The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding

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Abstract Thiol-activated cytolysins share a conserved hydrophobic, Trp-rich undecapeptide that is suggested to be involved in membrane binding and intercalation. The neutralizing monoclonal antibody PLY-5 recognizes all members of this toxin family and peptide mapping assigned its epitope to the undecapeptide motif. This antibody inhibited binding of the toxins to host cell membranes and the epitope was no longer available for binding when a preformed toxin/membrane complex was tested. These results confirm the model of cytolysin binding suggested by structural data.

Key words: Membrane binding; Cytolysis; Epitope mapping; Thiol-activated toxin; Anti-hemolytic antibody

1. Introduction

Thiol-activated, pore-forming cytolysins are produced by several species of Gram-positive bacteria (*Bacillus*, *Clostridium*, *Streptococcus* and *Listeria*). All these toxins are believed to bind to cholesterol as receptor in the host cell membranes which subsequently leads to insertion and oligomerization. As a consequence the membrane is permeabilized [1]. However, besides membrane permeabilization these cytolysins exhibit several other functions including induction of cytokine secretion and other modulators of the immune system [2,3]. Thus, most likely these cytolysins play essential roles as virulence factors in the progression of an infection by these bacteria [4].

The different thiol-activated cytolysins share sequence homology between 40 and 70%. They have in common a well-conserved Trp-rich undecapeptide in their C-terminal region which also contains the unique Cys that is found in all of these toxins. Recently, the crystal structure of perfringolysin O (PFO), one of the members of this toxin family, was resolved [5]. The monomer is folded into four discontinuous domains forming an elongated mushroom-like molecule. The putative membrane binding domain 4 comprises the last 110 C-terminal residues which includes the conserved Trp-rich undecapeptide. This hydrophobic motif forms a tip at the end of the molecule and presumably dips into the membrane to promote binding [6,7].

Monoclonal antibodies (mAbs) have been raised against several thiol-activated cytolysins. Some of them have been shown to neutralize hemolysis by preventing polymerization, whereas only a few were found to interfere with binding [8–11]. Here we report that PLY-5, a mAb that was raised against pneumolysin (PLY), recognizes an epitope in all thiol-activated cytolysins tested so far. Epitope mapping revealed that this antibody reacts with the conserved Trp-rich motif. Preincubation of toxins with PLY-5 prevented binding to membranes whereas the epitope was no longer accessible after membrane binding. These data corroborate the model obtained by X-ray diffraction [5], suggesting that the Trp-rich motif is responsible for binding by inserting into the host cell membrane.

2. Materials and methods

2.1. Antigens

Recombinant PLY was expressed in *Escherichia coli* and purified as described [12]. Listeriolysin O (LLO) was hyperexpressed in *Listeria innocua* and purified as described [9]. Perfringolysin O (PFO) and suilysin (SLY) were obtained as described [13,14]. Streptolysin O (SLO) was purchased from Sigma. Additional pore-forming toxins employed in immunoblot analyses were used unpurified from bacterial supernatants. Strains (*Bacillus cereus* 148, *Clostridium tetani* 829, *Listeria ivanovii* 913, *Listeria seeligeri*, *Bacillus alvei*, *Bacillus thuringiensis*, *Clostridium botulinum*) were obtained from the Spanish Type Culture Collection and were cultured in brain-heart infusion broth. As negative controls α-hemolysin from *Staphylococcus aureus* and *E. coli* (both from Sigma) were used.

2.2. Antibodies

Generation of the mouse monoclonal antibody PLY-5, the polyclonal rabbit anti-LLO antisera (pLLO) and a polyclonal anti-PLY antiserum has been described [8,9]. The neutralizing mouse mAb 2-5B specific for SLO was a generous gift of Dr. F. Hugo, Giessen, Germany [10].

2.3. Immunoblot assays

For immunoblot assays 500 ng of purified toxin or 15 μl of culture supernatants after TCA precipitation were applied per lane and separated by SDS-PAGE. After transfer to nitrocellulose, specific protein bands were developed using the BM chemiluminescence blotting substrate (POD) of Boehringer Mannheim according to the instructions of the vendor. Primary antibodies were used at 1 μg/ml; peroxidase-conjugated sheep anti-mouse IgG (Fab; Boehringer Mannheim) or goat anti-rabbit IgG (IgG; Jackson Laboratories) were used as secondary reagents.

2.4. Analyses of toxin-membrane interactions by flow cytometry

Two assays were employed. Two hemolytic units (HU) of SLO were incubated with 1 μg/ml of the mAbs PLY-5 or 2-5B for 30 min at 37°C, 50 μl of 1.6% sheep red blood cells (SRBC) in 1% bovine serum

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albumin in phosphate-buffered saline containing 0.1% NaN_3 (BSA-PBS-N_3) were added and further incubated for 30 min at 37°C. Then, cells were washed, resuspended in BSA-PBS-N_3 and stained with FITC-conjugated anti-mouse IgG (Sigma). Alternatively, SRBC were treated with 2 HU SLO or 4 HU LLO on ice. After washing, toxin-SRBC complexes were incubated with either PLY-5 and 2-5B (for SLO) or PLY-5 and polyclonal anti-LLO (for LLO). Binding of antibodies was revealed by staining with FITC-conjugated anti-mouse IgG or anti-rabbit IgG as secondary reagents. After staining, cells were washed, fixed in 100 μl of 1% paraformaldehyde and analyzed using a Cytotron Absolute flow cytometer (Ortho). At least 35 000 events were recorded per sample. Data were analyzed using WinList (Verity Software House).

2.5. Analyses of toxin-membrane interactions by immunoblot

SRBC were washed three times with PBS and lysed with 5 mM HEPES, pH 7.4. Membranes were washed with lysis buffer and further incubated with 10 mM HEPES, pH 7.4, 130 mM KCl, 10 mM NaCl, 3 mM $MgCl_2$ for 1 h to allow formation of vesicular ghosts. These were sedimented and resuspended in PBS, pH 7.4. Ghosts from 1×10^5 SRBC were incubated with 100 ng LLO or PLY or with the same amount of the particular toxin that was neutralized by addition of 1 μg PLY-5 for 15 min at 20°C in 200 μl PBS. Membranes were then sedimented by ultracentrifugation, washed and solubilized in SDS-PAGE loading buffer. Toxin from the supernatant was recovered by TCA precipitation. Subsequently, SDS-PAGE was performed on 12% polyacrylamide gels. Proteins were blotted and developed using the polyclonal rabbit anti-LLO antiserum which also detects PLY.

2.6. Epitope mapping

A library of overlapping peptides 15 amino acids in length and an offset of three was synthesized according to the primary sequence of PLY on cellulose paper as described [15]. This library was incubated with 1 μg PLY-5 in PBS containing 2.5% skim milk powder. After intensive washing a secondary peroxidase conjugated goat anti-mouse Ig antibody (Jackson Laboratories) was used. Spots were visualized using ECL (Amersham). As control, the library was probed with the secondary antibody only. For confirmation of the presumable epitope, a soluble peptide (ECTGLAWEWWRT) was synthesized using an ABI peptide synthesizer and Fmoc chemistry.

3. Results

The neutralizing mAb PLY-5 had been previously shown to recognize an epitope within a C-terminal fragment of pneumolysin [8]. To further characterize this epitope we tested PLY-5 for crossreactivity with other members of the thiol-activated toxin family. As shown in Fig. 1, PLY-5 reacted in immunoblot with purified PLY as well as SLO, LLO and SLY. Furthermore, PLY-5 detected PFO and the proteolytic fragments of PFO containing the conserved undecapeptide (data not shown). Reactivity of PLY-5 to additional thiol-activated toxins, such as cereolysin O, tetanolysin, alveolysin, thuringiolysin O, ivanolysin, seeligerolysin and botulinolysin, was studied using bacterial lysates. In all cases except botulinolysin, a band of the appropriate molecular weight was observed (data not shown) indicating reactivity against a determinant that is highly conserved amongst the thiol-activated toxins. As specificity control α-hemolysin of *Staphylococcus aureus* or supernatant from a culture of hemolytic *E. coli* was used. Both are known to produce other types of membrane-damaging bacterial toxins. As expected, no reactivity of PLY-5 was observed with these hemolysins (data not shown).

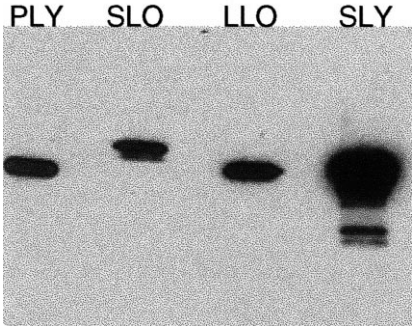
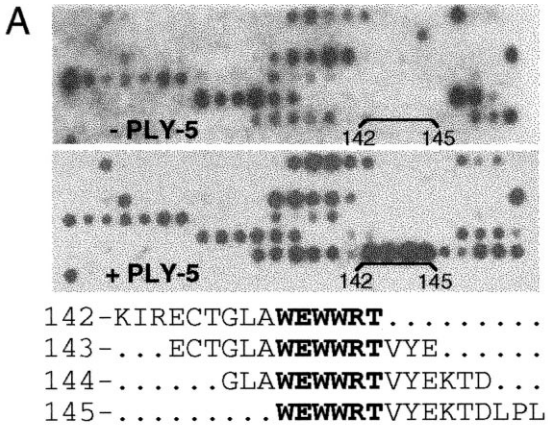


Fig. 1. Immunoblot assays of different thiol-activated toxins. 500 ng of purified PLY, SLO, LLO and SLY were incubated with 1 μg of PLY-5 followed by the respective peroxidase-labeled secondary antibody. Blots were developed by the chemiluminescence method.



B

PLY 50ng	PLY-5 100ng	peptide μg	lyis titer
+	–	–	3
+	+	–	0
–	+	–	0
+	+	1	1
+	+	3	3
+	+	10	3
+	–	10	3
–	+	10	0

Fig. 2. Epitope mapping of PLY-5. A: Pep-scan analysis of PLY-5. As a background control peptides covering the whole sequence of PLY bound on nitrocellulose paper were incubated with a peroxidase-labeled secondary antibody and developed by chemiluminescence (upper panel). The nitrocellulose paper was stripped afterwards and incubated with PLY-5 followed by the secondary antibody. The strongest signals were observed with peptides 142–145 (lower panel), these signals were absent in the control. Sequences of the respective peptides are given below. B: Blocking of anti-hemolytic activity of PLY-5 with the soluble form of the Trp-rich peptide.

In order to precisely map the epitope that is recognized by PLY-5 we used a library of overlapping peptides synthesized as spots on cellulose paper which covered the entire amino acid sequence of pneumolysin. Binding was detected with a peroxidase-conjugated secondary antibody and developed by chemiluminescence (Fig. 2). Spots of minor intensity were already visible when the library was probed with the secondary antibody alone (Fig. 2A, upper panel). These spots might represent unspecific binding or crossreactions of the secondary antibody. Despite this, the epitope of PLY-5 could be assigned unambiguously. Four strong spots were observed only when PLY-5 was included in the assay (142–145, Fig.

2A, lower panel). The amino acid sequences of these four spots are given below the images in Fig. 2A and the minimal epitope recognized by PLY-5 that was deduced from this sequence information should consist of WEWWRT. Thus, the mAb PLY-5 binds to an epitope within the conserved undecapeptide as already suggested by the reactivity with almost all thiol-activated toxins.

In order to confirm the epitope mapping we tested whether PLY-5 could be blocked by a peptide containing the presumable epitope (ECTGLAWEWWRT). In a conventional hemolysis neutralization assay 1 μg of PLY-5 was found to be neutralizing for 2 HU of PLY, 2 HU of SLO and 4 HU of LLO. This anti-hemolytic activity of PLY-5 could be blocked by preincubation with the soluble peptide (Fig. 2B and data not shown). Thus, the reactivity of PLY-5 with the soluble peptide confirmed the epitope mapping results obtained with the solid phase assay.

The undecapeptide was postulated to be involved in membrane binding of these toxins, therefore PLY-5 could be used as a probe to test this hypothesis. Since PLY-5 does not distinguish between members of the thiol-activated toxin family we could use toxins where the appropriate reagents were available. First, toxin binding to SRBC membranes was studied by flow cytometry. SLO was preincubated with PLY-5 or 2-5B, a neutralizing antibody that does not interfere with binding. Then SRBC were added under standard hemolysis assay conditions and the binding of the antibody-toxin complex was measured with FITC-conjugated secondary antibodies. Fig. 3A demonstrates that binding of SLO to SRBC was clearly inhibited by PLY-5, while the complex between 2-5B and toxin could still bind. This indicates that neutralization by PLY-5 is due to inhibition of toxin binding to membranes, which is consistent with the above hypothesis.

Upon binding and intercalation of the toxin into a membrane the undecapeptide should no longer be accessible to antibodies. This was found to be the case. When SLO was incubated with SRBC on ice to avoid polymerization and lysis, the toxin could no longer be recognized by PLY-5 whereas positive staining was obtained with 2-5B under these circumstances (Fig. 3B). Similar results were obtained using LLO (Fig. 3C and data not shown) or SLY (data not shown) as toxin.

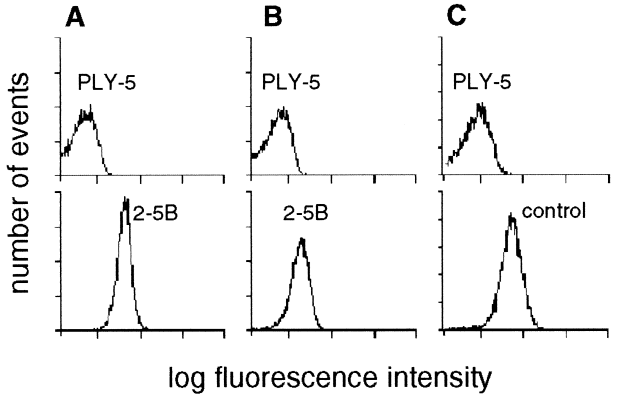


Fig. 3. Analysis of toxin-binding by flow cytometry. A: SRBC were incubated with SLO-antibody complexes obtained under standard neutralization conditions. Binding was observed with 2-5B but not when PLY-5 was used. B: SLO was preincubated with SRBC on ice followed by the respective antibody. Fluorescence staining was positive for the 2-5B antibody but not for PLY-5, indicating that the PLY-5 epitope is not recognized after the toxin had bound to SRBC membranes. C: LLO was incubated with SRBC on ice. Binding was analyzed with an anti-LLO rabbit polyclonal serum followed by FITC anti-rabbit IgG. When LLO was preincubated with PLY-5, no staining was observed, indicating that PLY-5 prevented toxin binding to SRBC membranes. In contrast, when LLO was not incubated with PLY-5 (control) binding could be detected.

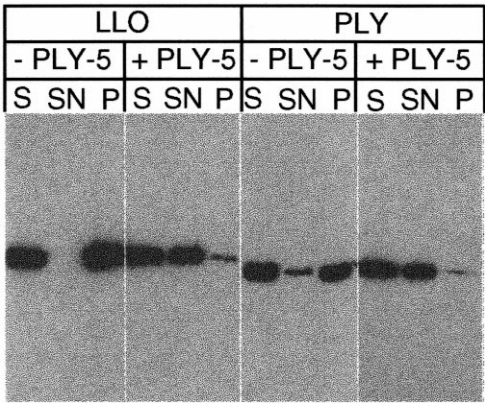


Fig. 4. Toxin binding analyses by immunoblot. 100 ng toxin (LLO or PLY) was incubated with SRBC ghosts without preincubation with PLY-5 (−PLY-5) or after preincubation with PLY-5 (+PLY-5). Membranes were harvested by ultracentrifugation. Supernatant (SN) and pellet (P) were applied onto a SDS polyacrylamide gel. Samples without added SRBC were used as control (S). The gels were blotted and stained with anti-LLO rabbit polyclonal IgG.

These findings were corroborated by quantitation of toxin binding in the presence or absence of PLY-5 to SRBC ghosts. Immunoblot revealed that thiol-activated toxins bind rapidly to the SRBC vesicles and can be recovered in the pellet fraction after centrifugation. In contrast, toxins preincubated with PLY-5 no longer bound to the membranes and remained in the supernatant fraction (Fig. 4) thus confirming the results obtained by cytofluorometry.

4. Discussion

The neutralizing antibody PLY-5 recognizes an epitope within the undecapeptide that is conserved amongst all the thiol-activated cytolysins. This was already suggested by the reactivity with most members of this toxin family tested so far. Only in immunoblots from supernatants of *C. botulinum* no reactivity with PLY-5 could be detected. This might be due to low expression of botulinolysin, which depends very strongly on culture conditions and also varies to a high extent in different stains. In addition, amino acid analysis of botulinolysin suggests that this protein might be very different from the other members of this toxin family [16]. Unambiguously, the epitope of PLY-5 could be identified by epitope mapping using overlapping peptides and blocking studies with a synthetic undecapeptide. This motif has been shown by mutagenesis experiments to be essential for the hemolytic activity [4,13].

Based on crystallographic data and other functional studies a model had been suggested that places this motif at the membrane proximal tip of the molecule during binding to the host cell membrane and subsequently intercalates into the lipid bilayer [5–7]. Based on the three-dimensional structure a model was proposed in which the undecapeptide is involved in the formation of a hydrophobic pocket that is

able to bind cholesterol. Upon binding, this motif is supposed to undergo a conformational change [7,17,18]. The inhibition of toxin binding to membranes by PLY-5 is consistent with this idea as is the fact that the epitope is no longer available to the antibody after the toxin has bound.

Our findings are in agreement with the model suggested by Rossjohn et al. on the binding of thiol-activated cytolysins [5] and extend previous studies using either mAbs or biophysical means [7–10]. They appear to be in contradiction with a study using monoclonal antibodies raised against the undecapeptide itself [11]. In this study no inhibition of hemolysis by these antibodies was found, whereas the toxin was recognized in immunoblot and ELISA. Antibodies against peptides might bind to native antigens with low affinities. Since a membrane-seeking molecule might undergo conformational changes when it is bound to the hydrophobic surface of plastic, reactivity of an antibody in ELISA and recognition in solution might be different. On the other hand, PLY-5 was raised against the native molecule and by definition has to bind to an area of the molecule that is exposed in solution. Therefore it is likely that the antibody is of high affinity. The property of PLY-5 to recognize such an epitope and to inhibit membrane binding of all thiol-activated toxins renders this antibody not only of biological but also of therapeutic interest.

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References

[1] Tweten, R.K. (1995) in: Virulence Mechanisms of Bacterial Pathogens, 2nd edn. (Roth, J.A. et al., Eds.), American Society for Microbiology, Washington, DC.
[2] Kayal, S., Lilienbaum, A., Poyart, C., Memet, S., Israel, A. and Berche, P. (1999) Mol. Microbiol. 31, 1709–1722.
[3] Sibelius, U., Schulz, E., Rose, F., Hattar, K., Jacobs, T., Weiss, S., Chakraborty, T., Seeger, W. and Grimminger, F. (1999) Infect. Immun. 67, 1125–1130.
[4] Michel, E., Reich, K.A., Favier, R., Berche, P. and Cossart, P. (1990) Mol. Microbiol. 4, 2167–2178.
[5] Rossjohn, J., Feil, S.C., McKinstry, W.J., Tweten, R.K. and Parker, M.W. (1997) Cell 89, 685–692.
[6] Gilbert, R.J.C., Jiménez, J.L., Chen, S., Tickle, I.J., Rossjohn, J., Parker, M., Andrew, P.W. and Saibil, H.R. (1999) Cell 97, 647–655.
[7] Nakamura, M., Sekino, N., Iwamoto, M. and Ohno-Iwashita, Y. (1995) Biochemistry 34, 6513–6520.
[8] de los Toyos, J.R., Mendez, F.J., Aparicio, J.F., Vazquez, F., Garcia Suarez, M.M., Fleites, A., Hardisson, C., Morgan, P.J., Andrew, P.W. and Mitchell, T.J. (1996) Infect. Immun. 64, 480–484.
[9] Darji, A., Niebuhr, K., Hense, M., Wehland, J., Chakraborty, T. and Weiss, S. (1996) Infect. Immun. 64, 2356–2358.
[10] Hugo, F., Reichwein, J., Arvand, M., Kramer, S. and Bhadki, S. (1986) Infect. Immun. 54, 641–645.
[11] Nato, F., Reich, K., Lhopital, S., Rouyre, S., Geoffroy, C., Mazie, J.C. and Cossart, P. (1991) Infect. Immun. 59, 4641–4646.
[12] Mitchell, T.J., Walker, J.A., Saunders, F.K., Andrew, P.W. and Boulnois, G.J. (1989) Biochim. Biophys. Acta 1007, 67–72.
[13] Sekino-Suzuki, N., Nakamura, M., Mitsui, K.I. and Ohno-Iwashita, Y. (1996) Eur. J. Biochem. 241, 941–947.
[14] Jacobs, A.A.C., Loeffen, P.L., van den Berg, A.J. and Storm, P.K. (1994) Infect. Immun. 62, 1742–1748.
[15] Frank, R. (1992) Tetrahedron 48, 9217–9232.
[16] Haque, A., Sugimoto, N., Horiguchi, Y., Okabe, T., Miyata, T., Iwanaga, S. and Matsuda, M. (1992) Infect. Immun. 60, 71–78.
[17] Palmer, M., Vulicevic, Y., Saweljew, P., Valeva, A., Kehoe, M. and Bhadki, S. (1998) Biochemistry 37, 2378–2383.
[18] Jacobs, T., Darji, A., Frahm, N., Rhode, M., Wehland, J., Chakraborty, T. and Weiss, S. (1998) Mol. Microbiol. 28, 1081–1089.