been proven to bind proteinaceous receptors in addition of their affinity to membrane lipids (Dal Peraro and van der Goot, 2016; DuMont and Torres, 2014). These pore-forming toxins include the intermedilysin (ILY) of *Streptococcus intermedius* which recognises CD59, an GPI-anchored protein, as the membrane receptor although it has been classified as a cholesterol-dependent cytolysin (Giddings et al., 2004). Hence, membrane proteins can be potential candidates as receptors for HALT-1.

Since the attempt to show sphingomyelin as an important membrane target site for HALT-1 was not successful (Glasser et al., 2014) and there has been no information on membrane protein binding, we used a combination of GST affinity purification and SILAC based quantitative mass spectrometry to identify potential membrane proteins that bind to HALT-1. This combination offered a powerful approach to identify complexes associated with signalling mechanisms (Ong and Mann, 2006), including those involving low abundant proteins and weak protein interactions (Bauer and Kuster, 2003). Using this approach we have identified a membrane protein, which appears to have a role in the binding of HALT-1 to membranes.

2. Materials and methods

2.1. Plasmid construction

For the construction of glutathione-S-transferase-tagged (GST-tagged) at N-terminus of HALT-1 fusion protein, the Gateway cloning system (Invitrogen-Life Technologies, USA) was performed according to the manufacturer's instruction. Prior to the gene cloning, *HALT-1* was amplified from *HALT-1*-pCR4-TOPO using Phusion® High Fidelity DNA Polymerase (New England BioLabs, USA) and a pair of primers (forward primer 5′-CACCGCCAGTGGAGCAGCTTTAG-3′ and reverse primer 5′- TTATCCAGAAAAAATAACTTTGAACTCAGCATGG-3′). The amplicon was then cloned into pENTR™/D-TOPO® Entry Vector using Directional TOPO cloning system to generate the Entry clone, and followed by the transformation into One Shot® TOP10 competent cells. To produce the expression vector containing GST-tagged at the N-terminus of HALT-1, the HALT-1 Entry clone was sub-cloned into pDEST™15 Destination vector using LR recombination reaction system and then transformed into Library Efficiency® DH5α cells. The construct of recombinant GST-tagged HALT-1 was finally verified by sequencing.

2.2. Protein expression and purification

The recombinant GST-tagged HALT-1 was expressed in BL21-AI™ One Shot® *E. coli* with L-(+)-arabinose induction (0.2%) for 4 h at 30 °C. After the expression, the cells were harvested by centrifugation at 10,000×*g* for 5 min. Subsequently, the arabinose-induced culture was re-suspended in equilibration buffer (50 mM Tris.HCl, 150 mM NaCl, pH 8.0) with additional protease inhibitor PMSF (0.1 mM), and the cells were lysed by sonication at 500 W and 20 kHz. The lysate was centrifuged 2,200×*g* for 15 min. For protein purification, a total of 50–200 mg soluble fraction of cell lysate were subjected to affinity chromatography of glutathione agarose (Thermo Fisher Scientific, USA). Purified GST-tagged HALT-1 fusion protein was concentrated and exchanged buffer with BupH Tris buffer using Amicon® Ultra-2 (Merck Millipore, USA). As based on the manufacturer's protocol of Quick Start Bradford Assay (Bio-Rad, USA), the final concentration of purified GST-tagged HALT-1 protein was measured as 10.8 μg/μL.

2.3. Cell culture and SILAC labelling

HeLa cells (ATCC® CCL-2™) were routinely maintained in Dulbecco's modified Eagle's medium (Gibco/Invitrogen-Life Technologies, USA) supplemented with 10% dialyzed fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in 5% CO_2 atmosphere. For SILAC labelling, the incorporation of “light” and “heavy” amino acids into cells was carried out using SILAC Protein Identification (ID) and Quantitation Kits (Gibco/Invitrogen-Life Technologies, USA) according to the manufacturer's instruction. Two sets of HeLa cells were grown separately in two different custom-made SILAC media, in which one group of cells was grown in normal or “light” medium containing 100 mg/mL of L-lysine HCl and 100 mg/mL of L-Arginine and the other group of cells was grown in labelling or “heavy” medium containing 100 mg/mL of [U-$^{13}C_6$]-L-Lysine HCl (*Lys) and 100 mg/mL of L-Arginine. Both “light” and “heavy” media were supplemented with 4500 mg/L of glucose, 292 mg/L of L-glutamine, 110 mg/L of sodium pyruvate, 15 mg/mL of phenol red, 1% of penicillin-streptomycin and 10% dialyzed FBS. “Light” and “heavy” labelled cell populations were passaged for at least 9 doubling times to achieve complete incorporation of [U-$^{13}C_6$]-L-Lysine HCl (*Lys) (Ong et al., 2002, 2003; Ong and Mann, 2006). Both cell cultures were grown at 37 °C in a humidified incubator of 5% CO_2 atmosphere. After cell adaptation, the efficiency of SILAC incorporation into the culture was determined to be >95% by mass spectrometry analysis. The percentage of SILAC incorporation was calculated manually using the following equation: Incorporation (%) = [Ratio (H/L)/(Ratio (H/L) + 1)] x 100 (Oeljeklaus et al., 2014).

2.4. Preparation of cell lysates

For sample preparation, both “light” and “heavy” labelled HeLa cell populations were separately grown to approximately 90% confluence in their respective SILAC media as described above. After that, the medium was removed and cells were washed with 0.9% sterile NaCl, scrapped in 0.9% sterile NaCl and centrifuged at 200×*g* for 5 min at 4 °C. Whole cell extracts were prepared by dissolving cells in Pierce IP lysis buffer (Thermo Fisher Scientific, USA) (25 mM Tris.HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA and 5% glycerol) with addition Halt™ Protease Inhibitor Single-Use Cocktail (Thermo Fisher Scientific, USA). Two cell suspensions were incubated for 7 min on ice with maximum agitation and