Specificity of *Loxosceles* α clade phospholipase D enzymes for choline-containing lipids: role of a conserved aromatic cage

Short title: Role of an aromatic cage for PLD lipid specificity

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ABSTRACT

Spider venom GDPD-like phospholipases D (*SicTox*) have been identified to be one of the major toxins in recluse spider venom. They are divided into two major clades: the α clade and the β clade. Most α clade toxins present high activity against lipids with choline head groups such as sphingomyelin, while activities in β clade toxins vary and include preference for substrates containing ethanolamine headgroups (*Sicarius terrosus*, St β IB1). A structural comparison of available PLDs structures reveals a conserved aromatic cage in the α clade. To test the potential influence of the aromatic cage on membrane-lipid specificity we performed molecular-dynamics (MD) simulations of the binding of several PLDs onto lipid bilayers containing choline headgroups; two *SicTox* from the α clade, *Loxosceles intermedia* α IA1 (Li α IA) and *Loxosceles laeta* α III1 (Li α III1), and one from the β clade, St β IB1. The simulation results reveal that the aromatic cage captures a choline-headgroup and suggest that the cage plays a major role in lipid specificity. We also simulated an engineered St β IB1,

where we introduced the aromatic cage, and this led to binding with choline-containing lipids. Moreover, a multiple sequence alignment revealed the conservation of the aromatic cage among the α clade PLDs. Here, we confirmed the membrane binding site of α and β clade PLDs on choline and ethanolamine-containing bilayers, respectively. Furthermore, our results suggest a major role in choline lipid recognition of the aromatic cage of the α clade PLDs. The MD simulation results are supported by *in vitro* liposome binding assay experiments.

KEYWORDS

Sphingomyelinase D, phospholipase D, *SicTox*, Loxoscelism, peripheral membrane enzymes, toxin, molecular dynamics simulation, aromatic cage

INTRODUCTION

Sphingomyelinase D/phospholipase D (SMaseD/PLD) enzymes are one of the major toxins in venom of spiders in the family Sicariidae (1–3) including *Loxosceles*, the recluse spiders. Sicariid spiders also include six-eyed sand spiders in the genera *Sicarius* and *Hexophthalma* (4). The gene family comprising the sicariid SMaseD/PLD toxins is called *SicTox* reflecting evidence that this venom toxin is a synapomorphy for the family Sicariidae and is not a homolog of other PLDs (5). Envenomation by recluse spiders causes necrotic tissue breakdown in mammals, a condition known as loxoscelism, and leads to skin degeneration and systemic reactions in the worst cases (2). *SicTox* enzymes are sufficient causative agents for this syndrome in mammals (6) and thus are pharmaceutical targets (7, 8). While sicariid spiders are found natively on all continents except Australia, treatments of loxoscelism are typically antibody based and available in some regions of the Americas (9).

SicTox enzymes catalyse the cleavage of lipid headgroups forming an alcohol and a cyclic phospholipid (10). The structures of two *Loxosceles* PLD have been resolved by X-ray (Figure 1): *Loxosceles laeta* L1_αIII1i (PDB ids: 1XX1 (11), 2F9R (11)) and *Loxosceles intermedia* Li-αIA1 (PDB id: 3RLH (12)). Their fold consists of a distorted TIM-barrel, a domain also found in many other phospholipases. Their membrane-binding region is called the i-face (for *interfacial* face) and is thought to consist of the loops β2α2 (also called *catalytic* loop), β5α5 and β6α6 (*flexible* loop) (11), all shown on Figure 1.

The *Loxosceles* SMaseD/PLD enzymes are classified in either the α clade or the β clade (13). Members of the α-clade have high catalytic activity against sphingomyelin (SM), while activity in the β clade is more variable (14–19). Only β clade enzymes have been found in *Sicarius* and *Hexopthalma* venoms while *Loxosceles* contain both α and β clade *SicTox* toxins paralogs. Using ³¹P NMR, Lajoie *et al.* recently showed that a β clade enzyme from *Sicarius terrosus* (St_βIB1) had a strong preference for substrates with ethanolamine headgroups vs. choline-containing lipids (14) in detergent micelles containing the lipid substrates. Lajoie *et al.* also measured the activity of an α and a β clade enzyme from *Loxosceles arizonica* La_αIB2bi and La_β1D1. La_αIB2bi presents a high activity against choline-containing lipids and interestingly La_β1D1 from the β clade shows little discrimination between ethanolamine and choline-containing substrates. The substrate specificity of the SMases is thought to be related to the relative abundance of ethanolamine-containing lipids in the cell membranes of arthropods (19), which are preferred prey of sicariid spiders (20). Lajoie *et al.* also resolved the X-ray structure of St_βIB1 (PDB ID 4Q6X, Fig.1); it resembles the structures of the α clade *SicTox* enzymes from *Loxosceles* and there are only subtle differences in the active or ligand binding sites. This led the authors to suggest that the i-face of the *SicTox* SMases/PLDs is likely to play a role in specific lipid recognition.

While the three previously mentioned loops are thought to constitute the i-face, there has not been any experimental investigation to identify which particular amino acids are involved in membrane binding. As demonstrated by many examples, molecular simulations have proven to be a useful tool to predict interfacial binding sites (IBS) of peripheral membrane proteins and map protein-lipid interactions (21–25). Of particular relevance are our efforts at characterizing the membrane binding site of a bacterial phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* (*BtPI-PLC*) (21, 25, 26). *BtPI-PLC* binds preferentially to PC-rich lipid bilayers, is folded as a distorted $\alpha\beta$ TIM barrel, and just as for the *SicTox* SMases/PLDs the *BtPI-PLC* IBS consists of a few loops located on the same side of the $\alpha\beta$ -barrel as the active site. Using a combination of MD simulations and *in vitro* K_d determination of the wild type enzyme and mutants, we could show that the preference of *BtPI-PLC* for PC lipids was the result of cation- π interactions between surface-exposed tyrosine residues and the choline groups of PC lipids (21, 25–27) and that those cation- π interactions between choline and interfacial tyrosine could contribute up to 2 to 3 kcal/mol to the protein-membrane affinity (28).

Closely observing the amino acid sequences and available X-ray structures of the *Sicarius* St_ β IB1 and the two α -clade *Loxosceles* enzymes Ll_ α III1i and Li_ α IA1, we noticed that the i-face of the α -clade enzymes is enriched in tyrosine and tryptophan residues. Li- α IA1 counts three tyrosines on the catalytic loop (Y44, Y46, Y60), Ll_ α III1 has two tyrosines (Y44, Y46, Y62) and a tryptophan (W60), while St_ β IB1 has only one tyrosine on this loop (Y44). In addition, Li- α IA1 has a tyrosine (Y98) on the β 3 α 3 loop and Ll_ α III1i has a tyrosine (Y169) on the β 5 α 5. Interestingly, the catalytic loop of La_ α IB2bi also has three tyrosines and a tryptophan at positions equivalent to Y44, Y46, Y62 and W60 in Ll- α III1i, according to a sequence alignment (14). The position and orientation of these conserved aromatic amino acids in the X-ray structures of Ll_ α III1i and Li- α IA1 (Figure 1) is

compatible with the formation of an aromatic cage as observed in proteins binding choline-containing ligands (29)(27, 30)(31). We pose the hypothesis that the tyrosine and tryptophan residues on the i-face of La_αIB2bi and other α-clade enzymes provides a mechanism to selectively recognize choline-containing lipids as ligands.

In this work, we evaluate this hypothesis using MD simulations and liposome binding assay experiments. As there is no X-ray structure of La_αIB2bi we chose to simulate Ll_αIII1i and Li_αIA1 instead. We identify the amino acids of the i-face involved in membrane binding by characterizing their interactions with lipid bilayers containing either only palmitoyl-oleyl-phosphatidyl choline lipids (POPC) or a mixture of POPC, sphingomyelin (SM) and cholesterol (CHOL). We also seek to understand how St_βIB1 interacts with phosphatidylethanolamine (PE) lipids and performed simulations of the β clade enzyme with bilayers consisting of both PC and PE lipids. Finally, we introduced aromatic amino acids in selected positions of the β clade enzyme to evaluate if it then acquires affinity towards choline-containing bilayers. We also performed *in vitro* liposome binding assay to evaluate the capacity of La_αIB2bi, wild type (WT) St_βIB1 and R44Y/S60Y St_βIB1 to bind to SM:CHOL (1:1) liposomes.

FIGURE 1 HERE

METHODS

Dataset for structural and multiple sequence alignment

We retrieved X-ray structures from the Protein Data Bank (PDB) (32) for Li_αIA1 (PDB id: 3RLH), Ll_αIII1 (PDB id: 1XX1, chain A) and St_βIB1 (PDB id: 4Q6X). The three

structures were aligned with MUSTANG (v 3.2.3) (33). Clustal Omega (34) was used to align multiple sequences alignment. Default parameters were used. We retrieved the sequences of 25 PLDs representatives of subgroups in the two clades (12 α - and 6 β -clade PLDs). Their UNIPROT identifiers are: P0CE83 (Li_ α IA2ai), P0CE80 (Li_ α IA1a), P0CE81 (Li_ α IA1bi), Q1W695 (Li_ α II1), P0DM60 (Li_ α IVA1), C0JB04 (Lru_ α IC2), A4USB4 (Li_ α V1), C0JAX7 (Ld_ α IB3ai), P0CE79 (Lr_ α IA1ii), Q5YD75 (Lr_ α IB1), Q5YD77 (Lb_ α IB1a), Q4ZFU2 (La_ α IB2bi), Q8I914 (LI_ α III1i), Q1KY79 (LI_ α III2), K9USW8 (Lg_ α IC1), A0A0D4WV12 (St β IB1), Q8I912 (LI_ β IA1), Q2XQ09 (Li_ β IA1i), Q1W694 (Li_ β ID1), C0JB41 (Lsp_ β IE2i), C0JB55 (Sd_ β IF1), C0JB68 (Spa_ β IIA1), C0JB92 (Lsp_ β III1), Q5YD76 (Lb_ β IA1a) and A0A0D4WTV1 (La_ β ID1).

System preparation

Lipid bilayers. Bilayers consisting of 256 lipid molecules (128 lipids in each leaflet) were built with the CHARMM-GUI (35) using the Membrane Builder module (36–38). POPC and POPC:POPE (50:50) bilayers were simulated for 200 ns (NPT) while the POPC:PSM:CHOL (70:20:10) bilayer was simulated for 500 ns (NPT) using the CHARMM36 force field (39–41). The calculated area per lipid for POPC is $65.0 \text{ \AA}^2$ in agreement with earlier simulations using the same force field ($65.5 \text{ \AA}^2$) (22, 39). The experimental value reported by Kuc□erka is $68.5 \text{ \AA}^2$ (42). The addition of POPE to construct a POPC:POPE (50:50) bilayer decreases the calculated area per lipid to $61.8 \text{ \AA}^2$, in good agreement with the $59.4 \text{ \AA}^2$ from recent simulation data (43). As expected (44), the addition of PSM and CHOL to POPC also reduces the area per lipid, measured to be $52.5 \text{ \AA}^2$ for the POPC:PSM:CHOL (70:20:10) mixture.

Protein-bilayer systems. We set up eight protein-bilayer systems: Li_ α IA1 and LI_ α III1 each with a POPC and a POPC:PSM:CHOL bilayer, and St_ β IB1 with these two

bilayers plus a POPC:POPE bilayer. In addition a mutant of St_ β IB1 was prepared with a POPC bilayer; two amino acids (R44 and S60) were replaced by tyrosines using UCSF Chimera (45). Protein structures were placed slightly above the surface of the relevant bilayers at a distance of 48 Å between the center of mass of bilayer and the center of mass of the protein. The protein were positioned in the binding orientation predicted by OPM (46) for Li_ α IA1 and Ll_ α III1. The initial configuration of St_ β IB1 WT and mutants are the binding orientation predicted by the PPM server (46). The systems were solvated and neutralized using VMD (v 1.9.1) (47). The composition of the eight simulated systems, included number of lipids, water molecules and ions, is reported in Table S1.

Molecular dynamics simulations

All simulations were performed using NAMD (v 2.13) (48) with the CHARMM36 force field (39–41) and its CHARMM-WYF extension for the treatment of aromatics-choline interactions (49, 50). The systems were first subjected to energy minimization with conjugate gradients (10000 steps) and an equilibration of 2 ns in the NPT ensemble. Next the production runs were run in the same ensemble for 300 ns using the last coordinates and velocities of the equilibration step. All the steps were done with an integration step of 2 fs. We ran two replicates for each of the systems listed in Table S1; replicas started from the same minimization step but differ by the equilibration step and therefore, different velocity distributions were used for the production run. Before equilibration and production runs, the mutant was subjected to extra equilibration in water for 1 ns. The temperature was set to 310 K and controlled using Langevin dynamics (temperature damping coefficient: 1.0). The pressure was set to 1 atm using the Langevin piston method with an oscillation period of 200 fs and a damping timescale of 50 fs. The SHAKE algorithm was applied in order to constraint all bonds between hydrogen and heavy atoms (51). Particle mesh Ewald was used for the

treatment of long range electrostatic interactions (52).

Trajectory analysis

The analysis of the trajectories was done on the window 150-300 ns, since all proteins were bound to the bilayers and the protein RMSD was stable for all 150 ns into the production run (Fig. S2). Trajectory analysis was done using CHARMM (v33b1) (53) for the inventory of lipid-protein interactions, VMD (v 1.9.1) (47) for the electron density plots and MDAnalysis (54, 55) for the calculation of the depth of insertion. We analysed three types of interactions: hydrophobic contacts, hydrogen bonds and cation- π interactions which we identified using the same definition as in Grauffel *et al.* (25). Briefly hydrophobic contacts are considered to exist if two non-bonded candidate atoms are within a distance of 3 Å. Candidate atoms are atoms in aliphatic groups of amino acids side chains (list provided in supporting information, Table S2). For hydrogen bonds the distance between the hydrogen and the donor of hydrogen bond atom should be below or equal to 2.4 Å, and the angle between the hydrogen bond donor, the hydrogen and the hydrogen bond acceptor should be higher than or equal to 130°. Cation- π interactions between the aromatic rings of tyrosines and tryptophans were considered to exist when all distances between the aromatic carbon and nitrogen atoms and the choline nitrogen were below 7 Å. In addition, these distances should not differ by more than 1.5 Å. Interactions were considered only if the criteria were met for at least 10ps and if the interaction was observed in both replicates. The depth of insertion for a given amino acid is the distance between its C α atom and the average upper phosphate plane. The position of the plane was calculated on the last frame of the simulation.

Liposome binding assays

T7 expression vectors (pHIS8) containing genes encoding La_ α IB2bi and St_ β 1B1 were constructed previously (56, 57). The R44Y/S60Y variant of St_ β 1B1 was constructed using

QuikChange mutagenesis (Agilent, Santa Clara, California, USA) of the wild-type construct. La_ α IB2bi, St_ β 1B1 and R44Y/S60Y St_ β 1B1 proteins were then expressed and purified by nickel affinity chromatography as described previously (10), followed by extensive dialysis into insect physiological saline (IPS; 5 mM potassium phosphate [pH 6.5], 100 mM potassium chloride, 4 mM sodium chloride, 15 mM magnesium chloride, 2 mM calcium chloride). Thin lipid films were prepared by dissolving equimolar amounts of egg sphingomyelin and cholesterol to a total lipid concentration of 9 mg/mL in 2:1 (v/v) chloroform:methanol, evaporating the solvent in a glass test tube with a weak stream of argon while rotating the tube, and drying the film under vacuum at 45 °C for at least 2 h. Films were converted to liposomes (large multilamellar vesicles) by suspending them at 36 mg/mL lipid concentration in 10 mM HEPES (pH 7.5), 150 mM sodium chloride, followed by heating at 65 °C for 1 h with occasional vortexing. For the liposome binding assay, 1.5-2 μ g protein in 10 mL IPS was mixed with 10 mL of an 18 mg/mL liposome solution and incubated at 25 °C for 30 min, followed by centrifugation for 10 min at ambient temperature at 16000 g. Supernatant and pellet were separated, converted to gel samples of equal volume, loaded onto Tris-tricine SDS-PAGE gels, and electrophoresed for at least 45 min at 150 V. Gels were stained with Coomassie Brilliant Blue R-250, destained, and photographed. Bands were quantified using ImageJ software (58). Values reported in the text are the average of three experiments; reported errors represent the standard error of the mean.

RESULTS

(1) Cation- π interactions between the aromatic cage in α clade enzymes and POPC

FIGURE 2 HERE

We performed molecular dynamics simulations of each of the three PLDs (Li_ α IA1, LI_ α III1 and St_ β IB1) in the presence of two different bilayers: POPC and POPC:PSM:CHOL (70:20:10). Each simulation was initiated with the protein positioned slightly above the bilayer. The RMSD of the protein backbone with respect to structures after equilibration remains low during the production runs (Cf Figures S1-S2) and show deviations of at most 1.2 Å.

The β clade *Sicarius* enzyme St_ β IB1 does not bind to the PC-containing bilayers and diffuses away rapidly (Fig. 2 and 3A), indicating a low affinity for POPC lipids. The α clade *Loxosceles* enzymes on the other hand, Li_ α IA1 and LI_ α III1, bind to bilayers composed of POPC (Fig. 2 and 3A) and POPC:SM:CHOL (Fig. 4A and S3) within 100 ns. Figure 4A shows the electron density profiles (EDP) and the depth of insertion below the phosphate planes calculated for each amino acid. The two enzymes are anchored rather superficially in the bilayer, irrespective of the type of bilayer, and only a few residues are inserted under the phosphate plane (Fig. 3D). Both Li_ α IA1 and LI_ α III1 interact with the lipids through their β 2 α 2 catalytic loop while the other loops, including β 6 α 6 remain higher above the interface (Fig. 3B-D). We observed hydrophobic contacts with the lipids chains for only a few residues; I49 and P50 from Li_ α IA1 and I58 from LI_ α III1 (Table 1).

FIGURE 3 AND TABLE 1 HERE

Amino acids involved in hydrogen bonds, hydrophobic contacts and cation- π interactions with the lipids are listed in Table 1. Numerous residues located in the catalytic loop establish a hydrogen bond network with the bilayer. Most occupancies are low (<50 %) and most hydrogen bonds are achieved by the side chains except for I49, C51, G54 in Li_ α IA1. We observe more hydrogen bonds in Li_ α IA1 than Ll_ α III1 but even in Li_ α IA1 the occupancies of hydrogen bonds are not high except for K58 (above 70%). The side chain of R59 in Ll_ α III1 also engages in long-lasting hydrogen bonds with the lipids (> 90% occupancy). Noteworthy are the hydrogen bonds achieved by Y44, Y46, Y60 and Y98 in Li_ α IA1 and Y62 from Ll_ α III1.

These tyrosines also establish cation- π interactions with the choline groups of the lipid heads (Table 1 and Figure 4). In Li_ α IA1 Y44, Y46 and Y60 form an aromatic cage occupied by a choline group. The occupancies of the cation- π interaction between those tyrosines and the bilayer are between 29 and 46%. In Ll_ α III1, Y44, Y46, W60 form an equivalent cage and the occupancies of the cation- π interactions are between 27.54 and 89%. In the course of the 300 ns of simulation, the cage can be occupied by either the same lipid or by up to 3 subsequent lipids during the simulation (Fig. S4-S6). Moreover, the two α clade PLDs contain an additional tyrosine establishing cation- π interactions with the bilayer: Y98 from the β 3 α 3 loop in Li_ α IA1 (46-60% depending on the bilayer) and Y62 from the catalytic loop in Ll_ α III1 (39-46%).

FIGURE 4 HERE

(2) Dense hydrogen bond network between aspartates in the β clade enzyme and POPE

FIGURE 5 and TABLE 2 HERE

Lajoie *et al.* showed that St_ β IB1 turns over ethanolamine-containing ceramides and lysophospholipids 2 to 3 orders of magnitude faster than corresponding choline-containing lipids. – The binding pocket for the lipid headgroup in PLDs has been identified using molecular docking and does not appear to account for the difference of lipid specificity. We ran simulations of St_ β IB1 in the presence of a POPC:POPE (50:50) bilayer to predict the binding site of the *Sicarius* enzyme and analyse the protein-lipid interactions.

Unlike the simulations of St_ β IB1 on the POPC and POPC:SM:CHOL bilayers (Fig. 3 and S3) the enzyme binds rapidly to the POPC:POPE bilayer (Fig. 5A). The density plots (Fig. 5B) show an anchoring slightly deeper than for the α -clade enzymes in the PC-containing bilayers and the orientation with respect to the bilayer surface is also slightly different with the flexible loop β 6 α 6 being involved in interactions with the lipids (Fig. 5C & D). This is reflected by the number of hydrophobic contacts and hydrogen bonds (Table 2). As a result of a deeper anchoring at the interface we observe more hydrophobic contacts with the lipid tails than between the α clade enzymes and PC bilayers. Amino acids of the highly conserved region of the flexible loop (I194, T195, L198, P199) all have on average more than 1.0 hydrophobic contacts per frame with the lipid tails in both replicas. The phenylalanine at position 54, which corresponds to a glycine in the two α clade enzymes, has a high count of hydrophobic contacts too.

We observe a dense hydrogen bond network between the lipids and several loops of St_ β IB1 (Table 2). In addition to the catalytic (β 2 α 2) and flexible (β 6 α 6) loops, the β 7 α 7 loop and the amino acids of the α 8 helix engage in long-lasting hydrogen bonds with lipid headgroups (occupancies between 50% and 100%). Loop β 5 α 5 and helix α 7 engage only in weak hydrogen bonds. Most of the hydrogen bonds involve the lipid phosphate group. However, six residues establish hydrogen bonds specifically with ammonium groups of the

ethanolamine headgroups: N169, D201, D202, D229, E235 and E259 (Table 2). Among those D201 and D202 establish long-lasting hydrogen bonds with POPE ammonium groups (Fig. 6). The hydrogen bonds between other amino acids - N169, D229, E235, E259 - and POPE headgroups have a relatively lower occupancy (< 40% of the simulation time). Interestingly in the two α clade enzymes, amino acids at position 202 are not acids but instead there is a leucine (L202) in Li_ α IA1 and a methionine (M202) in Ll_ α III1. Position 201 is an aspartic acid (D201) in Ll_ α III1 but a glycine (G201) in Li_ α IA1.

FIGURE 6 HERE

(3) Engineering the aromatic cage in St_ β IB1 recovers binding to pure POPC bilayer

FIGURE 7 AND TABLE 3 HERE

The aromatic cage present in the α clade Li_ α IA1 and Ll_ α III1 but not in St_ β IB1, a β clade enzyme, is likely to be important for the recognition of choline groups of PC and SM lipids. We engineered such an aromatic box on St_ β IB1 by substituting R44 and S60 by tyrosines and simulated the resulting structure on a POPC bilayer. We checked that the mutations did not alter the protein structure. During the simulations we observed no major structural differences compared to the X-ray structure of the wild-type (Fig. S7). The backbone RMSD between the starting structure and the R44Y/S60Y double mutant oscillates between 1.0 Å and 1.2 Å during the production run, and the RMSD between WT St_ β IB1 X-ray structure and R44Y/S60Y is equal to 1 Å at the end of the simulation. Unlike what we observed with the WT enzyme, and similar to Li_ α IA1 and Ll_ α III1, the double mutant binds quickly to the POPC bilayer (Fig. 8). The interaction with lipids happens mostly at the

catalytic loop (Fig. 7B-C). The aromatic cage interacts with choline headgroups and unlike for the wild type enzyme, few hydrogen bonds and hydrophobic contacts are established with the interfacial lipid groups (Table 3). This is in line with the shallow insertion of the enzyme. Overall, the interactions follow the same pattern as that obtained for Li_αIA1 with the POPC bilayer.

(4) Liposome Binding Assay

FIGURE 8 HERE

Binding assays with mixed sphingomyelin:cholesterol (SM:CHOL) liposomes (Figure 8) provide some experimental support for the aromatic cage effect found in the simulations. La_αIB2bi, an α clade protein possessing an aromatic cage and known to prefer substrates with choline head groups (14), is bound completely by 1:1 SM:CHOL liposomes. The β clade enzyme St_β1B1, which lacks an aromatic cage, is bound poorly, with an average of only $15 \pm 4\%$ of the protein sample pulled down by the liposomes relative to a negative control. The aromatic cage variant R44Y/S60Y St_β1B1 showed higher but still incomplete binding, with $28 \pm 4\%$ pulled down.

(5) The aromatic cage is mostly conserved in α clade *SicTox* PLDs

FIGURE 9 HERE

A multiple sequence alignment (MSA) of 25 PLDs from Lajoie *et al.* 2015 (14) and 7 additional samples that represent known diversity (list provided in methods section) shows that the sequences are fairly similar with the lowest sequence identity being around 37% (Li_αIA2ai vs Lsp_βIE2i) (Fig. 9B). Figure 9A shows the MSA in the region of the catalytic loop (from position 31 to 62, Fig. S8) and of the flexible loop (from position 192 to 204, Fig.

S8). The full MSA is given in supporting information (Fig. S8).

The aromatic cage is mostly conserved in the α clade but the complete cage is mostly absent from the β clade enzymes. Y44 and Y46 are strictly conserved in the α clade; position 60 can be either a tyrosine or a tryptophan except for Lg_alphaIC1 where it is a serine. The cage is not present in Lru_alphaIC2. In the β clade, only Y46 is mostly conserved (except in Li_betaID1 and La_betaID1) but position 44 is a charged amino acid (E, R or K) and position 60 is a polar amino acid (S, T or Q). Position 62 is an aromatic (Y) in about half of the α clade PLDs of our dataset, forming an aromatic cage of four residues. In the β clade sequences, this position is not occupied by aromatic residues. Only two representants of the β clade exhibit the aromatic cage (Lsp_betaIII1 and Sd_betaIF).

In the flexible loop, only two β clade enzymes present an aspartate at position 201 (G, I or V for the other β clade PLDs and G or D for the α clade PLDs). D202 is conserved among the β clade PLDs in our dataset (only two β clade PLDs present a methionine at this position). Interestingly, this position is occupied by a hydrophobic residue in the α clade PLDs (M or L). D201 and D202 are located in the $\beta 6\alpha 6$ loop (flexible loop). The C-terminus side of this loop is richer in aspartate in β clade PLDs than in the α clade PLDs towards the end of this loop (Fig. 9).

DISCUSSION AND CONCLUSIONS

The structures of Li_alphaIA1 and Ll_alphaIII1 reveal an aromatic cage formed by amino acids at positions 44, 46 and 60, but this cage is not present in the *Sicarius* PLD (St_betaIB1) structure, leading to the hypothesis that this cage is part of the mechanism by which the α clade enzymes achieve high affinity for choline-containing lipid headgroups. We discuss

below how our results support this hypothesis.

MD simulations showed that Li_αIA1 and Ll_αIII1 bind to choline-containing bilayers with the same binding orientation whereas St_βIB1 remained in solvent. The i-face of the two α clade enzymes on choline-containing bilayers involves the catalytic loop and cation-π interactions between the aromatic residues forming the cage and choline headgroup. The aromatic residues located at the i-face are engaged in strong cation-π interactions with choline headgroups. The liposome binding assays show that La_αIB2bi, an α clade enzyme with the aromatic cage, shows strong binding to SM:CHOL (1:1) liposomes whereas St_βIB1 binds poorly to those.

The structure of the aromatic cage resembles aromatic cages found in other proteins around choline groups (27, 30, 59–61). Such cages consist of 2 to 4 aromatic side chains, either only tyrosines as in Li_αIA1, or a combination of tyrosines and tryptophans as we observe Ll_αIII1. Roughly half the sequences in our datasets have a cage consisting of three aromatics and half have a cage of four. The aromatic amino acids arrange as a box forming a hydrophobic binding site for the methylated ammonium which positive charge is also stabilized by the π-system of the aromatics groups. The shortest distances between carbons of the aromatic ring and methyl groups are within 4 Å. Examples of cages around choline-containing lipids include the structure of the human phosphatidylcholine transfer protein (PDB IDs 1LN1 and 1LN3) complexed with 1,2-dilinoleoyl-*sn*-glycerol-3-phosphorylcholine (DLPC, 18:2(9,12)-18:2(9,12)) or 1-palmitoyl,2-linoleoyl-*sn*-glycerol-3-phosphorylcholine (PLPC, 16:0–18:2(9,12)) bound to a cage consisting of three tyrosines and a tryptophan. In the engineered N254Y/H258Y SaPI-PLC two tyrosines and one tryptophan bind a choline ion (PDB ID 4I90) or the choline group of diC4PC (PDB ID 4I9J)(27).

Inspired by the work of Cheng et al, we engineered an aromatic cage in the β clade

enzyme *in silico* (27). Starting from *Staphylococcus aureus* PI-PLC (*Sa*PI-PLC) which does not bind to PC-rich vesicles, Cheng et al increased its affinity for PC-rich liposomes by engineering an aromatic cage inspired by the sequence and structure of the PC-specific homologue *Bt*PI-PLC. X-ray structures of the modified *Sa*PI-PLC enzyme soaked with phosphocholine or DiC₇PC (1,2-Diheptanoyl-sn-Glycero-3-Phosphocholine) revealed that the choline groups were positioned in the aromatic cage. Our simulations of the engineered St β IB1 (R44Y/S60Y St β IB1) lead to a stable interaction with a POPC bilayer, unlike the results we obtained with the wild-type. Just as we observed for the α clade enzymes, a choline headgroup was observed residing in the aromatic cage during most of the simulation time. The liposome binding assay of R44Y/S60Y St β IB1 on SM:CHOL (1:1) liposomes shows significantly increased binding compared to WT St β IB1. This suggests that the aromatic cage is one important factor for the binding of membranes rich in choline head groups; on the other hand, the lack of complete pulldown suggests that other factors must also contribute to the weak binding of St β IB1 to choline-rich membranes, relative to α clade enzymes such as La α IB2bi.

An alignment of the sequences of 25 representative PLDs from different subgroups of the two clades shows that the aromatic cage is highly conserved in the PLDs from the α clade and not present in the well characterized PLDs from the β clade. Overall, the conservation of a cage in most α -clade enzymes, and its positive role in choline recognition based on simulations and *in silico* and *in vitro* mutagenesis data, support a significant role for the cage in targeting α -clade toxins to choline-rich membranes. Other things being equal, this targeting should increase access of the α -clade enzymes to the choline-containing substrates SM and lysophosphatidylcholine (LPC), toward which they have generally high activity. This likely contributes to the efficacy of α -clade toxins in triggering loxoscelism in humans, but presumably also plays an adaptive role in prey capture, the principal biological function of

Loxosceles venom (20). Meanwhile, St_ β IB1, a β clade paralog that strongly prefers substrates with ethanolamine head groups, lacks the cage, as do most other β clade enzymes. In both simulation and experiment, its binding to choline-rich membranes is increased by introduction of the cage. One caveat is that La_ β ID1, a second β clade enzyme that also lacks the cage, has fairly high activity against choline-containing substrates, though low preference relative to ethanolamine-containing substrates (14) suggesting that other determinants of head group preference must also contribute.

To better understand the specificity of St_ β IB1 for PE-rich membrane, we also ran simulations of WT St_ β IB1 in the presence of a POPC:POPE (50:50) bilayer. As opposed to the simulation on bilayers without POPE lipids, WT St_ β IB1 bound to the bilayer with an i-face composed of the catalytic (β 2 α 2) and flexible (β 6 α 6) loops, as well as loop β 7 α 7 and helix α 8. WT St_ β IB1 is anchored slightly deeper in the bilayer than the α -clade enzymes and in lieu of cation- π interactions, it engages in a dense hydrogen bond network with the lipid phosphates and headgroups. Interestingly two aspartates located on the flexible loop establish hydrogen bonds with the ethanolamine headgroup. Early assessments of sequence diversity in the MSA indicate that the-the-terminus region of the catalytic loop is richer in aspartate in the β clade than in the α clade.

Beyond suggesting the molecular basis for the specificity of α -clade PLDs for choline-rich membranes, this work is relevant for the design of drugs targeting PLDs from *SicTox* spiders. We suggest that the aromatic cage might be worth targeting to weaken protein-membrane interactions of the α clade enzymes. Last but not least the role of the evolutionary conserved aromatic cage in *SicTox* PLD enzymes, together with already existing evidence of interfacial choline-aromatics cation- π interactions in other protein families (24, 25, 60, 62), further confirms that such interactions could be a general mechanism for specific

recognition of the surface of eukaryotic cells or other choline-rich biological membranes.

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Tables

Table 1. Inventory of interactions between Li_αIA1 (3RLH) or Li_αIII1 (1XX1) and two types of bilayers: POPC and POPC:PSM:CHOL. Hydrophobic contacts are given as average number of contacts per frame. Hydrogen bonds and cation- π occupancies are shown in percentages. Residues in bold indicate hydrogen bonds involving the amino acid backbone. Asterix (*) indicates strictly conserved positions between Li_αIA1 and Li_αIII1.

SSE	residue	Li_αIA1				Li_αIII1			
		Bilayer A		Bilayer B		Bilayer A		Bilayer B	
		R1	R2	R1	R2	R1	R2	R1	R2
Hydrogen bonds (%)									
β2α2	K38	-	-	-	-	33.56	36.54	40.0	36.70
	Y44*	15.53	20.76	24.89	21.98	-	15	24.33	16.87
	Y46*	67.96	63.45	50.34	50.34	-	-	-	-
	I49	23.21	21.89	20.12	21.67	-	-	-	-
	C51*	45.93	40.78	38.56	35.56	-	-	-	-
	G54	19.98	26.37	35.90	35.77	-	-	-	-
	K58	72.49	79.90	83.21	77.45	-	-	-	-
	R59	-	-	-	-	91.45	89.45	100	92.45
	W60	-	-	-	-	15.45	11	15	20.56
	Y62	-	-	-	-	50.34	34.78	45.0	46.65
β3α3	Y98	36.96	46.89	35.90	32.87	-	-	-	-
Hydrophobic contacts (avg. contact per frame)									
β2α2	I49	4.0	4.2	5.0	4.0	-	-	-	-
	P50*	3.0	3.4	4.3	3.2	-	-	-	-
	I58	-	-	-	-	1.0	0.6	0.6	0.7
cation-π interactions (%)									
β2α2	Y44*	35.6	28.90	30.54	34.78	28.8	27.54	34.89	29.63
	Y46*	40.4	46.00	43.76	44.34	73.9	67.90	84.23	86.33
	Y60/W60	44.7	44.78	40.6	33.23	84.4	80.27	83.12	89.00
	Y62	-	-	-	-	41.2	45.89	39.45	41.67
β3α3	Y98	50.5	60.63	46.65	47.86	-	-	-	-

Table 2. Inventory of interactions between WT St_βIB1 and a POPC:POPE bilayer.

Hydrophobic contacts are given as average number of contacts per frame. Hydrogen bonds and cation- π occupancies are shown in percentages. Residues in bold indicate hydrogen bonds involving the amino acid backbone. Underlined residue names indicate hydrogen bonds involving the ethanolamine headgroup.

SSE	Residue	R1	R2
Hydrogen bonds (%)			
$\beta 2\alpha 2$	C51	99.83	57.94
	C53	17.25	19.70
	S56	99.67	99.85
	T58	40.63	41.76
$\beta 5\alpha 5$	R59	32.17	22.10
	<u>N169</u>	29.85	12.94
$\beta 6\alpha 6$	R200	12.44	49.26
	<u>D201</u>	80.54	85.38
	<u>D202</u>	58.87	65.67
$\beta 7\alpha 7$	<u>D229</u>	29.02	13.08
	K230	68.16	60.58
$\alpha 7$	E231	33.51	36.32
	S232	56.38	37.50
	S233	40.46	40.00
	<u>E235</u>	40.83	34.41
$\alpha 8$	Y249	76.12	84.85
	R252	85.41	81.47
	<u>E259</u>	27.36	32.50
	R260	30.51	36.18
Hydrophobic contacts (avg. contact per frame)			
$\beta 2\alpha 2$	P50	0.14	0.10
	C51	1.23	0.78
	C53	0.66	0.45
	F54	1.45	1.30
$\beta 6\alpha 6$	R55	1.03	0.78
	I194	1.02	1.11
	T195	1.13	1.22
	C197	0.75	0.60
	L198	1.53	1.67
$\alpha 7$	P199	1.62	1.53
	K230	1.34	1.22

Table 3. Inventory of interactions between R44Y/S60Y St_βIB1 and a POPC bilayer.

Hydrophobic contacts are given as average number of contacts per frame. Hydrogen bonds and cation- π occupancies are shown in percentages. Residues in bold indicate hydrogen bonds involving the amino acid backbone.

SSE	Residues	R1	R2
Hydrogen bonds (%)			
$\beta 2\alpha 2$	K38	39.085	68.49
	Y44	10.54	20.78
	Y46	60.00	54.34
	C51	53.33	67.34
	S56	46.67	58.34
	C57	20.60	10.32
	Y60	20.00	35.81
Hydrophobic contacts (avg. contact per frame)			
$\beta 2\alpha 2$	V49	3.0	4.3
	P50	2.5	1.9
cation-π interactions (%)			
$\beta 2\alpha 2$	Y44	43.62	39.89
	Y46	35.63	40.23
	Y60	44.81	20.33

Figure captions

Figure 1. Overview on the SicTox PLDs structure. A) Structures of phospholipases D used in this work. Li_αIII1, Li_αIA1 and St_βIB1 are represented in yellow, blue and green, respectively. The side chains of amino acids critical in substrate binding and catalysis are shown in orange. The aromatic residues located at the putative i-face are indicated in purple. B) PLD topology: strands (blue) and helices (red) of canonical (α/β)₈ barrel, other secondary structure elements (green), catalytic loop (cyan), flexible loop (green), disulphide bridges (purple). Residue numbering is based on the Li_αIA1 structure (PDB id: 3RLH). C) Structure and sequence similarity: pairwise RMSD (lower left triangle) and sequence similarity (upper right part).

Figure 2. Simulation trajectory snapshots of Li_αIA1, Li_αIII1 and St_βIB1 on a POPC bilayer. Snapshots were taken at time t=0, 80, 160, 270 and 300 ns of the production run.

Figure 3. Li_αIA1 and Li_αIII1 bound to POPC and POPC:PSM:CHOL (70:20:10). A) Electron density plots for Li_αIA1, Li_αIII1 and St_βIB1 on the POPC bilayer (top row) or on the POPC:PSM:CHOL bilayer (bottom row), replica 1 (solid lines) and replica 2 (dashed lines). B) Bound orientation of Li_αIA1 on a pure POPC bilayer. C) Binding orientation of Li_αIII1 on a POPC:PSM:CHOL bilayer. D) Depth of anchorage of Li_αIA1 into the POPC bilayer. The depth is calculated for each amino acid as the distance between its C-alpha and the phosphate plane in the last frame of simulation (relevant values are given in Tables S4 & S5).

Figure 4. Phosphatidylcholine headgroups trapped in the aromatic cage of Li_αIA1 (blue) and Li_αIII1 (yellow). Lipids carbon atoms are in purple. The snapshots are extracted from the last 150ns of the simulation trajectories.

Figure 5. Binding of St_ β IB1 to a POPC:POPE (50:50) bilayer. A) Simulation snapshots. B) Electron density plot for two replica (solid lines and dashed lines). C) Bound form. D) Depth of anchorage. The depth is calculated for each amino acid as the distance between its C-alpha and the phosphate plane in the last frame of simulation (relevant values are given in Table S6).

Figure 6. Hydrogen bonds (yellow) between residues D201 and D202 of St_ β ID1 and phosphatidylethanolamine headgroups: POPE lipid (carbon atoms in purple), protein (green). The two snapshots are taken during the last 50 ns of simulation.

Figure 7. Binding of R44Y/S60Y St_ β IB1 to a POPC Bilayer. A) Simulation snapshots. B) Electron density plot for R44Y/S60Y St_ β IB1 and the POPC bilayer for two replica (solid lines and dashed lines). C) Bound protein orientation. D) Depth of anchorage. The depth is calculated for each amino acid as the distance between its C-alpha and the phosphate plane in the last frame of simulation (relevant values are given in Table S4).

Figure 8. Liposome binding assay for WT La_ α IB2bi, WT St_ β IB1 and R44Y/S60Y St_ β IB1 on SM:CHOL (1:1) liposomes. P and S refer to pellet and supernatant, respectively. The “-” symbol indicates the control lanes (without liposomes) and the “+” symbol the lanes with the protein and the liposomes. Across three experiments the mutant R44Y/S60Y St_ β IB1 showed 10-17% higher levels of pulldown than the WT, giving a mean increase of $13 \pm 2\%$.

Figure 9. Amino acid sequences similarities in the catalytic and flexible loops. A) Multiple sequence alignment of the catalytic and the flexible loop. The full sequence alignment is given as supporting information (Fig S8). B) Sequence similarity matrix.

C)

Structure and sequence similarity

	LI_αIII1 (1XX1)	Li_αIAI (3RLH)	St_βIB1 (4Q6X)	La_αIB2bi
LI_αIII1 (1XX1)		59 %	49.28 %	60.40 %
Li_αIAI (3RLH)	1.3 Å		46.93 %	75.91 %
St_βIB1 (4Q6X)	1.5 Å	1.1 Å		47.46 %
La_αIB2bi				

St_βIB1

POPC

LI_αIII1

POPC

Li_αIA1

POPC

0ns

80ns

160ns

270ns

300ns

A)

B)

C)

D)