Abstract
A mixture of sphingomyelin (SM) and cholesterol (Chol) exhibits a characteristic lipid raft domain of the cell membranes that provides a platform to which various signal molecules as well as virus and bacterial proteins are recruited. Several proteins capable of specifically binding either SM or Chol have been reported. However, proteins that selectively bind to SM/Chol mixtures are less well characterized. In our screening for proteins specifically binding to SM/Chol liposomes, we identified a novel ortholog of Pleurotus ostreatus, pleurotolysin (PlyA), from the extract of edible mushroom Pleurotus eryngii, named PlyA2. Enhanced green fluorescent protein (EGFP)-conjugated PlyA2 bound to SM/Chol but not to phosphatidylcholine/Chol liposomes. Cell surface labeling of PlyA2-EGFP was abolished after sphingomyelinase as well as methyl-
forcyclodextrin treatment, removing SM and Chol, respectively, indicating that PlyA2-EGFP specifically binds cell surface SM/Chol rafts. Tryptophan to alanine point mutation of PlyA2 revealed the importance of C-terminal tryptophan residues for SM/Chol binding. Our results indicate that PlyA2-EGFP is a novel protein probe to label SM/Chol lipid domains both in cell and model membranes.—Bhat, H. B., T. Kishimoto, M. Abe, A. Makino, T. Inaba, M. Murate, N. Dohmae, A. Kurahashi, K. Nishibori, F. Fujimori, P. Greimel, R. Ishitsuka, and T. Kobayashi. Binding of a pleurotolysin ortholog from Pleurotus eryngii to sphingomyelin and cholesterol-rich membrane domains. J. Lipid Res. 2013. 54: 2933–2943.

Supplementary key words lipid binding protein • lipid raft • membrane lipids • sphingolipid • pore forming toxins
domains (15). Several nontoxic mutants of these toxins that specifically bind either SM (16–18) or Chol (19) have been employed to study the distribution, dynamics, and function of SM/Chol domains. Because both sphingolipids and Chol are required to assemble lipid rafts (6), a protein that binds to the SM/Chol complex would be a very useful tool to study lipid microdomain organization in cell membranes.

Two mushroom proteins belonging to the aegerolysin protein family (20) are reported to bind SM/Chol membranes, namely ostreolysin (Oly) (21) and pleurotolyson (PlyA) (22). Oly is an ~15 kDa acidic protein from the edible mushroom Pleurotus ostreatus (oyster mushroom) (21). The binding of Oly to SM/Chol and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)/Chol membranes has been reported (23). Electron paramagnetic resonance and Fourier-transformed infrared spectroscopy revealed the importance of highly ordered sterol-enriched membrane domains for the binding of Oly (24). When the Chol level is above 30 mol%, Oly exhibits cytotoxic effects by permeabilizing membranes composed of SM (23). PlyA, from P. ostreatus, binds to SM membranes containing ≥30 mol% Chol (22). PlyA requires formation of a complex with PlyB, an ~59 kDa protein, to exhibit cytotoxic effects (22). However, detailed characterization of PlyA has not yet been performed. Recently, erylysin (Ery), an ortholog of Oly, was isolated from Pleurotus eryngii (25). Similar to Ply, Ery is a two-component hemolysin composed of EryA and EryB. To date, the binding specificity of Ery has not been studied.

In this study, we identified a novel ortholog of PlyA, termed PlyA2, from the extracts of P. eryngii, capable of binding specifically to SM/Chol liposomes. Subsequently, we constructed an enhanced green fluorescent protein (EGFP)-PlyA2 chimera and characterized its lipid specificity in a model and cellular membranes. Our results demonstrate that PlyA2-EGFP is a novel probe to study SM/Chol-rich lipid rafts.

MATERIALS AND METHODS

Lipids

The following were purchased from Avanti Polar Lipids (Alabaster, AL): SM (brain, porcine); Lα-phosphatidylcholine (PC) (egg, chicken); 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); DPPC; 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[7-nitro-2-1,3-benzoxadiazol-4-yl] (N-NBD-PE); phosphocholine (POPC); DPPC; 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (POPE); Lα-phosphatidyldserine (PS) (brain, porcine); lactosyl ceramide (LacCer) (Galβ1-4Glcβ1-1Cer); and galactosyl ceramide (GalCer) (Galβ1-1Cer). From Sigma (St. Louis, MO): Chol; ergosterol; β-sitosterol; GM1 [Galβ1-3Galβ1NAcβ1-1(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer]; brain, bovine; sphingosine (SPH); and D-ribophytosphingosine (Phyto) (yeast). From Wako Pure Chemical Industries (Osaka, Japan): GM2 [Galβ1-4Glcβ1-1Cer]; brain, bovine. From Matreya (Pleasant Gap, PA): GM3 (Neu5Acα2-3Galβ1-4Glcβ1-1Cer); buttermilk, bovine). From Research Biochemicals International (Natick, MA): GD1a [Neu5Acα2-3Galβ1-3Galβ1NAcβ1-1(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer]. From Enzo Life Sciences (Farmingdale, NY): sphingosylphosphorylcholine (SPC).

Other materials

Sphingomyelinase (SMase) from Staphylococcus aureus and methyl-β-cyclodextrin (MβCD) were from Sigma. Anti-bis(monoacyl glycerol) phosphate/lysobisphosphatidic acid antibody was prepared as described (26). Anti-EEA1, anti-GM130 antibodies were from BD Bioscience (Franklin Lakes, NJ). Anti-CD59 was from Chemicon International, anti-GM3 from Seikagaku Corporation (Tokyo, Japan), and transferrin-Alexa Fluor 546 conjugate was from Molecular Probes. Two peptides of PlyA2 consisting of residues 45–60 and 99–115 were synthesized, and rabbit polyclonal antibodies against the cocktail of these peptides were prepared by Medical and Biological Laboratories Co., Ltd. (Japan).

Preparation of P. eryngii extract

P. eryngii (50 g) was homogenized in 50 ml of 50 mM Tris-HCl buffer (pH 8.5) containing protease inhibitor. The homogenate was centrifuged at 11,600 g for 30 min at 4°C. The supernatant was centrifuged further in a Beckmann Optima ultracentrifuge at 58,863 g for 30 min at 4°C. The final supernatant was aliquoted and kept at −80°C.

Liposome sedimentation assay

Multilamellar vesicles (MLVs) were prepared by hydrating lipid film with PBS (pH 7.5) and vortex mixing. P. eryngii extract or purified-proteins were incubated with MLVs in PBS for 30 min at 37°C. The mixture was centrifuged at 21,600 g for 10 min at room temperature. The liposomes were washed twice with PBS and were subjected to SDS-PAGE followed by Coomassie brilliant blue (CBB) staining.

 Determination of amino acid sequence of PlyA2

After blotting on polyvinylidene difluoride membranes, protein bands were stained with CBB. The protein bands were excised and subjected to Edman degradation using a Procise cLC or HT protein sequencing system (Applied Biosystems).

Molecular cloning of the cDNA encoding PlyA2

A coding sequence for PlyA2 was obtained from P. eryngii cDNA by PCR amplification.

Construction, expression, and purification of recombinant proteins

 Fragments of PlyA2 were cloned into the expression vector pET28 containing Hexa-histidine (His) fragment (Merck KGaA, Germany). The coding sequences of PlyA2 tryptophan mutants were optimized, and the DNA fragments were synthesized by GenScript through OptimumGene™ codon optimization analysis. The resulting fragments were cloned into pET-28 vectors. To construct the vectors for purification of EGFP-fused proteins, gene encoding EGFP was first cloned into pET-28 vectors, and then PlyA2 was recloned into the N terminal or C terminal of EGFP in pET-28-EGFP vectors, respectively. The resulting plasmids were transformed into Escherichia coli strain BL-21(DE3). Bacterial culture was grown at 30°C in lysozyme broth medium with 100 μg/ml kanamycin until an optical density (OD 600 nm) of 0.5 was reached. Expression of EGFP-protein was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside overnight and the cells were harvested by centrifugation at 5,000 g for 10 min at 4°C. Cell lysates were prepared by resuspending the pellet in binding buffer consisting of 20 mM sodium phosphate buffer (pH 7.4) containing 500 mM NaCl, 20 mM imidazole, and protease inhibitor cocktail set I (Galbiochem), followed by

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sonication (Misonix). Insoluble protein and cell debris were removed by centrifugation at 9,000 g at 4°C for 20 min. For purification of EGFP-proteins, a Ni-NTA (Qiagen) column was used according to the supplier’s protocol. The resulting fractions were concentrated, followed by exchanging buffer (PBS, pH 7.5) using an Amicon Ultra-15 Ultracel®-30K centrifugal unit (Millipore). The protein concentration was determined using the Bradford assay.

**Liposome flotation assay**

MLVs containing 0.05% N-NBD-PE were incubated with proteins in PBS at room temperature for 30 min. One-half milliliter of this suspension was mixed with 1 ml of 2.1 M sucrose in PBS, loaded at the bottom of an ultracentrifuge tube (MLS50; Beckman Coulter), and overlaid with 1.5 ml of 1.25 M sucrose and 2.25 ml of 0.8 M sucrose. The gradient was centrifuged for 1 h at 35,608 g using a Beckman Coulter Optima™ ultracentrifuge. Top fraction (200 μl) was collected and subjected to SDS-PAGE and Western blotting. Fluorescence intensity of N-NBD-PE was measured (λex, 483 nm; λem, 533 nm) to monitor the position and concentration of liposomes in the gradient.

**Cell culture**

HeLa cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (18).

**Cell surface labeling after SMase or MβCD treatment**

HeLa cells were treated with 1.25 IU/ml SMase or 5 mM MβCD in DMEM for 30 min at 37°C. Cells were then fixed with 4% paraformaldehyde, treated with 50 mM NH₄Cl and 0.1% BSA, and labeled with 1 μg/ml PlyA2-EGFP.

**Effect of preincubation with liposomes on PlyA2-EGFP labeling**

PlyA2-EGFP was preincubated with 1 mM (total lipid) brain SM/Chol (1:1) and POPC/Chol (1:1) MLVs in PBS for 30 min at 37°C. Then, the mixture was incubated with fixed HeLa cells for 30 min at room temperature. After washing, the specimen was observed under a confocal microscope.

**Colocalization of PlyA2-EGFP and lipid raft markers**

HeLa cells were fixed with 4% paraformaldehyde, blocked with 0.1% BSA, and doubly labeled with 1 μg/ml PlyA2-EGFP and anti-CD59 or anti-GM3. In the case of transferrin receptor staining, prior to fixation, the cells were washed with ice-cold DMEM and incubated with transferrin-Alexa Fluor 546 conjugate (250 μg/ml) on ice for 30 min. Cells were then labeled with 1 μg/ml PlyA2-EGFP.

**Confocal microscopy**

Confocal images were obtained on a Zeiss 510 or Zeiss 700 confocal microscope equipped with a C-Apochromat 63XW Korr (1.2 NA) objective.

**Intracellular labeling of HeLa cells with PlyA2-EGFP and organelle markers**

HeLa cells were fixed with 4% paraformaldehyde, permeabilized by freezing in liquid nitrogen then thawing, blocked with 0.1% BSA, and labeled with 1 μg/ml PlyA2-EGFP and various antibodies.

**Measurement of hemolysis**

The hemolytic activity of PlyA2-EGFP was measured as described (27). Sheep erythrocyte suspensions (1 ml, containing 3 × 10⁷ cells) were incubated with various concentrations of PlyA2-EGFP at 37°C for 30 min and then centrifuged at 500 g for 5 min to precipitate the erythrocytes. Aliquots of the supernatants were taken, and the optical densities at 415 nm were measured to determine the percentage of hemoglobin released from the erythrocytes. Total hemoglobin contents were determined by measuring hemoglobin released after freezing and thawing of the erythrocyte preparations.

**Expression of PlyA2 in cytoplasm**

The PlyA2 fragment was cloned into pAcGF-Hyg-N1 vector (Clontech) used for expression. Transient transfection was performed in DMEM containing FBS by using Lipofectamine LTX reagent (Invitrogen) according to the instructions provided by manufacturer.

**Homology modeling**

The amino acid sequence was submitted to the homology modeling and threading server I-TASSER with or without specified restraints (28–30). The protonation state of the resulting top-rated model was predicted utilizing the H++ server (31). Subsequently, the models were parameterized, solvated, and equilibrated for 10 ns at 310 K in the presence of 0.15 mM KCl utilizing the NAMD software package (32) and the CHARMM 36 force field (33). The bonded interactions were calculated every time-step, full range electrostatic interactions every other time-step, short-range nonbonded interaction cutoff was set to 12 Å and a smoothing function employed at 10 Å, and pair-lists were recalculated every 10 time-steps with a pair-list distance of 14 Å. The temperature and pressure (1 bar) were maintained utilizing Langevin dynamics and Langevin piston method, respectively. After reevaluation and adjustment of the protonation state by H++ server, the models were equilibrated for an additional 10 ns and subjected to a 10 ns production run. Molecular structures were visualized by visual molecular dynamics (34).

**RESULTS**

Two mushroom-derived aegerolysins, Ply and Oly, have been reported to bind SM/Chol membranes (22, 23). In order to find a new SM/Chol binding protein, we screened for proteins that bind liposomes composed of SM and Chol (1:1) using extracts of *P. eryngii*. The mushroom extract was incubated with SM/Chol (1:1) liposomes for 30 min at 37°C, centrifuged, and the pellet and supernatant were subjected to SDS-PAGE (Fig. 1A). The liposome-bound pellet gave a low molecular weight single band (Fig. 1A, lane 4, arrow). The bound protein was extracted from the gel and subjected to amino acid sequenc-
membranes in the liquid-ordered phase. Additionally, PlyA2-EGFP did not exhibit any binding affinity towards mixtures of Chol and other phospholipids such as PS, or glycosphingolipids such as GM1, GM2, GM3, GD1a, Lac-Cer, and GalCer, or degradation products of sphingolipids and intermediates of sphingolipid biosynthesis such as SPC, SPH, and Phyto (Fig. 2B).

The influence of sterol structure on PlyA2-EGFP binding specificity was characterized by incubating the protein with equimolar mixtures of SM and various sterols (Fig. 3). PlyA2-EGFP exhibited a similarly high binding affinity to SM/sterol mixtures containing lanosterol, 6-ketocholestanol, and epicholesterol completely abolished the binding of PlyA2-EGFP. This indicates the importance of the stereochemical configuration of the 3-hydroxyl group on SM/sterol domain organization and PlyA2-EGFP binding specificity, while modifications of the sterol tail exhibited only little influence. In contrast, the presence of bulky groups on the A- and B-ring of the sterol backbone completely abolished PlyA2-EGFP binding.

While our in vitro liposome results indicate that PlyA2-EGFP specifically binds to SM/Chol membranes, we cannot exclude the possibility that the protein also binds other cellular components, such as membrane proteins or glycoconjugates. Therefore, we examined the effect of PlyA2-EGFP preincubation with liposomes on cell surface staining.

(Oly) (80% identity). EryA and Oly share a high degree of sequence identity (98%) (25).

Next, we prepared recombinant PlyA2 which was conjugated to EGFP on the N terminus (EGFP-PlyA2) as well as the C terminus (PlyA2-EGFP). The new constructs were mixed with SM/Chol (1:1), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/Chol (1:1), and SM/POPC (1:1) liposomes to characterize their binding specificity. In all cases EGFP-PlyA2 was exclusively recovered in the supernatant (Fig. 2A). This strongly suggests that EGFP fusion to the N terminus of PlyA2 completely abolished the lipid binding capability. In contrast, PlyA2-EGFP was detected in the pellet fraction upon incubation with SM/Chol (1:1) liposomes (Fig. 2B). A considerable amount of the protein still remained in the supernatant, suggesting that the protein is weakly associated to the liposomes. Nevertheless, complete recovery of PlyA2-EGFP in the supernatant of POPC/Chol and SM/POPC liposomes strongly indicates that the presence of both SM and Chol are required for binding.

The SM and Chol mixture forms a characteristic liquid-ordered membrane (37). A liquid-ordered phase is also observed in the mixture of Chol and PC with saturated fatty acid (37). Thus, in Fig. 2B, we examined the binding of PlyA2-EGFP to the mixture of Chol and DPPC. The detected negligible binding to DPPC/Chol liposomes further supports the notion that PlyA2-EGFP specifically recognizes SM/Chol assemblies and does not simply associate with membranes in the liquid-ordered phase. Additionally, PlyA2-EGFP did not exhibit any binding affinity towards mixtures of Chol and other phospholipids such as PS, or glycosphingolipids such as GM1, GM2, GM3, GD1a, Lac-Cer, and GalCer, or degradation products of sphingolipids and intermediates of sphingolipid biosynthesis such as SPC, SPH, and Phyto (Fig. 2B).

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Protein that binds sphingomyelin/cholesterol domains

Preincubation with SM/Chol liposomes abolished cell labeling, whereas in the case of POPC/Chol liposome preincubation, cell labeling was not affected. Additionally, treatment of cells with SMase or M9252CD, to remove cell surface SM or Chol, respectively (18, 38), abolished PlyA2-EGFP binding (Fig. 4). This strongly indicates that PlyA2-EGFP selectively binds to a SM/Chol complex on the cell surface, rendering it a suitable probe to label SM/Chol-rich membrane domains.

We then compared the cell surface distribution of PlyA2-EGFP with established lipid raft markers (Fig. 5). Figure 5 indicates that there was partial colocalization between PlyA2-EGFP and raft-associated glycosylphosphatidylinositol-anchored protein CD59 (arrows) (39, 40). Based on dot-by-dot comparison, about 45% of CD59-positive dots were colocalized (images are shown in Fig. 5). In contrast to the protein raft marker, ganglioside GM3 was not significantly colocalized with SM/Chol domains labeled by PlyA2-EGFP (Fig. 5). GM3 antibody stained about 30% of our HeLa cell population. The different localization of raft lipids is consistent with our previous observation that lysenin did not colocalize with GM1-binding cholera toxin in Jurkat cells (16).

In order to visualize the intracellular distribution of SM/Chol (Fig. 6) domains, HeLa cells were fixed and permeabilized by freezing and thawing to avoid lipid loss prior to treatment with PlyA2-EGFP. Double labeling of PlyA2-EGFP with an antibody specific to bis(monoacylglycero)phosphate/lysobisphosphatidic acid, the late endosome marker (26, 41), indicates the enrichment of SM/Chol domains in the internal membranes of late endosomes. On the contrary, no significant colocalization of PlyA2-EGFP with EEA1 and GM130, organelle markers for early endosomes and cis-Golgi, respectively, was observed.

No hemolytic activity of PlyA2-EGFP against sheep red blood cells was detected up to a concentration of 10 µg protein/ml (Fig. 7A), akin to its homolog PlyA. Staining of the plasma membrane of living HeLa cells with PlyA2-EGFP revealed no cytotoxic effect (Fig. 7B). Similarly, expression of PlyA2-AcGFP in HeLa cells did not exhibit any obvious cytotoxic effect (Fig. 7C). Interestingly, PlyA2-AcGFP did not label the cytoplasmic leaflet of the plasma membrane, suggesting an asymmetric distribution of suitable SM/Chol-rich domains between the leaflets of the plasma membrane.

Previously we demonstrated the importance of conserved tryptophan residues for the SM binding specificity of lysenin family proteins (42). PlyA2 contains six tryptophan residues at positions 6, 28, 92, 96, 103, and 112 (Fig. 1B), and these tryptophan residues are well conserved among PlyA, PlyA2, Oly, and EryA. Mutations were introduced in PlyA2 by replacing individual tryptophan with alanine.

(1:1) liposomes; Phyto/Chol, D-ribo-phytosphingosine/cholesterol (1:1) liposomes.
The resulting mutants were designated as W6A, W28A, W92A, W96A, W103A, and W112A. These proteins were expressed in E. coli as C-terminus His-tag conjugates. Two proteins, W92A and W103A, could not be expressed in E. coli. The remaining mutants were subjected to flotation assays to evaluate their binding affinity to SM/Chol liposomes (Fig. 8A). The binding of the W28A and W112A mutants to SM/Chol liposomes was considerably reduced and in the case of the W96A mutant, completely abolished. In contrast, the W6A mutant retained its binding affinity and specificity. Similarly, only the W6A mutant exhibited a comparable staining pattern to that of wild-type PlyA2 in fixed HeLa cells, while the other clones failed to stain the cells (Fig. 8B).

Due to the absence of sufficiently homologous protein templates, the PlyA2 model was generated via an iterative process based on protein threading, amino acid charge prediction, and molecular dynamic simulation as described in Materials and Methods. The first model (model 1) was generated by unrestrained submission of the PlyA2 amino acid sequence to I-TASSER (supplementary Figs. I, II) and was based on chain A of 3VSF (exo-β-1,3-galactanase from Clostridium thermocellum). Model 1 achieved a confidence score (C-score) of −2.05 on a scale of −5 to 2 of low to high confidence respectively, where a C-score of about −1.5 and higher is considered to indicate a correct fold of the model (43).

Interestingly, the second ranked template structure, 3LIM_A (FraC from sea anemone Actinia fragacea), is a member of the actinoporin family of pore forming toxins. Actinoporins, such as equinatoxin II (Eqt2) [Protein Data Bank (PDB) code 1IAZ, 1O72] and sticholysin II (Stl2) (PDB code 1GWZ), are known to bind to SM-rich membranes.
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of the folding state as well as the binding mechanism be-

tween members of the aegerolysin and actinoporin fami-

lies has been proposed, based on circular dichroism (CD)

and fluorescence spectra (45). Resubmission of PlyA2 se-

quence aligned to FraC produced the second PlyA2 model

(44). Sequence alignment revealed an ~15% sequence

identity and up to 65% sequence similarity between PlyA2

and prominent members of the actinoporin family (Fig. 9).
The extended gap region corresponds to the complete α2-helix of the actinoporin templates. Still, a high similarity

of the folding state as well as the binding mechanism be-

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Fig. 5. Colocalization of PlyA2-EGFP with lipid raft markers. Cells were fixed and doubly labeled with PlyA2-EGFP and anti-CD59 or anti-GM3. Alternatively, cells were labeled with transferrin followed by fixation and PlyA2-EGFP labeling. Scale bar, 10 μm.

Fig. 6. Intracellular distribution of PlyA2-EGFP labeling in HeLa cells. Cells were fixed and permeabi-

lized by freezing and thawing. Cells were then doubly labeled with PlyA2-EGFP and various organelle mark-

ers. Scale bar, 20 μm.
During the initial equilibration period of the molecular dynamic simulation, only the low quality region after the extended gap of model 2 was significantly altered. Residue 112 moved nearly 2 nm from its initial position, until the favorable aryl/aryl interaction with F91 stabilized its position for the remaining simulation. The location of the relatively close C terminus was not altered due to a stabilizing salt bridge between K25 and D132. In both models, W92 and W103 exhibit an edge-to-face orientation, indicating their importance to stabilize aryl/aryl cross-strand interactions (46). This is in good agreement with the unsuccessful expression of W92A and W103A, possibly due to a failure of the mutants to acquire the correct folding state. W96 is located at the loop between two β-strands, namely β5-β6 (model 1) and β4-β5 (model 2), resembling W112 in FraC. Mutation studies of W112 of FraC revealed its importance during initial membrane binding due to primarily hydrophobic interaction (47). Mutation of W112 of FraC to alanine resulted in a complete loss of binding, similar to our W96A mutant. Abolishment of stabilizing W28-F90 aryl/aryl cross-strand interactions could be the cause for reduced binding affinity of the W28A mutant, according to model 2. In contrast, in model 1, the reduced binding affinity after W28 mutation is difficult to rationalize. Superimposition of the β-sheet core of both models with Stl2 (PDB code 1O72) in the presence of choline phosphate (CP) revealed the close proximity of W112 in model 2 to the superimposed CP binding site of Stl2. In contrast, loss of binding of the W112A mutant cannot be explained in terms of model 1. In summary, model 2 appears to be in better agreement with experimental results compared with model 1.

(model 2) with a C-score of −1.76 and a TM-score of 0.5 (Fig. 10). In line with CD spectral data of Oly (45), both models exhibit a high degree of β-structure. In contrast, only model 2 matched the α-helix content of the CD data, despite omission of the α2-helix of the template structure.

![Fig. 7](image_url)

**Fig. 7.** PlyA2-EGFP labels living cells. A: Hemolysis of sheep red blood cells was measured as described in Materials and Methods. B: Living HeLa cells were labeled with exogenously added PlyA2-EGFP. C: PlyA2-AcGFP was expressed in the cytoplasm of HeLa cells as described in Materials and Methods. Scale bar, 20 μm.

![Fig. 8](image_url)

**Fig. 8.** Tryptophan mutants fail to bind SM/Chol-rich membranes. A: Recombinant PlyA2 or tryptophan mutants were incubated with SM/Chol or PC/Chol liposomes for 30 min at 37°C. Proteins bound to liposomes were recovered by a sucrose gradient centrifugation and detected by Western blotting using anti-His antibodies. B: Fixed HeLa cells were incubated with PlyA2 or tryptophan mutants, followed by treatment with anti-His antibodies and the secondary antibodies conjugated with Alexa 488. Scale bar, 20 μm.
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Fig. 9. Alignment of amino acid sequence of PlyA2 with prominent members of the actinoporin family. Protein names are given on the left. Sequence numbers are indicated above and refer to Eqt2. Choline binding site residues of the actinoporin family are indicated by arrows. Single letters, amino acids; –, alignment gap; black background, identical; gray background, conserved substitution.

Fig. 10. Cartoon representation of model 2. Position of displayed residues as indicated. N, N terminus; C, C terminus.
cytoplasmic leaflet of the Golgi apparatus (17), indicating the presence of different pools of SM in cell membranes (53). Utilizing domain 4, a Chol-binding probe, and photoactivation localization microscopy, we demonstrated that SM-rich and Chol-rich domains do not always give a similar cell surface labeling pattern (38). Additionally, we recently identified a novel SM/Chol binding protein from the mushroom Grifola frondosa (Makino et al., in preparation). This highlights the importance of multiple probes capable of recognizing different assemblies of SM and Chol to understand the detailed organization of cellular membranes.

In conclusion, PlyA2-EGFP is a nontoxic lipid binding protein capable of selectively associating with SM/Chol-rich membrane domains. Cell surface labeling was achieved by exogenous addition, while expression in the cytoplasm did not result in labeling of internal membranes. Partial colocalization of PlyA2-EGFP and raft-resident protein indicates that PlyA2-EGFP labels a sub-population of lipid rafts. Consequently, PlyA2-EGFP is a complementary addition to the lipid probe toolkit to study lipid distribution and dynamics in situ.

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