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Essential Role of Domain 4 of Pneumolysin from *Streptococcus pneumoniae* in Cytolytic Activity as Determined by Truncated Proteins

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Pneumolysin (PLY), an important virulence factor of *Streptococcus pneumoniae,* is one of the members of thiol-activated cytolysins (TACYs) consisting of four domains. TACYs commonly bind to membrane cholesterol and oligomerize to form transmembrane pore. We have constructed full-length and various truncated PLYs to study the role of domains of PLY in the cytolytic activity. Full-length PLY had binding ability to both cell membrane and immobilized cholesterol. A truncated PLY which comprised only domain 4 molecule, the C-terminal domain of PLY, sustained the binding ability to cell membrane and cholesterol, whereas domain 1–3 molecule had no binding ability to them. Furthermore, the domain 4 molecule inhibited both the membrane binding and the hemolytic activity of full-length PLY. Accordingly, the present results provided the direct evidence that domain 4 was essential for the initial binding to membrane cholesterol and the interaction led to the subsequent membrane damage process.

Key Words: **pneumoylsin; *Streptococcus pneumoniae;* cytolytic activity; hemolysis; membrane cholesterol; thiol-activated cytolysins.**

Pneumolysin (PLY), produced by *Streptococcus pneumoniae,* is a 53 kDa protein toxin consisting of 471 amino acids (1). PLY has been regarded as one of the essential virulence factors of this bacterium causing pneumonia, bacterial meningitis, otitis media, and so on (2–6). This protein toxin may be one of the candidates for vaccine development against *S. pneumoniae* infection since it is produced by virtually all clinical isolates of *S. pneumoniae* irrespective of their serotypes (7–9).

PLY is a pore-forming toxin and one of the members of the family of structurally related thiol-activated cytolysins (TACYs). TACYs are produced by over 20 species of different gram-positive bacteria and are characterized by the presence of highly conserved undecapeptide in the C-terminal region (10, 11). These toxins are also known to bind commonly to cholesterol on cell membrane and oligomerize to form transmembrane pore. A recent study using X-ray crystallography revealed that perfringolysin O (PFO), another member of TACYs, is an elongated molecule consisting of four domains rich in β-sheet (12). It has been suggested that the structure of PLY is conformationally similar to that of PFO (13).

In terms of the lytic activity of TACYs, several steps are known to be involved in the lytic process. First, TACY binds to membrane of erythrocytes via cholesterol. Next self-association of monomeric molecules results in the oligomerization and the formation of ultimate pore, which leads to the membrane lysis (11). In this process, de los Toyos *et al.* showed by using various PLY-specific monoclonal antibodies that C-terminal portion is critical for the binding to the membrane (14). Truncation of the C-terminal portion also led to cause a defect of the binding activity of PLY (15) and PFO (16). These reports emphasized that the binding site to the membrane was in the C-terminal portion and that C-terminal domain (domain 4) was functionally associated with the initial binding of PLY to the erythrocytes. However, there has been so far no direct evidence indicating the binding of domain 4 to the membrane.

In the present study, we constructed full-length and various truncated forms of PLY to analyze the structure-function relationship with special reference

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TABLE 1

Oligonucleotide Primer Sets

Recombinant protein	Sequence of primers
PLY1–471 (full-length)	forward 5′-CGATGGATCCTATGGCAAATAAAGCAGTAAAT-3′ reverse 5′-ACGCGGTACCCTAGTCATTTTCTACCTTATC-3′
PLY1–437	forward 5′-CGATGGATCCTATGGCAAATAAAGCAGTAAAT-3′ reverse 5′-ACGCGGTACCCTAACGCCACCATTCCCAGG-3′
PLY1–426	forward 5′-CGATGGATCCTATGGCAAATAAAGCAGTAAAT-3′ reverse 5′-ACGCGGTACCCTATCTAATTTTGACAGAGAGAT-3′
PLY22–471	forward 5′-CGATGGATCCTACCCATCAGGGAGAAAGT-3′ reverse 5′-ACGCGGTACCCTAGTCATTTTCTACCTTATC-3′
PLY1–359 (domain 1–3)	forward 5′-CGATGGATCCTATGGCAAATAAAGCAGTAAAT-3′ reverse 5′-ACGCGGTACCCTATCTGTAAGCTGTAACCTTAG-3′
PLY360–471 (domain 4)	forward 5′-CGATGGATCCTAACGGAGATTTACTGCTGG-3′ reverse 5′-ACGCGGTACCCTAGTCATTTTCTACCTTATC-3′

to the role of the domain 4. The results obtained in this study show that domain 4 molecule of PLY was capable of binding to the membrane and cholesterol, and blocking the binding of full-length PLY to erythrocytes, and that direct binding of PLY to membrane cholesterol via domain 4 was the essential process to exert the lytic activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Streptococcus pneumoniae IID553 (serotype 2), one of the standard strains of Japanese Society for Bacteriology, was obtained from Laboratory Culture Collection, Institute of Medical Science, University of Tokyo, Tokyo, Japan, and grown in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C for preparation of chromosomal DNA. *Escherichia coli* M15 (Qiagen, Hilden, Germany) harboring pREP4 plasmid which contains *lacI* and kanamycin resistant genes was used as a host cell. Expression vector pQE-31 (Qiagen) which is designed to place a tag of six histidines (6 × His) at the N-terminus of the protein of interest was used. In order to express recombinant proteins *E. coli* harboring recombinant plasmid was grown in tryptic soy broth (Difco) containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin.

Production and purification of recombinant PLYs. Chromosomal DNA was extracted from *S. pneumoniae* IID553. The gene encoding full-length and truncated PLYs was amplified by PCR using specific primer sets described in Table 1. The primers were designed according to the sequence of *ply* gene (1) and contained modifications to add appropriate restriction enzyme sites for insertion into the vector, where *Bam*HI site in the forward primer and the *Kpn*I site in the reverse primer are underlined, respectively. The sequences of all PCR products were confirmed by DNA sequencing using ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). Both PCR product and pQE-31 expression vector were cut with *Bam*HI and *Kpn*I enzymes (New England Biolabs, Beverly, MA) and ligated with T4 ligase (New England Biolabs). The recombinant plasmid was transformed into *E. coli* M15 by electroporation using Gene Pulser II Electroporation System (Bio-Rad Laboratories, Hercules, CA). Transformants were cultured in tryptic soy broth (Difco) containing ampicillin and kanamycin, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the expression of recombinant protein at the middle of log phase of the culture. Purification was performed under native condition according to the manufacturer's instructions with slight modifications. Briefly, transformants of 1 liter culture were harvested by centrifugation at 4°C after an appropriate period of the induction and resuspended in 50 ml of phosphate buffer (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl) containing 20 mM imidazole. The cells were lysed on ice by homogenization with zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) after treatment with 1 mg/ml lysozyme. Lysate was centrifuged at 10,000*g* for 30 min at 4°C. The clarified supernatant was mixed with 3 ml of nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) and incubated for 60 min at 4°C with rotation. The agarose was packed into a 15 ml column and washed with 10 volumes of phosphate buffer containing 40 mM imidazole. Bound protein was eluted with 250 mM imidazole in phosphate buffer. Purified recombinant protein was desalted by a passage through PD-10 column of Sephadex G-25 M (Amersham Parmacia Biotech, Buckinghamshire, UK). The purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting using anti-His-tag monoclonal antibody (Penta · His Antibody; Qiagen).

Hemolytic activity of recombinant PLYs. Hemolytic activity of full-length and truncated PLYs was measured as follows. Sheep erythrocytes were washed three times with PBS and resuspended at 1.0% (v/v) in PBS. Fifty microliter of the suspension was added to the equal volume of serially diluted recombinant PLY and the suspension was incubated at 37°C for 30 min. Hemolysis was estimated by the amount of hemoglobin released in the supernatant as measured by absorbance at 415 nm. Hemolytic unit (HU) was defined as the amount of hemolysin required for 50% lysis of 1 ml of 0.5% sheep erythrocytes. Based on HU/mg of full-length PLY, the relative hemolytic activity of each hemolysin was calculated as follows. The relative hemolytic activity = (HU/mg of truncated PLY)/(HU/mg of full-length PLY) × 100.

Binding of recombinant PLYs to sheep erythrocytes. Binding ability of full-length and truncated PLYs to erythrocytes was determined by the method of Owen *et al.* (15) with slight modifications. Each recombinant PLY was incubated at the concentration of 5 nM with 0.5% (v/v) sheep erythrocytes in PBS at 4°C for 30 min. To determine the inhibitory effect of domain 4 molecule on the binding of full-length PLY to the erythrocytes, cells were incubated with domain 4 at 37°C for 60 min, and then with 5 nM full-length PLY at 4°C for 15 min. The cells were washed three times with ice-cold PBS and then lysed in distilled water. The membrane was harvested by centrifugation at 15,000*g*, washed twice with distilled water and treated at 95°C for 5 min in SDS–PAGE loading buffer. Proteins were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA). The membrane was treated with Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) for 60 min to block the non-specific binding, washed twice with PBS containing 0.1% Tween 20 (PBS-T), and incubated with mouse anti-His-tag monoclonal antibody in PBS-T with 10%

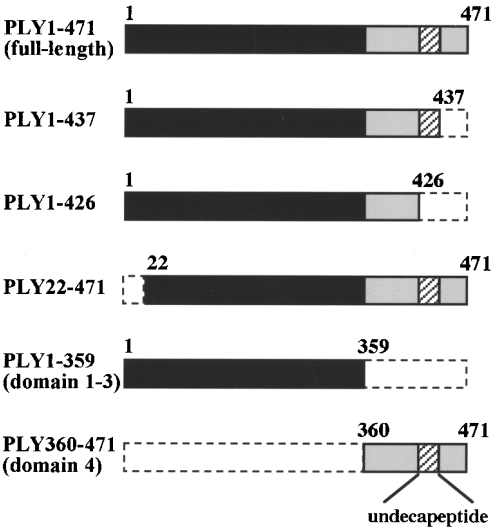

FIG. 1. The construction of full-length and five truncated PLYs. Filled boxes indicate the portion of PLY comprising domain 1 through domain 3 and gray boxes indicate the domain 4 region of PLY. Hatched boxes indicate the region of conserved undecapeptide. The area surrounded by dotted line indicates the region deleted from full-length PLY.

Block Ace for 60 min. The membrane was washed twice and incubated with horseradish-peroxidase-conjugated rabbit anti-mouse IgG antibody for 60 min. After extensive washes, the hemolysin bound to the erythrocytes was detected by ECL Western blotting detection reagent (Amersham) according to the manufacturer's instructions.

Binding of recombinant PLYs to cholesterol on TLC plate. Binding ability of full-length and truncated PLYs to cholesterol was determined by the method of Sekino-Suzuki *et al.* (17) with slight modifications. Graded amounts (0.125 μg, 0.5 μg, and 2.0 μg) of free cholesterol were spotted at a regular interval on plastic thin layer chromatography (TLC) plates (Polygram Sil G; Machery-Nagel, Düren, Germany). The plate was dried and soaked in buffer A (50 mM Tris–HCl, 200 mM NaCl, pH 7.4, 3% BSA, and 0.3% gelatin) at 4°C for 60 min and then the plate was incubated with each recombinant PLY diluted at 100 nM in buffer A for 60 min at room temperature. The plate was washed six times with buffer A and incubated with mouse anti-His-tag monoclonal antibody in buffer A for 60 min at room temperature. After being washed, the plate was incubated with horseradish-peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed Laboratories, Inc., San Francisco, CA) for 60 min and washed six times with buffer A. The spots were developed with Peroxidase Stain Kit for Immuno-blotting (Nacalai tesque, Inc., Kyoto, Japan).

Inhibition of hemolysis caused by full-length PLY. Inhibitory effect of domain 4 molecule on the hemolysis caused by full length PLY was determined as follows. Sheep erythrocytes were suspended in PBS to give 1% (v/v) and incubated with domain 4 or domain 1–3 of PLY at 37°C for 60 min. The suspension was added with the same volume of full-length PLY and incubated at 37°C for 30 min. Inhibition of hemolysis was estimated by measurement of hemoglobin release in the supernatant.

RESULTS

Construction of full-length PLY and truncated PLYs. In order to determine the structure-function study of PLY, various truncated PLYs were used in this study. Schematic illustration of full-length and truncated PLYs was depicted in Fig. 1. PLY1–471 is the full-length protein of PLY consisting of 471 amino acids. Since conserved undecapeptide which is located close to C-terminus of PLY has been shown to be important for the membrane lytic activity (18), two truncated proteins with the deletions of C-terminal amino acids were designed. One was named as PLY1–437 which was deleted for 34 amino acids and contained undecapeptide. The other, PLY1–426 was deleted for 45 amino acids of C-terminus including undecapeptide. To know the role of N-terminus in the function of PLY, a truncated protein with a deletion of N-terminal 21 amino acids (PLY22–471) was also produced. Furthermore, based on the tertiary structure of PFO (12) and comparison of the alignment of amino acid sequence between PFO and PLY (13), we designed two recombinant proteins. One comprised 1 to 359th amino acid of PLY (PLY1–359), which represented domain 1–3 of PLY and the other comprised 360 to 471st amino acid (PLY360–471), which represented the domain 4. Genes encoding full-length and these parts of PLY were amplified and inserted into the pQE-31 vector. The recombinant protein expressed in *E. coli* host was extracted and purified by Ni-agarose column chromatography. SDS–PAGE analysis showed that highly purified recombinant PLYs were obtained by the one step chromatography (Fig. 2).

Hemolytic activity of recombinant PLYs. Hemolytic activity of recombinant PLYs against sheep erythrocytes was measured. The purified full-length PLY exhibited a strong hemolytic activity (Fig. 3A). The complete hemolysis was observed by the treatment with at least 1 nM of full-length PLY and the hemolytic activity was estimated to be 3.9×10^4 HU/mg. PLY22–471 deleted for 21 amino acids at N-terminus also caused hemolysis, whereas the activity decreased to 0.06% of

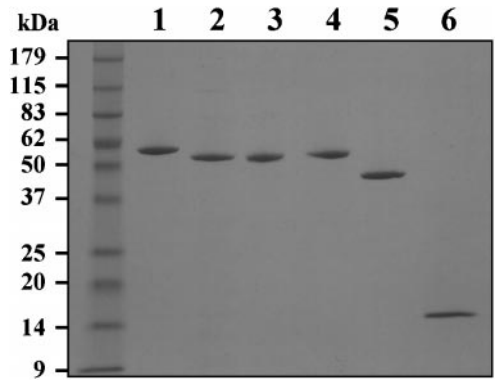

FIG. 2. SDS–PAGE analysis of the recombinant PLYs. An equal amount of the purified recombinant PLYs was loaded in 10–20% gradient SDS–PAGE gel. After electrophoresis the gel was stained with coomassie brilliant blue solution. Lane 1, PLY1–471; lane 2, PLY1–437; lane 3, PLY1–426; lane 4, PLY22–471; lane 5, PLY1–359; lane 6, PLY360–471.

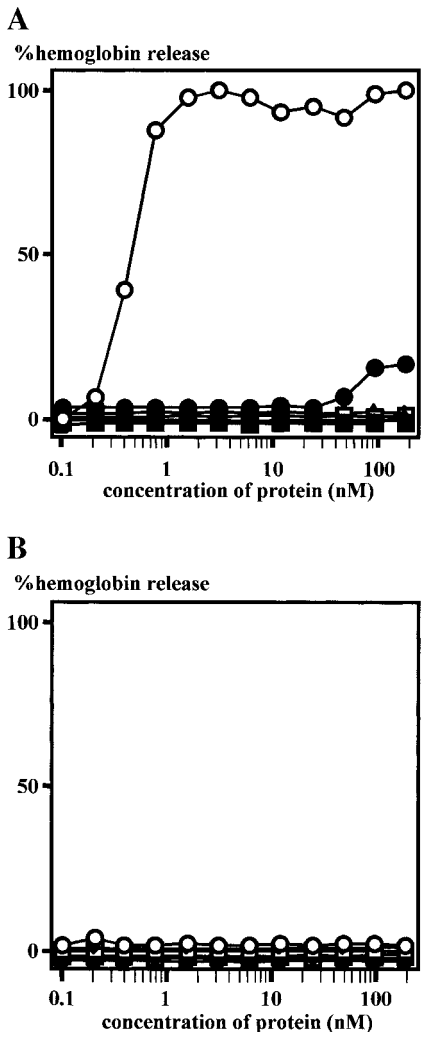

FIG. 3. Hemolytic activity of full-length and truncated PLYs. Recombinant PLYs were incubated in the absence (A) or presence of 10 μg/ml of cholesterol (B) and mixed with sheep erythrocytes. After incubation at 37°C for 30 min, hemolytic activity was measured for PLY1–471 (open circles), PLY1–437 (closed squares), PLY1–426 (open triangles), PLY22–471 (closed circles), PLY1–359 (open squares), and PLY360–471 (closed triangles).

that of full-length PLY. These hemolytic activities were completely inhibited by the treatment with 10 μg/ml of cholesterol, indicating that recombinant PLY retained the actual hemolytic activity of native PLY (Fig. 3B). In contrast, neither PLY1–437 nor PLY1–426, the truncated PLYs with C-terminal deletions, exhibited the hemolytic activity. The hemolytic activity was not observed in PLY1–359 (domain 1–3). These data indicated that domain 4, especially C-terminal portion of PLY may be critical for the lytic activity as shown previously (15). However, PLY360–471 (domain 4) did not cause hemolysis by itself. These data indicated that the whole molecule of PLY was required for the complete hemolysis and domains of PLY played a distinct role in the course of hemolysis.

Binding ability of recombinant PLYs to sheep erythrocytes and cholesterol. From the previous findings that cholesterol pretreatment abrogated the hemolysis of PLY, binding of PLY to cell membrane via cholesterol is believed to be essential for the lytic activity. Here we examined the binding ability of each recombinant PLYs to both erythrocytes and cholesterol. After incubation of erythrocytes with 5 nM recombinant PLYs that caused complete hemolysis, cells were lysed and PLYs bound to erythrocyte membrane were detected by immunoblotting (Fig. 4A). The band on lane C of each sample represented recombinant PLYs before application to the experiment. The intensity of those bands was almost at the same level. After incubation of recombinant PLYs with the erythrocytes, the band of full-length PLY was detected in the membrane fraction (lane B), indicating that full-length PLY has the binding ability to the erythrocyte membrane. Both PLY22–471 and PLY360–471 (domain 4) also bound to the membrane. However, three truncated PLYs with C-terminal deletions, PLY1–437, PLY1–426, and PLY1–359 (domain 1–3), had no binding ability to erythrocyte membrane at all, which was consistent with their loss of hemolytic activity.

We next determined the binding ability of recombinant PLYs to cholesterol spotted on TLC plate. Because the sensitivity of this assay was rather low, higher concentration (100 nM) of PLYs was applied. Full-length PLY, PLY22–471, and PLY360–471 (domain 4) all of which had the binding ability to the erythrocytes did bind to cholesterol, while the ability of two truncated PLYs (PLY22–471 and PLY360–471) appeared to be slightly weaker than that of full-length PLY (Fig. 4B). However, PLY1–437, PLY1–426, and PLY1–359 (domain 1–3) in which the C-terminal portion has been truncated could not bind to cholesterol at all. The results clearly showed that the complete C-terminal amino acid sequence was required for the binding to membrane and cholesterol, and truncation of N-terminal peptide affected the consequence of hemolysis, but not the binding ability.

Inhibitory effect of domain 4 molecule on hemolysis caused by full-length PLY. Based on the fact that domain 4 molecule did bind to erythrocyte membrane and cholesterol, we examined whether domain 4 molecule exerts an inhibitory effect on the hemolysis caused by full-length PLY. Erythrocytes were preincubated with graded doses of PLY360–471 (domain 4), and challenged with a varying concentration of full-length PLY. Complete hemolysis was caused by the treatment with over 1 nM of full-length PLY. The hemolysis was inhibited by pretreatment with PLY360–471 (domain 4) in a dose-dependent manner (Fig. 5A). Treatment with 400 nM of PLY360–471 (domain 4) was required for complete inhibition of the hemolysis. To define the role of PLY360–471 (domain 4) in the

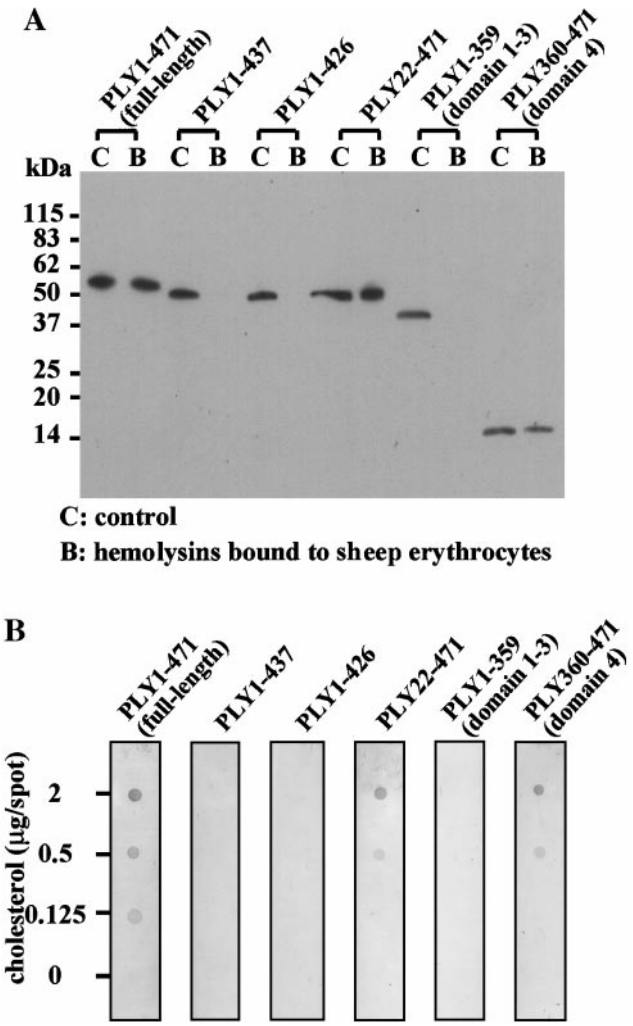

FIG. 4. Binding ability of recombinant PLYs to erythrocytes and cholesterol. (Panel A) Binding ability of PLYs to sheep erythrocytes. Full-length and truncated PLYs (5 nM) were incubated with 0.5% (v/v) sheep erythrocytes at 4°C for 30 min, washed in PBS, and lysed in water. The membranes were harvested and washed by centrifugation. Each recombinant PLY preparation before incubation with erythrocytes (C) or after incubation (B) was analyzed on SDS–PAGE. The protein was detected by immunoblotting. (Panel B) Binding of recombinant PLYs to cholesterol immobilized on TLC plates. Cholesterol was spotted on TLC plates at the indicated doses. The plate was incubated with 100 nM of each recombinant protein for 60 min and protein bound to cholesterol was detected by immunostaining.

hemolytic process, PLY1–359 (domain 1–3), the other N-terminal fragment of PLY, was applied to the same experiment. Pretreatment of PLY1–359 (domain 1–3) did not exert any inhibition even if erythrocytes were treated with 400 nM (Fig. 5B). These data indicated that domain 4, but not domain 1–3, interfered with the hemolytic process to be induced by full-length PLY.

Domain 4 molecule inhibits binding of full-length PLY to sheep erythrocytes. To confirm that the inhibition of full-length PLY-induced hemolysis by domain 4 molecule was due to the blocking of binding, we examined the effect of domain 4 molecule on the binding of full-length PLY to erythrocytes. The binding of 5 nM of full-length PLY to sheep erythrocytes in the absence of PLY360–471 (domain 4) was observed by SDS–PAGE and immunoblotting (Fig. 6). By pretreatment with increasing doses of PLY360–471 (domain 4), additional band corresponding to the PLY360–471 (domain 4) bound to the erythrocytes became detectable in an increasing manner. Interestingly, treatment with 400 nM of PLY360–471 (domain 4) caused block of the binding of full-length PLY to the erythrocytes. This dose of PLY360–471 (domain 4) was consistent with that required for the complete block of hemolysis. Consequently, this study demonstrated direct evidence

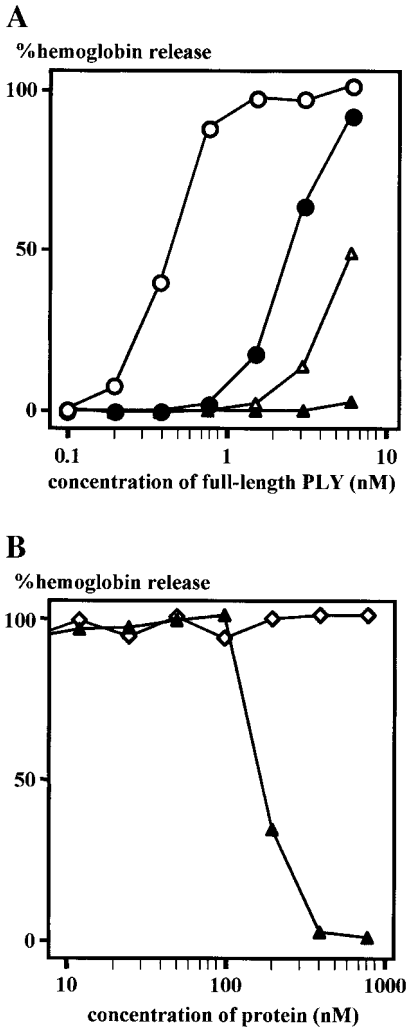

FIG. 5. Inhibitory effect of domain 4 molecule on the hemolysis induced by full-length PLY. (Panel A) Sheep erythrocytes were treated with 100 nM (closed circles), 200 nM (open triangles), and 400 nM (closed triangles) of PLY360–471 (domain 4) or PBS (open circles). (Panel B) Erythrocytes were treated with graded concentrations of domain 4 (closed triangles) and domain 1–3 (open squares). Degree of hemolysis induced by 5 nM full-length PLY was determined.

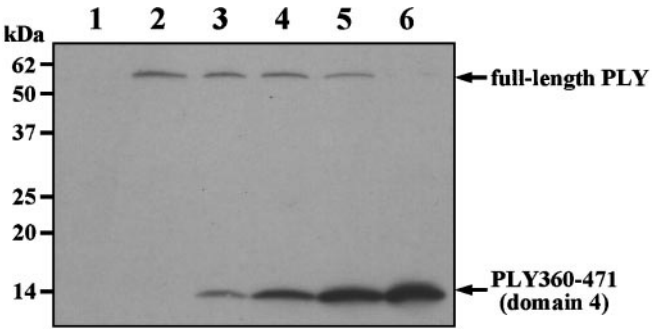

FIG. 6. Inhibitory effect of domain 4 molecule on the binding of full-length PLY to erythrocytes. Sheep erythrocytes were incubated in PBS (lane 2) or with 50 nM (lane 3), 100 nM (lane 4), 200 nM (lane 5), and 400 nM of PLY domain 4 (lane 6). The erythrocytes were then incubated with 5 nM of full-length PLY for 15 min, washed three times with PBS, and lysed in water. The membrane fraction was collected and washed twice with water. The PLY bound to membranes was detected by SDS–PAGE and immunoblotting. The sample obtained by an incubation of the erythrocytes with 5 nM full-length PLY for 0 min was loaded on lane 1.

that domain 4, but not domain 1–3, was the primary binding site to cell membrane and this initial binding was essential to complete the lytic process.

DISCUSSION

One of the unique characteristics common to all the TACYs is the abrogation of cytolytic activity by a low concentration of cholesterol (10). Recombinant full-length PLY with His-tag at the N-terminus exhibited a high level of cytolytic activity as well as binding to both erythrocyte membrane and cholesterol immobilized on TLC plate (Table 2). The binding of recombinant full-length PLY to solid phase cholesterol and the blockade of lytic activity by free cholesterol were consistent with the activity of native PLY.

Based on the homology in amino acid sequence and the structural relatedness to PFO of which the tertiary structure has been demonstrated, PLY is proposed to be consisting of four domains (13). It has been shown that several steps are included in the process of membrane lysis caused by PLY (binding to cell membrane, insertion into the membrane, and oligomerization) and that each domain plays the distinct role in this process to form transmembrane oligomer. Domain 1 is structurally associated with domain 3 and is suggested to be important for the oligomerization. Domain 2 has β-sheet structure and is a junctional region between domain 1 and domain 4. Domain 4 is distant from domain 1 and domain 3 and has been regarded as the site for binding to the membrane (14–16, 19). Morgan *et al.* reported that 37 kDa, 329 amino acid fragment obtained by proteolytic cleavage of PLY, which represented C-terminal part of PLY, was able to bind liposomes containing cholesterol (20). Iwamoto *et al.* (21) and Tweten *et al.* (22) reported that an inhibitory effect of C-terminal fragment of PFO on hemolysis by using a 196 amino acid fragment prepared by the proteolytic cleavage of full-length PFO. Though their reports implied that C-terminal part was important for the binding to cell membrane, it was not still clear whether the binding ability of the fragment was attributable only to domain 4 because the fragments included the part other than domain 4. In this study, we constructed His-tagged domain 4 molecule to determine the role for the expression of membrane binding and subsequent hemolysis.

The results of this study with recombinant proteins of domain 1–3 (PLY1–359) and domain 4 (PLY360–471) have shown that domain 4 is capable of binding to cholesterol as well as erythrocyte membrane by itself while domain 1–3 never binds to them, indicating that domain 4 is a critical part for the membrane binding.

TABLE 2

Characteristics of Full-length and Truncated PLYs

Recombinant protein	Hemolytic unit/mg[a] (relative hemolytic activity)	Sheep erythrocytes-binding ability[b]	Cholesterol-binding ability[c]
PLY1–471 (full-length)	3.9×10^4 (100)	+	+
PLY1–437	No hemolysis (0)	−	−
PLY1–426	No hemolysis (0)	−	−
PLY22–471	2.3 ± 10 (0.06)	+	+
PLY1–359 (domain 1–3)	No hemolysis (0)	−	−
PLY360–471 (domain 4)	No hemolysis (0)	+	+

[a] Sheep erythrocytes suspended to give 0.5% (v/v) were incubated with recombinant PLYs at 37°C for 30 min. Hemolytic unit was defined as the amount of protein required for 50% lysis of 1 ml of 0.5% sheep erythrocytes. The relative hemolytic activity of each recombinant protein was calculated.

[b] Each recombinant protein was incubated with 0.5% (v/v) sheep erythrocytes and then washed by centrifugation repeatedly. The protein bound to cell membrane was detected by SDS–PAGE and immunoblotting.

[c] The plates on which cholesterol was spotted were incubated with recombinant PLYs and the protein bound to cholesterol was detected by immunostaining.

Based on the crystallographic analysis, Rossjohn *et al.* proposed that cholesterol bound to a hydrophobic pocket which was formed by the long side-chains of Tyr376, Lys424, Asp426, and Gln374 of PLY (13). The fact that all these four residues are present in the domain 4 molecule (PLY360–471) is consistent with the present finding that domain 4 is the portion required for binding to membrane cholesterol.

On the other hand, it has been reported that truncated PLY deleted for 21 amino acids at the C-terminus lacked the binding ability to membrane (15). A reduced activity of binding to membrane and cholesterol has also been reported in PFO truncated for the last 21 amino acids (16). In the present study, cytolytic activity was completely lost when C-terminal region was deleted as shown by the truncated proteins, PLY1–437 and PLY1–426 (Table 2). It appears that truncation of C-terminal region of PLY may lead to the conformational change of overall domain 4 resulting in a loss of the binding ability, as suggested for PFO (16).

Pretreatment of erythrocytes with domain 4 (PLY360–471) resulted in a dose-dependent inhibition of the hemolysis caused by full-length PLY. The complete inhibition was observed when the erythrocytes were pretreated with high dose (400 nM) of domain 4 (PLY360–471) (Fig. 5). Concomitantly, the complete inhibition of the binding of full-length PLY to the membrane was also accomplished with 400 nM of domain 4 (PLY360–471) (Fig. 6). The requirement of a much higher dose of domain 4 (PLY360–471) relative to the dose of full-length PLY may be due to the presence of a large number of binding sites (membrane cholesterol). It is highly plausible that even if most of the binding sites have been occupied by domain 4 (PLY360–471) added first, the binding of any minute amount of full-length PLY to remaining binding sites may cause the membrane lysis. Alternatively, there is a possibility that the affinity to membrane cholesterol is different between full-length PLY and domain 4 (PLY360–471), since the result of spot blot (Fig. 4B) shows a difference to some extent in the ability to bind to solid phase cholesterol on TLC plate.

Domain 4 (PLY360–471) did not inhibit the binding of full-length PLY apparently at the concentrations up to 200 nM, whereas the hemolysis was inhibited at that concentration of domain 4 (PLY360–471). This finding may indicate that domain 4 which bound to cell membrane interfere with the formation of the intact pores which are usually formed by full-length PLY as suggested for the C-terminal fragment of PFO (21, 22).

PLY22–471 is one of the truncated toxins in which 21 amino acids, a part of domain 1, have been deleted from the N-terminus. The hemolytic activity of this protein was significantly reduced while the binding ability to cell membrane and cholesterol was not (Table 2 and Fig. 4). One paper has also reported that the proteolytic cleavage of 142 amino acids from the N-terminus resulted in the loss of the cytolytic activity but not the binding to liposome containing cholesterol (20). These data indicated that truncation of N-terminal peptides did not affect the binding of domain 4 to cholesterol whereas it caused a significant reduction in the hemolytic activity. Studies using X-ray crystallography or monoclonal antibody specific to domain 1 have suggested the important role of domain 1 for the oligomerization of monomeric toxin (12–14), therefore, the observed reduction in the hemolytic activity of PLY22–471 could be ascribed to the impaired oligomerization after the binding to membrane. However, it cannot be ruled out that the deletion of only 21 amino acids caused some conformational change of domain 1–3 because overall molecular structure is quite complicated due to the discontinuous folding of polypeptides.

Thus, the present study clearly demonstrated that the domain 4, which is approximately $\frac{1}{4}$ the size of full-length PLY molecule, is capable of binding to the membrane. The cholesterol binding ability and blocking effect have confirmed that domain 4 itself is essential for the binding of the entire molecule to be followed by oligomerization and pore formation. Though the cytolytic activity of PLY has been implicated in the virulence expression of *S. pneumoniae* from the study of *in vivo* virulence of mutants in *ply* encoding PLY, a precise mechanism for the involvement of PLY in the virulence remains to be determined (4). In addition to the cytolytic activity, PLY is known to exert complement activation (23) or stimulation of immune system at a sublytic concentration (24–27). The various mutant proteins constructed in our study should be useful for the further study of the biological function of PLY other than hemolytic activity.

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REFERENCES

1. Walker, J. A., Allen, R. L., Falmagne, P., Johnson, M. K., and Boulnois, G. J. (1987) Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae. Infect. Immun.* **55,** 1184–1189.
2. Alexander, J. E., Berry, A. M., Paton, J. C., Rubins, J. B., Andrew, P. W., and Mitchell, T. J. (1998) Amino acid changes affecting the activity of pneumolysin alter the behaviour of pneumococci in pneumonia. *Microb. Pathog.* **24,** 167–174.
3. Berry, A. M., Alexander, J. E., Mitchell, T. J., Andrew, P. W., Hansman, D., and Paton, J. C. (1995) Effect of defined point mutations in the pneumolysin gene on the virulence of *Streptococcus pneumoniae. Infect. Immun.* **63,** 1969–1974.

4. Berry, A. M., Ogunniyi, A. D., Miller, D. C., and Paton, J. C. (1999) Comparative virulence of *Streptococcus pneumoniae* strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. *Infect. Immun.* **67,** 981–985.
5. Berry, A. M., and Paton, J. C. (2000) Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. *Infect. Immun.* **68,** 133–140.
6. Berry, A. M., Yother, J., Briles, D. E., Hansman, D., and Paton, J. C. (1989) Reduced virulence of a defined pneumoylsin-negative mutant of *Streptococcus pneumoniae. Infect. Immun.* **57,** 2037–2042.
7. AlonsoDeVelasco, E., Verheul, A. F. M., Verhoef, J., and Snippe, H. (1995) *Streptococcus pneumoniae:* Virulence factors, pathogenesis, and vaccines. *Microbiol. Rev.* **59,** 591–603.
8. Mitchell, T. J. (1999) Pneumolysin: Structure, function, and role in disease. *in* The Comprehensive Sourcebook of Bacterial Protein Toxins, 2nd ed. (Alouf, J. E., and Freer, J. H., Eds.), pp. 476–495, Academic Press, London.
9. Paton, J. C. (1996) The contribution of pneumolysin to the pathogenicity of *Streptococcus pneumoniae. Trends Microbiol.* **4,** 103–106.
10. Alouf, J. E. (1999) Introduction to the family of the structurally related cholesterol-binding cytolysins ('sulfhydryl-activated' toxins). *in* The Comprehensive Sourcebook of Bacterial Protein Toxins, 2nd ed. (Alouf, J. E., and Freer, J. H., Eds.), pp. 443–456, Academic Press, London.
11. Billington, S. J., Jost, B. H., and Songer, J. G. (2000) Thiol-activated cytolysins: Structure, function, and role in pathogenesis. *FEMS Microbiol. Lett.* **182,** 197–205.
12. Rossjohn, J., Feil, S. C., McKinstry, W. J., Tweten, R. K., and Parker, M. W. (1997) Structure of a cholesterol-binding, thiol-activated cytolysin, and a model of its membrane form. *Cell* **89,** 685–692.
13. Rossjohn, J., Gilbert, R. J. C., Crane, D., Morgan, P. J., Mitchell, T. J., Rowe, A. J., Andrew, P. W., Paton, J. C., Tweten, R. K., and Parker, M. W. (1998) The molecular mechanism of pneumolysin, a virulence factor from *Streptococcus pneumoniae. J. Mol. Biol.* **284,** 449–461.
14. de los Toyos, J. R., Mendez, F. J., Aparicio, J. F., Vazquez, F., del Mar Garcia Suarez, M., Fleites, A., Hardisson, C., Morgan, P. J., Andrew, P. W., and Mitchell, T. J. (1996) Functional analysis of pneumolysin by use of monoclonal antibodies. *Infect. Immun.* **64,** 480–484.
15. Owen, R. H. G., Boulnois, G. J., Andrew, P. W., and Mitchell, T. J. (1994) A role in cell-binding for the C-terminus of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae. FEMS Microbiol. Lett.* **121,** 217–222.
16. Shimada, Y., Nakamura, M., Naito, Y., Nomura, K., and Ohno-Iwashita, Y. (1999) C-terminal amino acid residues are required for the folding and cholesterol binding property of perfringolysin O, a pore-forming cytolysin. *J. Biol. Chem.* **274,** 18536–18542.
17. Sekino-Suzuki, N., Nakamura, M., Mitsui, K., and Ohno-Iwashita, Y. (1996) Contribution of individual tryptophan residues to the structure and activity of θ-toxin (perfringolysin O), a cholesterol-binding cytolysin. *Eur. J. Biochem.* **241,** 941–947.
18. Korchev, Y. E., Bashford, C. L., Pederzolli, C., Pasternak, C. A., Morgan, P. J., Andrew, P. W., and Mitchell, T. J. (1998) A conserved tryptophan in pneumolysin is a determinant of the characteristics of channels formed by pneumolysin in cells and planar lipid bilayers. *Biochem. J.* **329,** 571–577.
19. Jacobs, T., Cima-Cabal, M. D., Darji, A., Mendez, F. J., Vazquez, F., Jacobs, A. A. C., Shimada, Y., Ohno-Iwashita, Y., Weiss, S., and de los Toyos, J. R. (1999) The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding. *FEBS Lett.* **459,** 463–466.
20. Morgan, P. J., Harrison, G., Freestone, P. P. E., Crane, D., Rowe, A. J., Mitchell, T. J., Andrew, P. W., and Gilbert, R. J. C. (1997) Structural and functional characterisation of two proteolytic fragments of the bacterial protein toxin, pneumolysin. *FEBS Lett.* **412,** 563–567.
21. Iwamoto, M., Ohno-Iwashita, Y., and Ando, S. (1990) Effect of isolated C-terminal fragment of θ-toxin (perfringolysin O) on toxin assembly and membrane lysis. *Eur. J. Biochem.* **194,** 25–31.
22. Tweten, R. K., Harris, R. W., and Sims, P. J. (1991) Isolation of a tryptic fragment from *Clostridium perfringens* θ-toxin that contains sites for binding and self-aggregation. *J. Biol. Chem.* **266,** 12449–12454.
23. Mitchell, T. J., Andrew, P. W., Saunders, F. K., Smith, A. N., and Boulnois, G. J. (1991) Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. *Mol. Microbiol.* **5,** 1883–1888.
24. Ferrante, A., Rowan-Kelly, B., and Paton, J. C. (1984) Inhibition of *in vitro* human lymphocyte response by the pneumococcal toxin pneumolysin. *Infect. Immun.* **46,** 585–589.
25. Houldsworth, S., Andrew, P. W., and Mitchell, T. J. (1994) Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1β by human mononuclear phagocytes. *Infect. Immun.* **62,** 1501–1503.
26. Nandoskar, M., Ferrante, A., Bates, E. J., Hurst, N., and Paton, J. C. (1986) Inhibition of human monocyte respiratory burst, degranulation, phospholipid methylation, and bactericidal activity by pneumolysin. *Immunology* **59,** 515–520.
27. Paton, J. C., and Ferrante, A. (1983) Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. *Infect. Immun.* **41,** 1212–1216.