Fatty acyl chain-dependent but charge-independent association of the SH4 domain of Lck with lipid membranes

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The SH4 domain of Src family of nonreceptor protein tyrosine kinases represents the extreme N-terminal 1–16 amino acid region which mediates membrane association of these proteins and facilitates their functions. The SH4 domains among Src members lack well-defined sequence consensus and vary in the net charge. However, they readily anchor to the cytoplasmic face of the plasma membrane upon fatty acid acylation. Here, we report the membrane association of differentially acylated SH4 domain of Lck kinase, which has net negative charge at physiological pH. Our results suggest that despite the net negative charge, the SH4 domain of Lck associates with membranes upon fatty acid acylation. While myristoylation at the N-terminus is sufficient for providing membrane anchorage, multiple acylation determines orientation of the peptide chain with respect to the lipid bilayer. Hence, fatty acylation serves more than just a lipid anchor. It has an important role in regulating the spatial orientation of the peptide domain with respect to the lipid bilayer, which could be important for the interaction of the other domains of these kinases with their partners.

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1. Introduction

The Src family of tyrosine kinases is a group of nine non-receptor tyrosine-specific multi-domain protein kinases that are bound to membranes. They interact with diverse classes of membrane receptors, affect an array of downstream targets and regulate a variety of cellular functions (Resh 1993; Brown and Cooper 1996; Thomas and Brugge 1997). Despite the absence of a transmembrane domain, they associate with both plasma membranes as well as intracellular membranes (Courtneidge *et al*. 1980; Krueger *et al*. 1983). Membrane association is a crucial requirement for the function of these kinases (Cross *et al*. 1984, 1985; Kamps *et al*. 1985, 1986; Spencer *et al*. 1995; Kabouridis *et al*. 1997). The membrane association and microdomain segregation of these kinases is mediated by the SH4 domain (Src homology 4; Resh 1993), a 1–16 amino acid residue region in the N-terminus of the proteins that have covalently linked fatty acyl chains (Shenoy-Scaria *et al*. 1994; Robbins *et al*. 1995; McCabe and Berthiaume 2001). A close examination of the sequences of the SH4 domains from these kinases reveals that the net charge on the domains varies widely among different members. In the SH4 domain of Fgr, the net charge is positive, in Fyn, the net charge is 0, while in the case of Lck, the SH4 domain is negatively charged (Resh 1994). The SH4 domains of all Src kinases are enzymatically myristoylated by N-myristoyltransferase on the N-terminus Gly residue (Garber *et al*. 1985; Wilcox *et al*. 1987; Towler *et al*. 1988). The SH4 domains of Src kinases are further modified by one (in case of Lyn, Yes, and Hck) or two (in case of Fgr, Fyn, and Lck) palmitoyl chains (Paige *et al*. 1993; Shenoy-Scaria *et al*. 1993, 1994; Koegl *et al*. 1994).

While studies on myristoylated short peptides have revealed that myristoyl moiety alone provides only weak membrane affinity to the short peptides (Peitzsch and McLaughlin 1993), other studies demonstrate that contribution of basic residues promotes membrane association of the peptides (Kim *et al*. 1991; Silverman and Resh 1992).

Keywords. Fatty acid acylation; membrane association; model membranes; tyrosine kinases

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Studies on the interaction of isolated SH4 domain of Src myristoylated at the N-terminus with model membranes suggest that the contribution of myristate and the electrostatic interactions act synergistically to confer stable membrane anchorage (Buser *et al*. 1994). Such electrostatic contribution to the membrane association of Src members, along with N-myristoylation, is unlikely in the case of Lck, which although devoid of basic residues in the SH4 domain, remains membrane bound (Marchildon *et al*. 1984). A covalently attached palmitoyl chain was subsequently identified in the SH4 domain (Paige *et al*. 1993; Shenoy-Scaria *et al*. 1993), which is crucial for membrane attachment of Lck. The apparent lack of sequence consensus beyond N-terminal 'Met-Gly-Cys' motif in the SH4 domains among Src family members indicates that biophysical characteristics rather than sequence specificity could be important for membrane association (McCabe and Berthiaume 1999; Bhatnagar and Gordon 1997). It has been suggested that sub-membrane topology of Src kinase may influence conformation of the kinase and affect modular interaction (Ilangumaran *et al*. 1999). In an earlier report, we have shown that although N-myristoylation of the SH4 domain of Fyn is sufficient for membrane association, dual acylation with an additional thioester-linked palmitoyl chain pushes the peptide chain away from the membrane bilayer (Rawat and Nagaraj 2010).

The study presented here details investigation of the membrane interaction of the SH4 domain from Lck, which has net negative charge, modified with only N-terminal myristic acid as well as N-myristoyl and two palmitoyl chains at the Cys side-chains (table 1).

2. Materials and methods

2.1 *Materials*

The Fmoc-(9-fluorenylmethoxycarbonyl) protected amino acids for solid phase peptide synthesis were from Novabiochem AG, Switzerland, and Advanced ChemTech, Louisville, KY. The solid support resin PAL (5-[4-(9-fluorenylmethyloxycarbonyl) aminomethyl-3,5-dimethoxy-phenoxy] valeric acid) (substitution value 0.3 mmol/gm) was from Advanced ChemTech, Louisville, KY. The coupling reagents *N*-hydroxybenzotriazole hydrate (HOBt) and 2-(1-H-benzotriazole-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Advanced ChemTech, Louisville, KY, while *N*,*N*-diisopropylethylamine was from Sigma-Aldrich, St. Louis, MO. Piperidine was from Loba-Chemie Pvt Ltd, India. Thioanisole, ethanedithiol, and acetic anhydride were either from Fluka AG Chemical Corp, Switzerland, or Pierce Chemical Company, USA. 1-Palmitoyl-2-oleoyl-phosphatidylcholine (PC), 1-palmitoyl-2-oleoyl-phosphotidylglycerol (PG), cholesterol (CHL), sphingomyelin (SM), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(5-dimethylamino-1-naphthalenesulfonyl) (DNS-PE) were from Avanti Polar Lipids, Alabaster, AL. Solvents such as dimethylformamide (DMF), *N*-methyl pyrrolidone (NMP), dichloromethane (DCM), chloroform, methanol, diethyl ether, isopropanol, and dimethyl sulfoxide (DMSO) were from Spectro Chem Pvt Ltd, India. All reagents and solvents used were of the highest purity commercially available.

Table 1. Sequence of the SH4 domain of Lck and the peptides synthesized for this study

Peptide	Sequence
SH4 domain of Lck	GCGCSSHPEDDWMEN
Plck	Ac-GCGCSSHPEDDWMEN-amide
Pmlck	Myr-GCGCSSHPEDDWMEN-amide
Pmpplck	Myr-GC(Pal)GC(Pal)SSHPEDDWMEN-amide

Ac: acetyl; Myr: myristoyl, Pal: palmitoyl

2.2 *Synthesis, acylation and characterization of peptides*

All peptides (table 1) were synthesized using standard Fmoc chemistry (Atherton and Sheppard 1989; Wellings and Atherton 1997). The peptides were assembled on PAL resin. The free N-termini of the peptides were either acetylated or fatty acylated. For acetylation, resin-bound peptides were treated with a mixture containing acetic anhydride/diisopropylethylamine (5 equivalents of each per equivalent of peptide) in minimal volume of DMF for a period of 1 h (Stewart and Young 1984). The N-myristoylated peptide was obtained by on-resin acylation procedure described elsewhere (Harishchandran *et al*. 2002). Triply acylated peptide was generated by performing first acylation on-resin at the N-terminus with myristic acid followed by palmitoylation at two Cys residues using the activated fatty acids as described by Harishchandran *et al*. (2002). The free –SH groups on Cys residues for palmitoylation were exposed by treating N-myristoylated resin-bound peptide first with 10 equivalents of iodine per Cys residue in DMF for 10 min and then with 20 equivalents of dithiothreitol (DTT) per Cys for 1 h and finally washing the resin thoroughly in DMF containing excess of DTT to remove traces of mercuric sulfide sticking to resin. The entire acylation procedure was carried out on-resin. The peptides were cleaved and deprotected from the resin by treating with a mixture containing trifluoroacetic acid (TFA), m-cresol, thioanisole, and ethanedithiol (10:1:1:0.5, v/v). All peptides were purified by reversed-phase HPLC on Hewlett-Packard 1100 series system using Zorbax 300 SB C18 column, using the 0.1% TFA in deionized water as solvent A and 0.1% TFA in acetonitrile (for unacylated and N-myristoylated peptide) or 0.1% TFA in acetonitrile:isopropanol (7:3, vol/vol) mixture (for triply acylated peptide) as solvent B such that the percentage of solvent B increases from 5% to 100% in 60 min. The

purified peptides were characterized by matrix assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) on Voyager DE-STR, Perseptive Biosystems (Proteomics facility, CCMB, Hyderabad) using recrystallized CHCA (α-cyano-4-hydroxycinnamic acid) as matrix. The peptide stocks were prepared by dissolving the weighed quantities of dry peptides in DMSO immediately before use.

2.3 *Preparation of lipid vesicles*

For preparing lipid vesicles of various compositions, including those doped with fluorophore-tagged lipids, appropriate volumes of lipid stocks were drawn from respective chloroform stocks and transferred into glass tubes and dried either under nitrogen stream or in a rotary evaporator system to yield uniform lipid films. The lipid films were desiccated under vacuum for 6 h and then hydrated for 10 h in 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, pH 7.4, containing 150 mM NaCl. The hydrated films were vortexed vigorously to give turbid suspensions of lipid in buffer, which were subsequently used for preparation of large unilamellar vesicles (LUV) by extruding the suspension through 200 nm pore size polycarbonate filter membrane (MacDonald *et al.* 1991). Homogeneity of lipid vesicles was verified by dynamic light scattering (DLS).

2.4 *Interaction of peptides with lipid vesicles*

Steady-state tryptophan fluorescence intensity of peptides was recorded in absence and presence of lipid vesicles of PC, PC:PG (1:1) and PC:SM:CHL (1:1:1) compositions. Fluorescence spectra were recorded for each peptide at a fixed concentration of 2 μM in 5 mM HEPES buffer, pH 7.4, containing 150 mM NaCl in a Hitachi F4500 Fluorescence Spectrometer (Hitachi, Tokyo, Japan). The excitation wavelength was 280 nm, and emission was recorded between 300 and 400 nm with excitation and emission slit-widths of 2 and 5 nm respectively. The solvent exposure of Trp residue in different peptides in absence and presence of lipid vesicles was assessed by monitoring fluorescence quenching by aqueous quencher iodide. Increasing amounts of 4 M KI (containing 1 mM $Na_2S_2O_3$) were added from 0 to 50 mM to the fixed peptide concentration of 2 μM. The data for fluorescence quenching were analysed by Stern-Volmer equation, $F_0/F = 1 + K_{sv}[Q]$, where, F_0 and F are the fluorescence intensities in absence and presence of quencher, respectively, at quencher concentration [Q], while K_{sv} is the Stern-Volmer quenching constant. The normalized accessibility factor (NAF) (De Kroon *et al.* 1990) was calculated from the ratio of values of K_{sv} obtained in presence and in absence of lipid vesicles.

2.5 *Fluorescence resonance energy transfer*

Energy transfer from donor Trp in peptides to acceptor DNS in lipid vesicles of PC, PC:PG (1:1) and PC:SM:CHL (1:1:1) compositions labelled with 2 mol% of DNS-PE was monitored in 5 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, in a Hitachi F4500 Fluorescence Spectrometer (Hitachi, Tokyo, Japan). To a fixed concentration of labelled lipid vesicles (50 μM), increasing concentration of peptide (peptide–lipid molar ratio (P/L)) from 1:100 to 1:10 was added and the energy transfer monitored by scanning the emission from 300 to 550 nm. At P/L of 1:10, when the energy transfer nearly saturated, excess of unlabelled vesicles (final concentration 500 μM) were added and the decrease in energy transfer was recorded. Appropriate blanks were subtracted from the sample data before plotting the fluorescence resonance energy transfer (FRET) spectra.

2.6 *90° Scatter*

The 90° scatter of Pmpplck was recorded at 2 μM concentration in 5 mM HEPES buffer, pH 7.4, containing 150 mM NaCl in a Hitachi F4500 Fluorescence Spectrometer (Hitachi, Tokyo, Japan) by keeping both excitation and emission monochromators at 450 nm (bandwidth 5 nm) and recording the scatter as a function of time for a period of 30 min after incubating the sample for 5 min at room temperature.

3. Results and discussion

We have synthesized unmodified (Plck), N-terminal myristoylated (Pmlck), and triply fatty acylated (Pmpplck, containing N-myristoyl moiety and two palmitoyl moieties at Cys-3 and Cys-6 side-chains) peptides corresponding to the SH4 domain of Lck (table 1). All three peptides have a net negative charge of −4 at neutral pH. The mono- and triply-acylated peptides were synthesized to probe the difference in membrane binding ability of peptides with varying numbers of fatty acyl chains.

The interaction of synthetic peptides with lipid vesicles was investigated by monitoring the fluorescence of the single tryptophan residue. The emission spectra of the peptides recorded in buffer are shown in figure 1. The unmodified and N-myristoylated Lck-series peptides Plck and Pmlck exhibited λ_{max} at 350–354 nm (figure 1), indicating that Trp residue was exposed to polar environment in these peptides. The triply acylated peptide Pmpplck shows a blue-shifted emission with λ_{max} around 345 nm, indicating a moderate hydrophobic environment of Trp residue. Scatter at 90° for Pmpplck recorded in the buffer condition used in this study (5 mM HEPES, pH 7.4, containing 150 mM NaCl) over 30 min shows no significant change in scatter compared

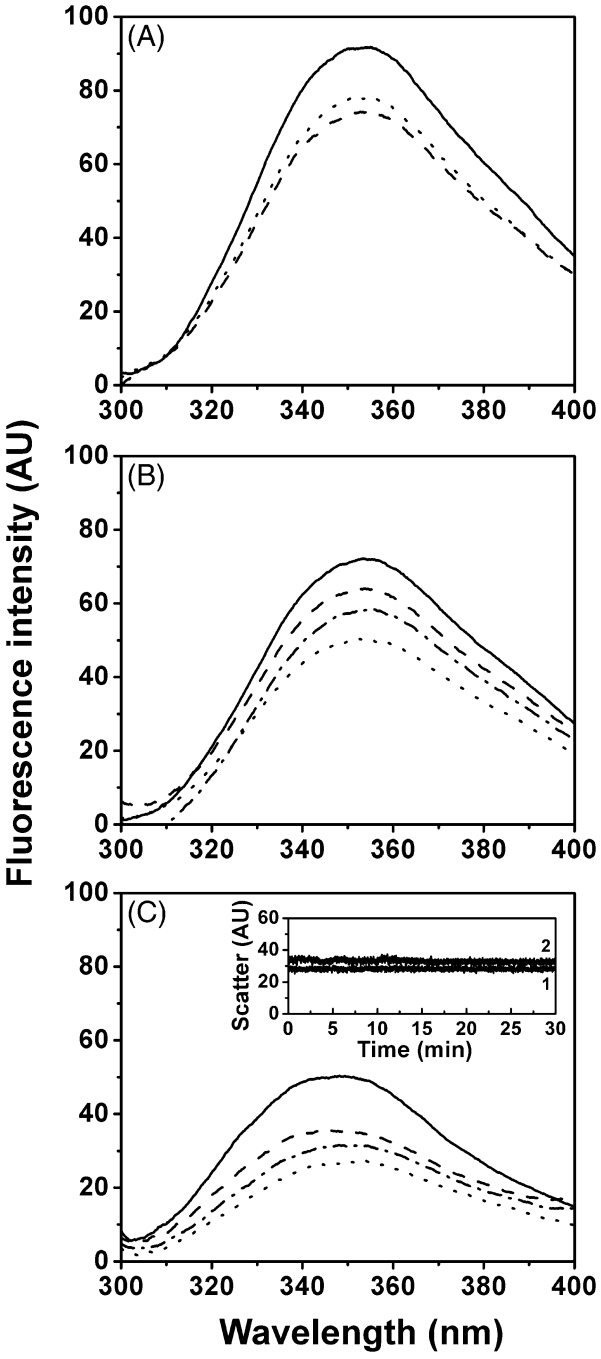

Figure 1. Tryptophan fluorescence emission spectra of Lck-related peptides (**A**) Plck, (**B**) Pmlck and (**C**) Pmpplck, in the absence and presence of lipid vesicles of varying compositions as indicated. The spectra were recorded in buffer alone (continuous line) and in the presence of large unilamellar vesicles of PC (dashed line), PC:PG (dotted line) and PC:SM:CHL (dash-dot-dash) compositions. The peptide-to-lipid ratio in all cases was 1:100 (molar ratio). The inset of panel (**C**) shows the 90° scatter of the triply acylated peptide Pmpplck recorded as a function of time. The traces in inset show scatter profile of (1) buffer blank and (2) 2 μM of Pmpplck.

to the buffer (inset of figure 1C), thus ruling out the possibility of aggregation of Pmpplck under these experimental conditions and timescales.

Change in fluorescence when the peptides were titrated with increasing amounts of lipid vesicles are shown in figure 2. All peptides show a decrease in fluorescence intensity with increasing amount of lipid vesicles, the decrease being more pronounced for fatty acylated peptides Pmlck and Pmpplck (figure 2). The Trp residue in these peptides, in the presence of lipid vesicles, experiences quenched emission probably from the phospholipid head groups of lipid bilayer. Such decrease in the quantum yield of fluorescence emission in the presence of lipid vesicles has also been reported for the Trp analog *N*-acetyl-L-tryptophanamide (NATA) (Mishra and Palgunachari 1996). Such quenching can be attributed to the excited-state energy transfer from indole ring of Trp residue to the phospholipid head groups in lipid bilayer. The data suggest that despite the absence of blueshift in Trp emission in presence of lipid vesicles, Trp residue in all three peptides remains accessible to the lipid head groups during excited-state lifetime. Pmlck and Pmpplck interact with the lipid vesicles, and the presence of net negative charge on these peptides does not appear to hinder Trp residue associating with lipid vesicles. Changes in F/F_0 are dependent on the lipid composition for Pmlck with maximum decrease observed in the presence of PC and PC:PG vesicles (figure 2B). The increase in F/F_0 beyond a lipid concentration of 150 mM observed with Pmlck suggests multiple modes of association with PC vesicles.

The accessibility of Trp residue to aqueous quencher I^- was monitored by recording Trp emission spectra after adding KI to the peptide solutions without or with lipid vesicles. The results of iodide quenching are shown in figure 3 as normalized accessibility factor (NAF). The data indicate that despite the lipid-vesicle-induced quenching of Plck fluorescence (figure 2), the Trp residue remains accessible to aqueous quencher iodide. Even though the changes in fluorescence intensity for mono- and triply-acylated peptides follow similar trends (figure 2), the data from NAF plots show that the Trp residue in acylated peptides remains significantly protected against quenching by iodide in presence of lipid vesicles (figure 3). Trp residue in Pmlck experiences higher degree of protection as compared to Pmpplck, indicating that Trp in triply fatty acylated peptide Pmpplck is more accessible to iodide compared to the myristoylated peptide Pmlck. These results indicate different orientations of Trp residue with respect to the lipid bilayer in peptide chains varying in the number of acyl chains attached to the peptide chain. The significantly low NAF value for Pmlck in the presence of PC:SM:CHL vesicles suggests preference of N-myristoylated peptide for vesicles with raft-like lipid composition.

To gain further insights into the location of Trp residue in lipid bilayers, FRET study was carried out by monitoring FRET from Trp in peptides to DNS-PE-doped lipid vesicles.

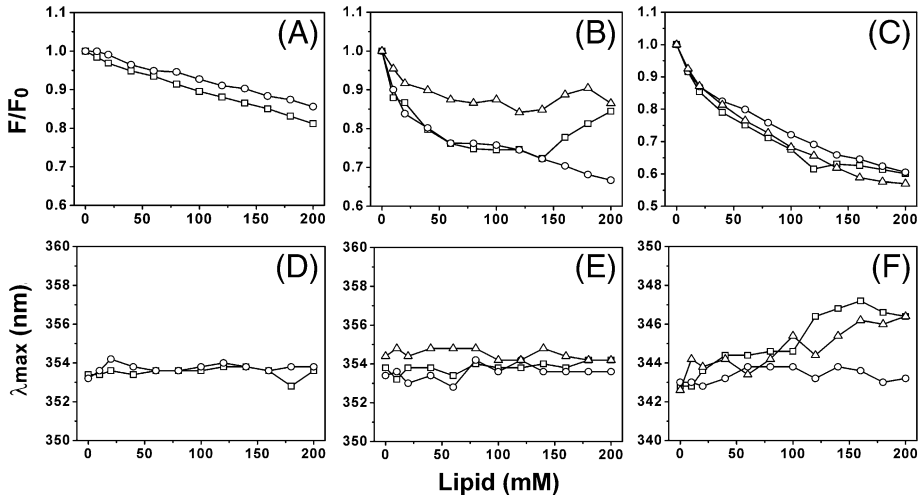

Figure 2. Changes in the fluorescence characteristics of Lck and fatty acylated Lck peptides as a function of lipid concentration. Changes in fluorescence intensity and position of emission maximum respectively are shown for the unacylated peptide Plck (**A** and **D**), the N-myristoylated peptide Pmlck (**B** and **E**), and the triply acylated peptide Pmpplck (**C** and **F**). The lipid vesicles used were of PC (—□—), PC:PG (—○—) and PC:SM:CHL (—△—) compositions.

In addition to fatty acylated Lck peptides, energy transfer from Trp in melittin to DNS-PE was also monitored to serve as a control as melittin binds strongly to lipid vesicles. The data (figure 4) show no energy transfer in peptide Plck in the presence of lipid vesicles. Extensive energy transfer from Trp in N-myristoylated peptide Pmlck and in melittin is observed with lipid vesicles. No energy transfer is observed when the peptide contains palmitoyl chains. The data suggest that N-myristoylation of the SH4 domain of Lck leads to Trp localization in the surface of the lipid bilayer such that energy transfer to DNS is possible. The Trp residue is not proximal to DNS for energy transfer to occur when the peptide chain is further acylated with additional palmitoyl chains. In order to assess the avidity of binding of peptides to lipid vesicles, large excess of unlabelled vesicles were added to peptide–labelled vesicles system (figure 5). The data show significantly decreased DNS emission intensity for Pmlck, indicating that a large fraction of peptide is stripped off from the labelled liposomes. The DNS fluorescence in the case of melittin on the other hand, although drops in the presence of PC:PG vesicles, remains largely unaffected in the presence of PC and PC:SM:CHL vesicles. These results suggest that although Trp residue in N-myristoylated peptide Pmlck inserts into lipid bilayer, such insertion is peripheral, unlike melittin. DNS is covalently linked to the head group of PE. Hence, the fluorophore will be localized in the interfacial region of the lipid bilayer. The observation that unlabelled vesicles are able to attenuate energy transfer between Pmlck and DNS further supports our argument that Trp in Pmlck is localized on the surface of the bilayer and not in the hydrophobic core. Both Pmlck and Pmpplck are anchored to the bilayer via their acyl chains. In Pmlck, the peptide chain is associated with the bilayer. In the triply acylated peptide Pmpplck, the peptide chain is oriented away from the bilayer. Differential acylation thus governs the orientation of the

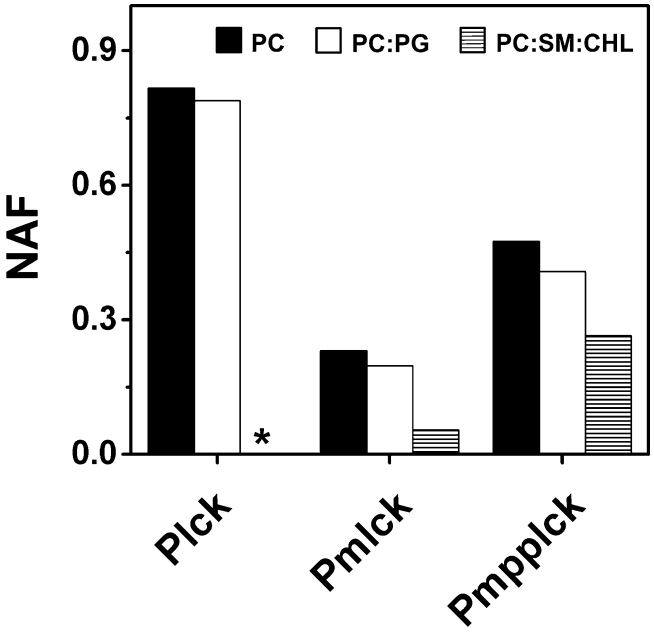

Figure 3. Exposure of Trp residue in unacylated and fatty acylated peptides to the aqueous quencher iodide in the presence of lipid vesicles expressed as net accessibility factor (NAF). The asterisk mark indicates that the NAF value for Plck in the presence of PC:SM:CHL vesicles was not determined.

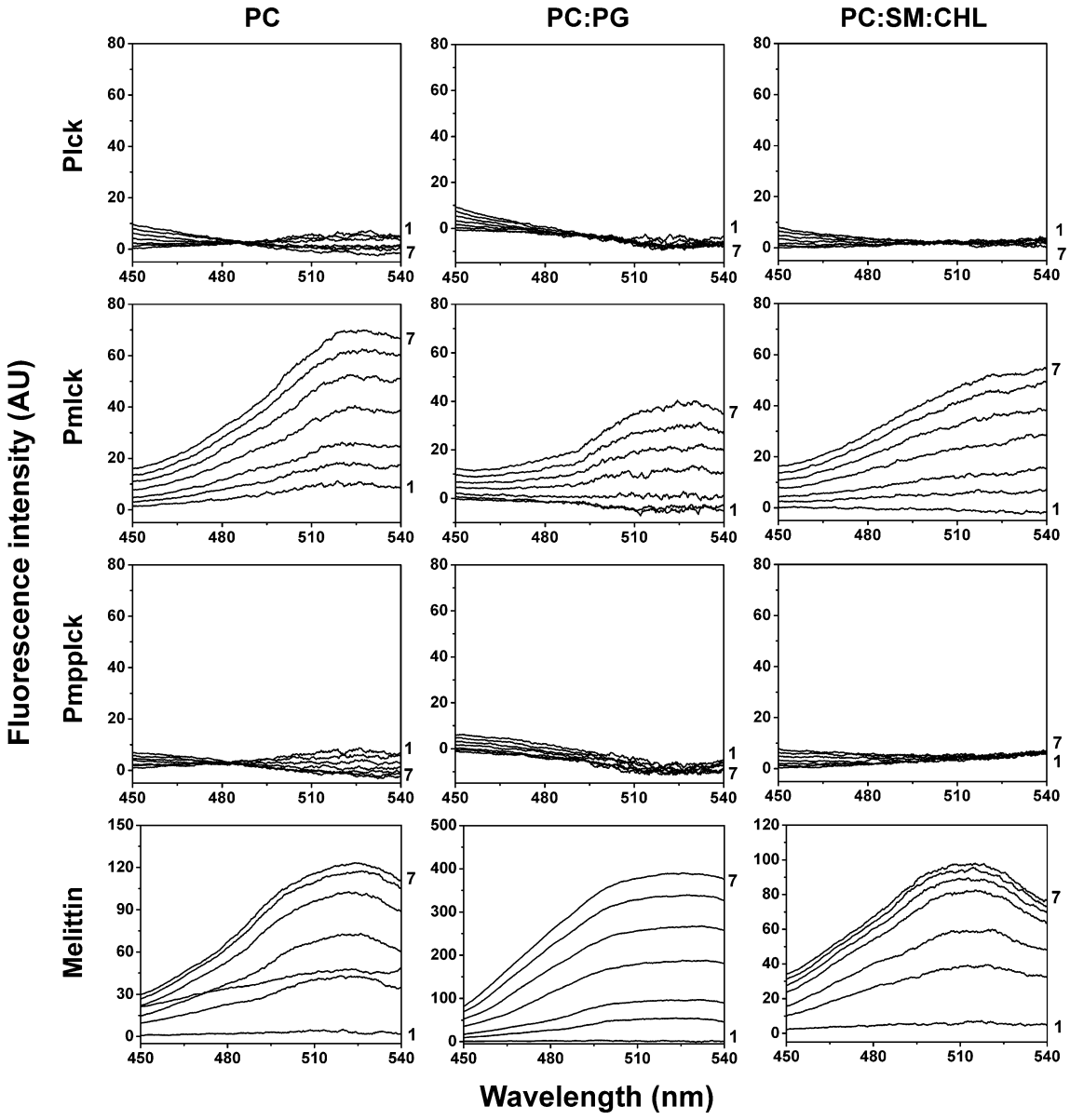

Figure 4. Fluorescence resonance energy transfer (FRET) from tryptophan residue in unacylated, fatty acylated Lck peptides and melittin to DNS-PE incorporated in LUV of PC, PC:PG and PC:SM:CHL compositions. Increasing amounts of peptides were added to the fixed concentration (50 μM) of LUV labelled with 2 mol% DNS-PE and spectra recorded for excitation at 280 nm. The numbers in traces indicate vesicle blank (1) and peptide–lipid ratios: 1:100 (2), 1:50 (3), 1:25 (4), 1:17 (5), 1:12.5 (6) and 1:10 (7).

peptide chain with respect to the bilayer plane as in the case of Fyn peptides (Rawat and Nagaraj 2010). The data also demonstrates that despite having net negative charge, the SH4 domain of Lck interacts even with negatively charged lipid membranes when myristoylated at the N-terminus, suggesting that myristoyl chain provides sufficient hydrophobicity for membrane association of this domain even though the peptide chain has a net negative charge and overcomes repulsive interaction with the negatively charged bilayer surface in PG vesicles. In fact, no head group preference is discernible between PC- and PG-containing vesicles. The presence of SM and CHL favours deeper insertion of the peptide chain into the lipid bilayer.

In an earlier study (Rawat and Nagaraj 2010), it was reported that dual acylation of the SH4 domain of Fyn, which carries a net neutral charge at physiological pH, leads to reorientation of the peptide chain with respect to the membrane plane. Multiple acylation of Lck sequence, too, like Fyn sequence (Rawat and Nagaraj 2010), results in the peptide chain being forced away from the bilayer surface,

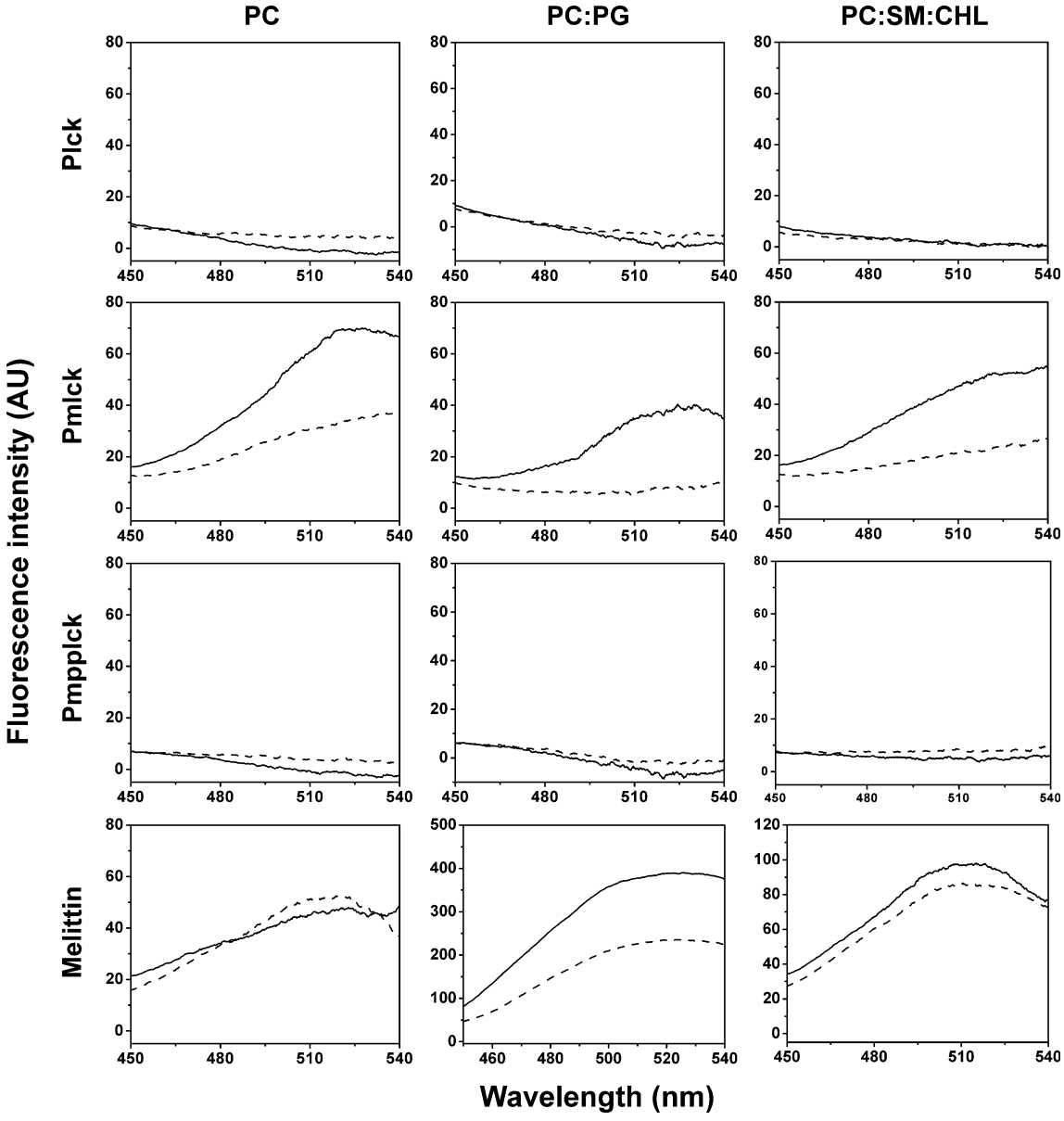

Figure 5. Effect of adding unlabelled vesicles to DNS-PE-labelled vesicles and peptides. A fixed concentration (50 μM) of vesicles of PC, PC:PG and PC:SM:CHL compositions labelled with 2 mol % of DNS-PE were titrated with increasing amounts of peptides, and to the end point of the titration, excess (500 μM) of unlabelled vesicles of PC, PC:PG and PC:SM:CHL compositions were added. Excitation was at 280 nm and spectra from 450–540 nm were recorded. Traces shown with continuous curves represent the DNS emission at the end point of titration (peptide–lipid ratio=1:10), while those shown with broken curves represent DNS emission after addition of unlabelled vesicles to the end point of titration.

Table 2. A comparison of NAF values between multiply acylated Lck- and Fyn-related peptides in different lipid environments

Lipid vesicles	Pmpplck (this study)	Pmpfyn (Rawat and Nagaraj 2010)
PC	0.48	0
PC:PG	0.41	0.13
PC:SM:CHL	0.26	0.75

unlike modification with single fatty acyl chain. In this case, the SH4 domains of both Fyn as well as Lck are associated with the bilayer. However, a comparison of NAF values between the multiply acylated Fyn and Lck sequences (table 2) suggests that there are differences in the manner the two SH4 domains (that is, neutral SH4 domain of Fyn and anionic SH4 domain of Lck) orient with respect to membrane plane upon fatty acylation. While in PC and PC:PG vesicles, Pmpplck is more accessible to iodide as compared to

Pmpfyn, in SM-containing vesicles, the peptide chain in Pmpplck is less accessible to iodide as compared to Pmpfyn. It is evident that depending on the nature of charged residues, there could be variations in the orientations of the peptide chain towards or away from the bilayer surface. While Fyn and Lck are involved in the activation of T lymphocytes, they interact with different cell surface receptors (Ley *et al.* 1994) and also differ in their biochemical activity (Zamoyska *et al*. 2003). Hence, variations in the orientation of the SH4 domains on the membrane surface could be relevant to the biological functions of Fyn and Lck.

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