Biol. Chem., Vol. 393, pp. 1173–1181, October 2012 • Copyright © by Walter de Gruyter • Berlin • Boston. DOI 10.1515/hsz-2012-0129

Biochemical and functional characterization of human phospholipid scramblase 4 (hPLSCR4)

Vincent Gerard Francis and Sathyanarayana N. Gummadi*

Department of Biotechnology, Applied Industrial Microbiology Laboratory, Indian Institute of Technology-Madras, Chennai 600 036, India

* Corresponding author
e-mail: gummadi@iitm.ac.in

Abstract

Human phospholipid scramblase 4 (hPLSCR4), an isoform of the scramblase family, is a type II single-pass transmembrane protein whose function remains unknown. To understand its role, recombinant hPLSCR4 was obtained by cloning the ORF into a pET28 a(+) vector and overexpressed in *Escherichia coli*. Functional assay showed that Ca^{2+}, Mg^{2+}, and Zn^{2+} activate hPLSCR4 and mediate scrambling activity independent of the phospholipid head group. Far-UV-CD and fluorescence spectroscopy revealed that Ca^{2+} and Mg^{2+} binding induces conformation change in hPLSCR4, exposing hydrophobic patches of the protein, and Ca^{2+} has more affinity than Mg^{2+} and Zn^{2+}. Stains-all studies further confirm that hPLSCR4 is a Ca^{2+}-binding protein. Point mutation (Asp^{290}→Ala) in hPLSCR4 decreased the Ca^{2+}-binding affinity as well as Tb^{3+} luminescence, suggesting residues of the predicted Ca^{2+}-binding motif are involved in Ca^{2+} binding. Functional reconstitution with (Asp^{290}→Ala) mutant led to ~50% and ~40% decrease in scramblase activity in the presence of Ca^{2+} and Mg^{2+}, respectively.

Keywords: ANS binding; Ca^{2+} binding; fluorescence; phospholipids; scramblase; spectroscopy; Stains-all.

Introduction

Human phospholipid scramblases (hPLSCRs) are a class of type II integral membrane proteins belonging to the ATP-independent class of phospholipid (PL) translocators (Sahu et al., 2007). Four isoforms of the scramblase family hPLSCR1-4 have been identified so far, which are highly conserved from *Caenorhabditis elegans* to humans (Wiedmer et al., 2000). hPLSCR1, the most widely studied isoform, has been shown to mediate nonspecific, bidirectional, and head group independent transbilayer movement of PL across the lipid bilayer in response to increased cytosolic Ca^{2+} concentration (Basse et al., 1996). hPLSCR1 has been shown to be activated during processes such as cellular activation, injury, and apoptosis, during which a 1000-fold increase in intracellular Ca^{2+} levels was observed (Sahu et al., 2007). Deduced amino acid sequences of hPLSCR2-4 share approximately 59%, 47%, and 46% homology with hPLSCR1. Sequence alignment revealed the presence of conserved domains among the members of the scramblase family, which includes a cysteine-rich domain, a Ca^{2+}-binding EF-hand domain and a single-pass transmembrane domain (Sahu et al., 2007).

Palmitoylation of hPLSCR1 localizes it to the plasma membrane (PM) and mutation of the palmitoylation motif localizes all the expressed protein completely to the nucleus (Chen et al., 2005). Both hPLSCR1 and hPLSCR4 have been shown to localize to the nucleus *via* the importin α/β pathway (Chen et al., 2005; Lott et al., 2011). Cytokines and interferon α have been shown to regulate the expression of hPLSCR1. Adult $PLSCR1^{-/-}$ mice had normal hemostasis but defective hematopoietic proliferation and differentiation in response to growth factors, indicating the protein's role in cellular signaling pathways (Zhou et al., 2002). hPLSCR4 has been shown to interact with CD4 at the PM, and its interaction is modulated by secretory leukocyte protease inhibitor (SLP1) (Py et al., 2009). It has been reported that hPLSCR1 acts as a transcription regulator of *IP3R* gene as well as enhancing the decatenation activity of human topoisomerase IIα in the nucleus (Zhou, 2005; Wyles et al., 2007).

Calcium signaling mechanisms regulate a wide variety of cellular processes such as gene expression, signal transduction, and enzyme activities among a wide variety of other cellular processes (Clapham, 2007). Ca^{2+}-binding proteins can either function as Ca^{2+} pools or as effector molecules of Ca^{2+} signaling (Niki et al., 1996). Ca^{2+} binding to proteins is characterized by the presence of helix-loop-helix motif called the EF-hand motif (Gifford et al., 2007). The loop region of this motif is involved in metal ion coordination and in most proteins Ca^{2+} binding is associated with conformation change (Yap et al., 1999). The members of the scramblase family possess a single nonclassical EF-hand-like Ca^{2+}-binding motif having 80–90% homology with Ca^{2+}-binding motif (Sahu et al., 2007). Mutations within the predicted EF-hand-like motif had been shown to disrupt the scramblase activity of hPLSCR1 (Zhou et al., 1998). Although the scrambling activity of hPLSCR1 is known, no reports exist whether other members of the scramblase family also mediate transbilayer movement of PLs in the presence of Ca^{2+}. In this article, for the first time, we demonstrate the scrambling activity of hPLSCR4 in the presence of Ca^{2+}. In addition, we also show metal ion-induced conformational change of hPLSCR4 using various spectroscopic techniques.

Results

Overexpression and purification of the recombinant hPLSCR4 and Asp^{290}→Ala mutant

To characterize hPLSCR4, we subcloned the hPLSCR4 ORF from a mammalian vector into pET-28 a(+) vector and overexpressed in *Escherichia coli* BL21 (DE3) as 6XHis-hPLSCR4. The Asp^{290}→Ala mutant of hPLSCR4 was generated by site-directed mutagenesis and also expressed as 6XHis-(Asp^{290}→Ala)-hPLSCR4 mutant. Results showed that both the overexpressed proteins were exclusively found in inclusion bodies (IBs), and low levels of expression were observed in the soluble fraction (Figure 1A) (data not shown for the Asp^{290}→Ala mutant). The molecular weight of the overexpressed protein was ~37 kDa and matched with the reported molecular weight (Basse et al., 1996). Hence, IBs were used as a protein source to purify hPLSCR4 using *N*-lauroyl sarcosine (NLS), an anionic detergent, to solubilize IBs overnight. After NLS treatment, ~50% of the target protein was solubilized, and the supernatant was pulse dialyzed to remove NLS. As membrane proteins require the presence of low levels of detergent for stability, we replaced NLS with 0.025% (w/v) Brij-35, a non ionic detergent. The supernatant was then loaded on to a Ni-NTA column and the bound protein was eluted with 250 mM imidazole. The final yield of protein was ~2 mg/g cell mass and the purity was 98% (Figure 1B). The Asp^{290}→Ala mutant of hPLSCR4 was also purified in the same way as described above (data not shown).

Biochemical reconstitution and functional scramblase assay

To determine whether hPLSCR4 (an isoform of hPLSCR1) is involved in rapid scrambling of PLs across the bilayer in the presence of elevated levels of calcium (Basse et al., 1996; Sahu et al., 2008), we reconstituted hPLSCR4 in to artificial lipid bilayers (proteoliposomes), and scramblase activity was initiated by the addition of Ca^{2+}. No significant scramblase activity was observed in the absence of Ca^{2+} (Figure 2A, trace a), but in the presence of Ca^{2+}, ~4% NBD-PC scrambling activity was observed (Figure 2A, trace b). This suggests that scramblase activity of hPLSCR4 is calcium dependent. Interestingly, hPLSCR4 showed more scrambling activity (~10%) for NBD-PS in the presence of Ca^{2+} (Figure 2B, trace b). Scramblase activity performed in the presence of other divalent metal ions such as Mg^{2+} and Zn^{2+} showed transbilayer movement of PLs independent of the PL head group (Figure 2A, traces b and c). However, the percentage of scramblase activity was higher for proteoliposomes labeled with NBD-PS compared with NBD-PC. To characterize whether the predicted Ca^{2+}-binding motif of hPLSCR4 is important for PL translocation activity of hPLSCR4, a point mutation (Asp^{290}→Ala) in the predicted Ca^{2+}-binding segment was generated. The functional reconstitution of the Asp^{290}→Ala mutant was assayed for activity in the presence of Ca^{2+} and Mg^{2+}. The mutant showed ~50% and ~40% decrease in scrambling activity, indicating the importance of this motif in metal ion-induced activation of scramblase (Figure 2C).

Intrinsic tryptophan fluorescence

The affinity of metal ions (Ca^{2+}, Mg^{2+}, and Zn^{2+}) to hPLSCR4 was studied by measuring the intrinsic tryptophan fluorescence. To confirm whether the predicted Ca^{2+}-binding motif is indeed the Ca^{2+}-binding site in the protein, the binding constant for the Asp^{290}→Ala mutant was also measured. Figure 3A–C shows the quenching of intrinsic tryptophan fluorescence in the presence of metal ions, where trace a is

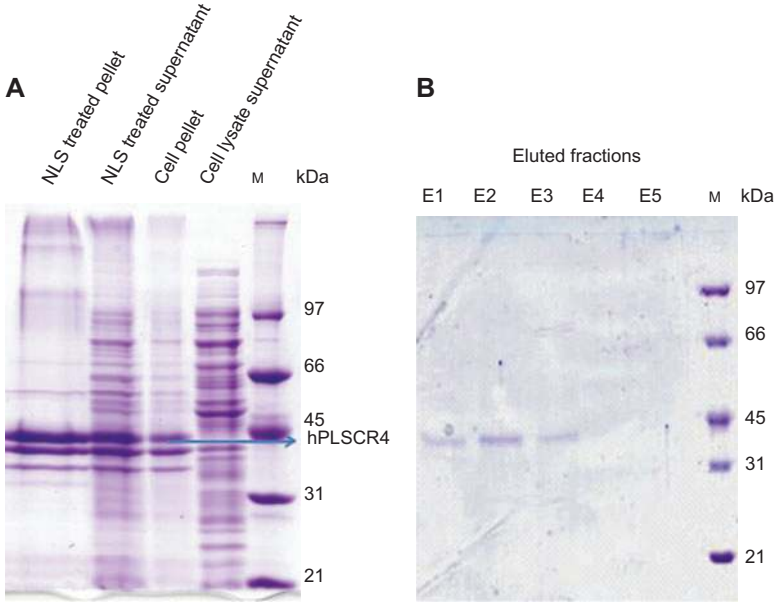

Figure 1 Overexpression and purification of hPLSCR4.
(A) Cells were lysed and checked for the expression of the protein on 12% SDS-PAGE. (B) Ni-NTA purification of 6X His tag hPLSCR4.

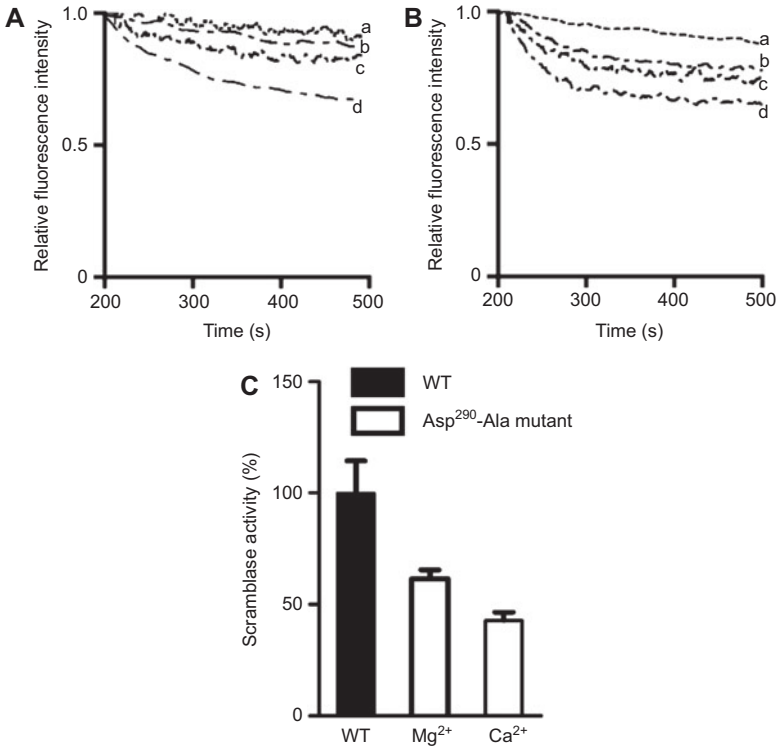

Figure 2 Scramblase assay.
(A) Scramblase activity using proteoliposomes reconstituted with NBD-PC. (B) Scramblase activity using proteoliposomes reconstituted with NBD-PS. Trace a, proteoliposomes incubated with 2 mM EGTA; trace b, proteoliposomes incubated with 2 mM Ca^{2+}; trace c, proteoliposomes incubated with 2 mM Mg^{2+}; trace d, proteoliposomes incubated with 2 mM Zn^{2+}. (C) The comparative rate of scramblase activity with NBD-PC as tracer using proteoliposomes reconstituted with Asp^{290}→Ala mutant in the presence of Ca^{2+} and Mg^{2+}, calculated by normalizing it with respect to wild-type scramblase activity. Results are representative of at least three sets of experiments ($p<0.05$).

the intrinsic tryptophan fluorescence of apoprotein and traces b, c, and d in the presence of 2, 5, and 10 mM of metal ion, respectively. A slight change in the emission maxima (~4 nm) toward shorter wavelength was observed in the presence of metal ions. This suggests that hPLSCR4 undergoes conformation change in the presence of metal ions. Binding constants (K_a) were determined by Scatchard plot using nonlinear curve fitting (Prism 3.0 GraphPad software Inc., San Diego, USA). Curve fitting was done for the fraction of ligand sites occupied $[(F_o\text{-}F)/(F_o\text{-}F_{sat})]$ vs. ligand concentration using the one-site binding nonlinear regression analysis, where F_o is the fluorescence in the absence of a ligand, F_{sat} is the fluorescence at saturation of ligand concentration, and F is the fluorescence in the presence of a particular ligand concentration. It has been found that hPLSCR4 has a greater affinity for Ca^{2+} than Mg^{2+} and Zn^{2+} (Table 1). Furthermore, in the case of the Asp^{290}→Ala mutant, an approximately 30% decrease in affinity of hPLSCR4 for Ca^{2+} was observed, indicating the predicted Ca^{2+}-binding motif is indeed the Ca^{2+}-binding site in the protein (Table 1).

Stains-all assay

Stains-all was used as a Ca^{2+} mimic dye to probe the Ca^{2+}-binding properties of hPLSCR4. In the free form, the dye produces two distinct absorption bands at 535 and 575 nm, which correspond to the monogenic and dimeric forms of the dye, respectively. However, when the dye binds to proteins containing Ca^{2+}-binding motifs, it induces a distinct J band at 650 nm (Caday et al., 1986). No J band was observed in the free form of the dye (Figure 4A and B, trace a). Stains-all binds to hPLSCR4 and induces an intense J band at 650 nm (Figure 4A and B, trace b). However, the intensity of the J band decreased when Ca^{2+} or Mg^{2+} (2 mM) was added to the saturated hPLSCR4-Stains-all complex, indicating displacement of Stains-all by the metal ions, which have a higher affinity for the protein (Figure 4A and B, trace c). These results confirm that the metal ions bind to the putative Ca^{2+}-binding motif of hPLSCR4 and induce a conformational change.

Terbium fluorescence

Tb^{3+} is used as a luminescent isomorphous replacement probe for Ca^{2+} and was used here to study Ca^{2+} interaction with hPLSCR4. Tb^{3+} excitation at 285 nm induces luminescent peaks at 491 and 547 nm (Brittain et al., 1976). Tb^{3+} luminescence is enhanced due to fluorescence resonance energy transfer from aromatic amino acids located close to the binding site. Figure 4C shows that addition of Tb^{3+} to hPLSCR4

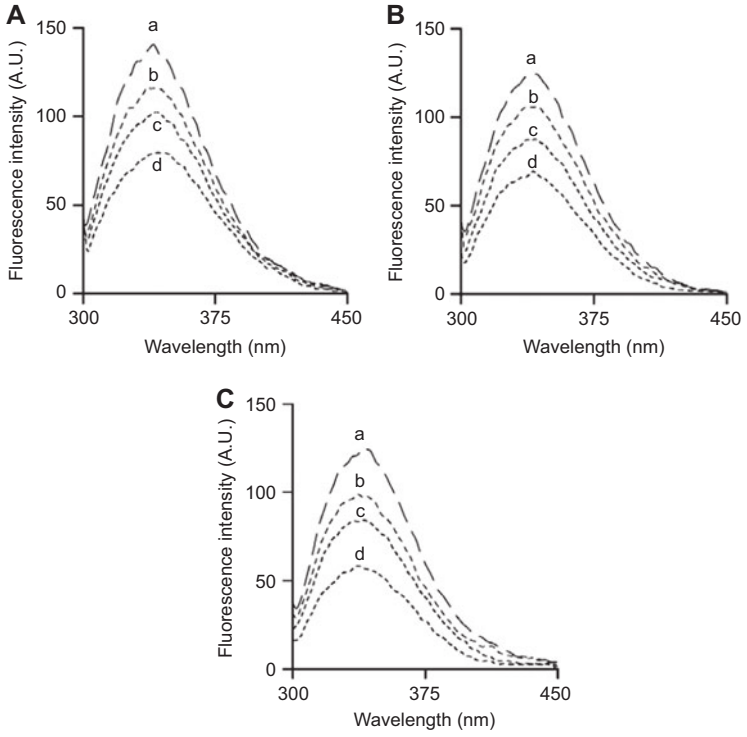

Figure 3 Intrinsic tryptophan fluorescence.
(A) Effect of Ca^{2+} on the intrinsic tryptophan fluorescence of hPLSCR4. (B) Effect of Mg^{2+} on the intrinsic tryptophan fluorescence of hPLSCR4. (C) Effect of Zn^{2+} on the intrinsic tryptophan fluorescence of hPLSCR4. Trace a, apoprotein; trace b, protein with 2 mM metal ion; trace c, protein with 5 mM metal ion; trace d, protein with 10 mM metal ion. Results are representative of at least three sets of experiments ($p<0.05$).

leads to an increase in emission intensity of two luminescent peaks, accompanied by decreased Trp fluorescence emission. Luminescence peaks indicate movement of Trp residues toward the metal ion-binding site. To validate the predicted Ca^{2+}-binding site of hPLSCR4, the Tb^{3+} luminescence of the $Asp^{290}\rightarrow Ala$ mutant was also measured. Decreased Tb^{3+} luminescence was observed in the case of the $Asp^{290}\rightarrow Ala$ mutant compared with wild-type hPLSCR4 indicating the importance of this residue in Ca^{2+} coordination (Figure 4D). Furthermore, when Ca^{2+} was added to this saturated complex of hPLSCR4-Tb^{3+}, there was a decrease in the Tb^{3+} luminescence peaks along with an increase in Trp fluorescence, indicating displacement of bound Tb^{3+} by Ca^{2+} (Supplementary Figure S1).

Table 1 Binding constants of the hPLSCR4 and $Asp^{290}\rightarrow Ala$ mutant interaction with Ca^{2+}, Mg^{2+}, and Zn^{2+}.

Metal ion	K_a (μM)	R^2
hPLSCR4 affinity for Ca^{2+}	23.0±3.2	0.95
hPLSCR4 ($Asp^{290}\rightarrow Ala$) for Ca^{2+}	16.6±2.6	0.94
hPLSCR4 affinity for Mg^{2+}	10.5±1.6	0.98
hPLSCR4 affinity for Zn^{2+}	16.1±0.8	0.98

The binding constants of the protein ligand interaction were determined from the Scatchard plot using nonlinear regression analysis.

Far-UV-CD spectroscopy

The metal ion-induced conformation change of hPLSCR4 was further investigated by far-UV-CD spectroscopy. The apo form of the hPLSCR4 was characterized by two distinct minima at 208 and 223 nm, which are characteristic of proteins containing α-helix conformational elements (Figure 5A and B, trace a). These results showed that in the presence of Ca^{2+} and Mg^{2+}, spectral changes are observed in the CD spectra of hPLSCR4 (Figure 5A and B, trace b). These results indicate that metal ion binding induces conformational changes.

Probing conformational (surface hydrophobicity) changes by 8-anilino-1-naphthalenesulfonic acid fluorescence

8-Anilino-1-naphthalenesulfonic acid (ANS) was used to study metal ion-induced changes in the hydrophobicity of hPLSCR4. ANS is fluorescent only in a nonpolar environment, and its fluorescence is quenched in the presence of a polar environment (Daniel and Weber, 1966). In Figure 5C, trace a represents the fluorescence emission of the ANS-protein complex in the absence of metal ions. In the presence of metal ions, enhanced ANS fluorescence with a blue shift of ~10 nm was observed (Figure 5C, traces b, c, and d). This indicates that the apo-hPLSCR4 is in an ordered state,

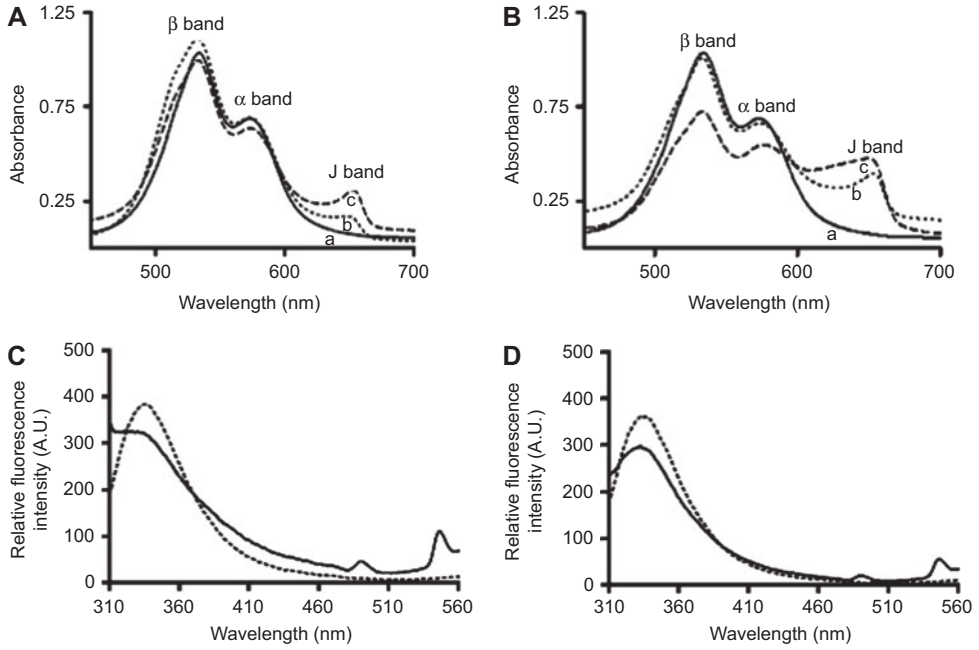

Figure 4 Stains-all binding and Tb^{3+} luminescence spectroscopy.
(A) Effect of Ca^{2+} on the Stains-all-hPLSCR4 complex. Trace a, Stains-all dye spectra; trace b, Stains-all-hPLSCR4 complex; trace c, effect of Ca^{2+} on the Stains-all-hPLSCR4 complex. (B) Effect of Mg^{2+} on the Stains-all-hPLSCR4 complex. Trace a, Stains-all dye spectra; trace b, Stains-all-hPLSCR4 complex; trace c, effect of Mg^{2+} on the Stains-all-hPLSCR4 complex. (C) Tb^{3+} luminescence of apo-hPLSCR4 (dashed line) and Tb^{3+}-bound hPLSCR4 (solid line). (D) Tb^{3+} luminescence of apo $Asp^{290}\rightarrow Ala$ (dashed line) and Tb^{3+}-bound $Asp^{290}\rightarrow Ala$ mutant (solid line). Results are representative of at least three sets of experiments ($p<0.05$).

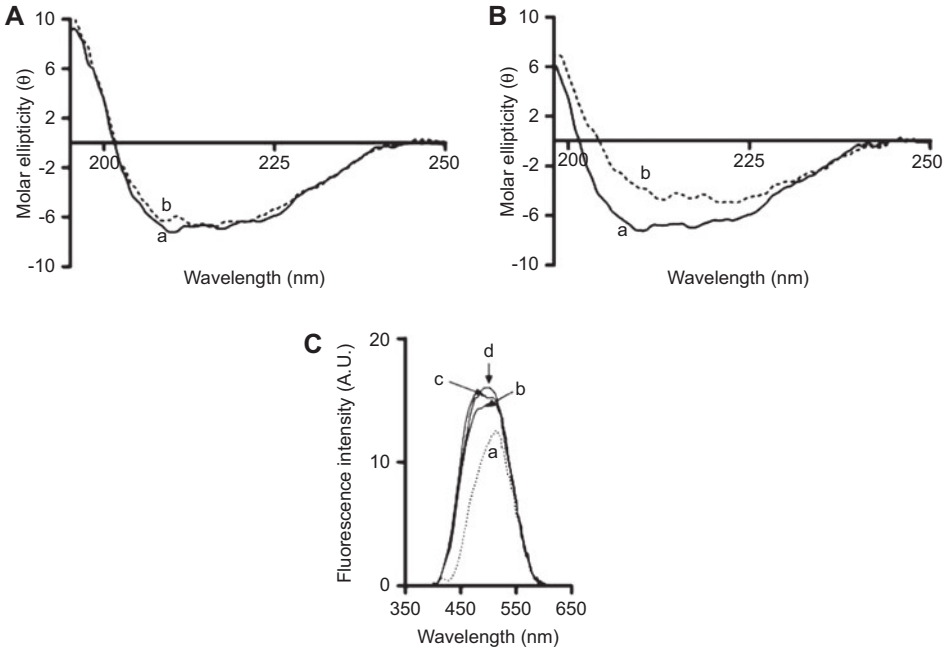

Figure 5 Far-UV-CD and ANS binding.
(A) Trace a, far-UV-CD of apo-hPLSCR4; trace b, far-UV-CD of hPLSCR4 in the presence of 2 mM Ca^{2+}. (B) Trace a, far-UV-CD of apo-hPLSCR4; trace b, far-UV-CD of hPLSCR4 in the presence of 2 mM Mg^{2+}. (C) ANS binding to hPLSCR4. Trace a, ANS-hPLSCR4 complex (control); trace b, 2 mM Ca^{2+} addition to ANS-hPLSCR4 complex; trace c, 2 mM Mg^{2+} addition to ANS-hPLSCR4 complex; trace d, 2 mM Zn^{2+} addition to ANS-hPLSCR4 complex. Results are representative of at least three sets of experiments ($p<0.05$).

and in the presence of metal ions, this state was altered, leading to increased surface exposure of hydrophobic residues on hPLSCR4.

Discussion

Four homologs of PLSCR1 have been identified in humans so far and are involved in a wide range of cellular processes (Sahu et al., 2007). Recently, the fifth homolog of the scramblase family has been identified but has not yet been characterized at the protein level. Initially, hPLSCR1 was identified as a protein capable of mediating transbilayer movement of PLs when activated by Ca^{2+} (Basse et al., 1996). However, studies have also shown the involvement of hPLSCR1 in cellular signaling, suggesting that this protein also functions as a signaling molecule (Zhou et al., 2002). Although hPLSCR1 has been widely characterized, other isoforms of scramblase are not well characterized. To elucidate the possible function of hPLSCR4, we characterized the ion binding, scramblase activity, validation of the predicted Ca^{2+}-binding site, and metal ion-induced conformation changes because it possesses a putative EF hand-like Ca^{2+}-binding motif similar to hPLSCR1. Hence, we cloned the ORF of hPLSCR4, overexpressed it in *E. coli*, and purified it from IBs using NLS.

To examine whether hPLSCR4 mediates the transbilayer movement of PLs similar to hPLSCR1, we reconstituted hPLSCR4 into NBD-labeled vesicles. We have shown that in the presence of Ca^{2+}, hPLSCR4 gets activated and mediates transbilayer movement of PLs. The scrambling activity of hPLSCR4 was also checked in the presence of Mg^{2+} and Zn^{2+} and was also found to activate the protein. However, higher scramblase activity by hPLSCR4 was observed in the presence of Zn^{2+} and Mg^{2+} than Ca^{2+}. This difference could be attributed to the difference in the protein-folding pattern of a bacterial-expressed protein as well as the protein conformation in a detergent solution vs. membrane-inserted polypeptide. Similar differences in hPLSCR1 (overexpressed and purified from *E. coli*) activity were observed for Ca^{2+} vs. other metal ions, where Zn^{2+} showed more activity than calcium (Stout et al., 1998). Additionally, for the EF-hand peptide of hPLSCR4, the Mg^{2+}-binding affinity was higher than Ca^{2+} binding (Sahu et al., 2009).

As hPLSCR4 belongs to an ATP-independent class of PL translocators, we also checked for PL head group specificity. Unlike multidrug-resistant proteins and aminophospholipid translocators, which show selectivity for PLs and mediate vectorial transport, members of the scramblase family show head group-independent translocation of PL across the bilayer. hPLSCR4-mediated transbilayer movement of PLs was found to be independent of the PL head group; however, the rate of scrambling for NBD-PS PLs was found to be higher than NBD-PC. Similar rates of scrambling for NBD-PC and NBD-PS PLs were observed with hPLSCR1-reconstituted proteoliposomes (Sahu et al., 2008). This could be justified because Ca^{2+}-activated scramblase mediates the movement of PL from the inner leaflet to the outer leaflet of the PM and the inner leaflet is selectively enriched in anionic PL. These results are in agreement with that of hPLSCR1 (Sahu et al., 2008). Reports have indicated that the rate of scrambling by scramblase depends on the head group size (Dekkers et al., 2002). However, scramblases are relatively nonspecific and mainly serve to translocate PS on the outer leaflet of the PM upon activation.

We further characterized the Ca^{2+}-binding properties of hPLSCR4 using Stains-all; the dye produces a series of characteristic spectra depending on the interaction and conformation of the binding region (Caday et al., 1986). Addition of Ca^{2+} and Mg^{2+} leads to the displacement of Stains-all dye, indicating that the metal ions bind to the putative EF-hand motif. This result is in agreement with earlier reported data on Stains-all binding to hPLSCR1 (Sahu et al., 2008). We further investigated whether metal ion binding induces conformation change in hPLSCR4 using various spectroscopic techniques. Stout et al. (1998) have proposed a change in the conformation of hPLSCR1 due to Ca^{2+} binding. Far-UV-CD studies have revealed spectral changes in the CD spectra of hPLSCR4, which is predominantly an α-helical protein, indicating metal ion-induced conformational changes. Similar spectral changes are generally observed during metal ion binding to Ca^{2+}-binding proteins and EF-hand peptides (Kobayashi et al., 2005; Zhao et al., 2012).

To validate whether the predicted Ca^{2+}-binding motif of hPLSCR4 is indeed the true Ca^{2+}-binding site in the protein, we generated the $Asp^{290}\rightarrow Ala$ mutant because residues such as Asp are highly conserved in the EF-hand motif and are important for the octahedral coordination of Ca^{2+}. Similar mutation of Asp^{275} in the predicted Ca^{2+}-binding motif of hPLSCR1 has been shown to decrease the scramblase activity by ~90% compared with wild type (Stout et al., 1998). The affinity of Ca^{2+} for hPLSCR4 was found to be the highest and is consistent with earlier reported data on metal ion binding to EF-hand peptide of hPLSCR4 (Sahu et al., 2009). The decreased binding affinity of Ca^{2+} for $Asp^{290}\rightarrow Ala$ mutant indicates the importance of this residue for Ca^{2+} binding and also the decreased rate of PL scrambling by $Asp^{290}\rightarrow Ala$ mutant confirms that PL-mobilizing activity of hPLSCR4 is metal ion dependent. Binding affinity values in the micromolar range are of physiological relevance, as intracellular Ca^{2+} concentrations within the cell are in the nanomolar range and activation of scramblase requires a 1000-fold increase in intracellular Ca^{2+} concentrations, i.e., micromolar range. Generally, Ca^{2+}-binding proteins act as buffers for storage and release of Ca^{2+} ions or as Ca^{2+}-signaling molecules (Niki et al., 1996; Mikhaylova et al., 2011). Binding affinity values reveal that hPLSCR4 is a weak Ca^{2+}-binding protein and may be the reason behind its requirement for a 1000-fold increase in intracellular Ca^{2+} concentration to get activated. Such increase in intracellular Ca^{2+} is generally seen during apoptosis, which may be required for the activation of scramblases, which are known to be involved in the same (Olofsson et al., 2008). These results indicate that hPLSCR4 probably functions as a Ca^{2+} sensor and is activated in the presence of high Ca^{2+} concentrations.

We further validated the Ca^{2+}-binding site of hPLSCR4 using Tb^{3+} luminescence spectroscopy. Tb^{3+} binding was associated with enhanced luminescence, indicating energy

transfer from Trp residues. Decreased luminescence of the Asp^{290}→Ala mutant compared with the wild-type hPLSCR4 further confirms the importance of these residues in Ca^{2+} coordination (Figure 4C and D). Addition of Ca^{2+} to the Tb^{3+}-hPLSCR4 complex leads to the displacement of Tb^{3+}, indicating that both the metal ions bind at the same site in the protein. Metal ion-induced conformation changes in hPLSCR4 were further characterized by monitoring surface hydrophobicity changes using ANS – a fluorescent probe. Metal ion binding to hPLSCR4 leads to increased surface exposure of hydrophobic patches, indicating ion-induced conformation change. Generally, Ca^{2+} sensor proteins undergo conformation change in the presence of Ca^{2+}, leading to exposure of hydrophobic residues, whereas Ca^{2+} buffer proteins do not expose any hydrophobic residues in the presence of Ca^{2+} (Berggård, 2002; Venkitaramani et al., 2005). Exposed hydrophobic patches may possibly serve as binding sites for other proteins or may undergo oligomerization. Metal ion-induced exposure of hydrophobic residues of hPLSCR4 may be required for the interaction with its binding partners. Similar conformational changes have been observed during metal ion binding to calbindin D_{28k} (Berggård et al., 2000).

In conclusion, we report for the first time, metal ion-induced transbilayer movement of PLs mediated by hPLSCR4. Point mutation confirms that residues in the predicted Ca^{2+}-binding site of the protein is important for Ca^{2+} coordination and are important for the PL translocation activity of hPLSCR4. Furthermore, metal ions bind to the Ca^{2+}-binding site and induce conformation change in the protein, which may be required for the activation of the protein.

Materials and methods

Materials

NLS, egg phosphatidyl choline (egg PC), phosphatidyl serine (PS), and phenyl methane sulfonyl fluoride (PMSF) were obtained from Sigma (USA); *E. coli* DH5α and *E. coli* BL21 (DE3) strains were obtained from ATCC (USA). The cDNA of hPLSCR1 was purchased from Invitrogen (USA) and pET-28 a(+) was obtained from Novagen (USA). Fluorescent-labeled lipid, 7-nitrobenz-2-oxa-1,3-diazol-4-yl-phosphatidyl choline (NBD-PC), 7-nitrobenz-2-oxa-1,3-diazol-4-yl-phosphatidylserine (NBD-PS) were purchased from Avanti Polar Lipids, Inc. (USA). SM2 Bio-Beads, Chelex-100 resin, and protein molecular weight markers were obtained from BioRad, (USA). The Ni^{2+}-NTA matrix was purchased from Qiagen (USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG), dithiothreitol (DTT), ethylene glycol tetraacetic acid (EGTA), molecular biology-grade $CaCl_2$, and other routine chemicals were purchased from Himedia (India).

Plasmid construction

hPLSCR4 ORF was directionally cloned between the *Nde*I *and Xho*I sites of the pET-28 a(+) expression vector maintaining the reading frame. The ORF was amplified using forward primer 5′-GGC CCA TAT GAT GTC AGG TGT GGT ACC C-3′ and reverse primer 5′-CCA CAA CGT TCA AGA TAG AGC TCG AGC GG-3′ containing *Nde*I and *Xho*I sites, respectively. PCR-amplified product was then digested with *Nde*I and *Xho*1 and ligated into pET-28 a(+) vector digested with the same enzymes. The cloned insert was further verified by sequencing both the strands. Site-directed mutagenesis was done using the QuikChange® II XL site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. To generate the Asp^{290}→Ala mutant of hPLSCR4, the following primers were used 5′-GCA ATG GCA GAT GCT **GCC** CAT TTT GAC ATT CAC-3′ and 5′-GTG AAT GTC AAA ATG **GGC** AGC ATC TGC CAT TGC-3′ were used, and mutation was confirmed by sequencing.

Overexpression of the recombinant hPLSCR4 and Asp^{290}→Ala mutant

Protein overexpression was achieved by the transformation of the hPLSCR4-pET-28 a(+) and Asp^{290}→Ala mutant hPLSCR4 plasmids into the BL21 (DE3) strain of *E. coli* by chemical transformation. The transformants were then selected on an LB agar plate containing 100 µg/ml kanamycin. A single colony was then inoculated into the LB broth containing kanamycin (100 µg/ml) and was grown at 37°C and 180 rpm until the A_{600} reached ~0.5. The cells were then induced with 0.1 mM IPTG, and these cells were maintained in the broth at 37°C and 180 rpm for another 4–6 h. Induced cells (1 g) were then harvested and resuspended in 10-ml buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM PMSF, 1 mM EDTA, and 1 mM DTT. The cell suspension was then subjected to probe sonication for 2 min (1 s on/2 s off) at 30% amplitude. The soluble and insoluble fractions were separated by centrifugation at 4°C for 20 min at 18 000 *g*, and the localization of the overexpressed protein was analyzed on 12% SDS-PAGE.

Purification of 6X His tag-hPLSCR4

The insoluble fraction was resuspended in 10 ml of buffer A (20 mM Tris-HCl, 200 mM NaCl, pH 7.5) with 0.3% (w/v) NLS. The suspension was agitated for 24 h at 130 rpm and 20°C followed by centrifugation at 4400 *g* for 15 min at 4°C. Subsequently, NLS was replaced with 0.025% (w/v) Brij-35 (buffer B) in all the steps of purification. The supernatant was then subjected to dialysis with 20-kDa cutoff membrane against buffer B, maintaining the protein concentration at 100 µg/ml with three buffer changes after every 12 h. Absorbance at 215 nm was used to determine the amount of the residual NLS present after dialysis. The protein solution was then filtered through a 0.22-µm membrane filter and loaded onto a Ni-NTA column, equilibrated with buffer B before use. Binding was carried out for 10 min at 25°C, and the protein was finally eluted out with buffer B containing 250 mM imidazole. The eluted protein was then repeatedly dialyzed against buffer B made from water treated with Chelex-100 resin (BioRad) to completely remove the imidazole and make the protein free of metal ions. The purification of the Asp^{290}→Ala mutant of hPLSCR4 was done similarly as described above.

Scramblase assay

Symmetrically labeled proteoliposomes reconstituted with the hPLSCR4 and Asp^{290}→Ala mutant were prepared as described earlier (Rajasekharan and Gummadi, 2011). Schematic representation of scramblase assay is shown in Supplementary Figure S2. Egg PC and PS (9:1) along with 0.3 mol% NBD-PLs were briefly dried under a stream of N_2 gas and solubilized in buffer C (10 mM HEPES/NaOH-7.5, 100 mM NaCl) with 1% (w/v) Triton-X100 to solubilize the lipids. For proteoliposome preparation, 0.1 mg of each protein was added to the solubilized lipids followed by the addition of SM2 Bio-Beads to completely remove Triton-X100. Inside-labeled proteoliposomes were prepared from symmetrically labeled proteoliposomes

as described earlier (Sahu et al., 2008). For scramblase assay, inside-labeled proteoliposomes were incubated for 2 h at 37°C in buffer B with various metal ions (2 mM) or 4 mM EGTA (control). Sample fluorescence (excitation at 470 nm and emission at 532 nm) was monitored continuously at 25°C with constant low speed stirring and slit widths of 3 nm (excitation) and 5 nm (emission). The initial fluorescence was recorded in a spectrofluorometer for 100 s until the values stabilized. Then, 20 mM dithionite (freshly prepared in 1 M Tris base) was added, and the fluorescence was monitored for the next 300 s. The difference between the residual fluorescence in the presence and absence of metal ions after dithionite addition is attributed to metal ion-induced scrambling of the NBD-PC located in the inner leaflet of proteoliposomes. Scramblase activity was calculated as

$$\text{Scramblase activity (\%NBD-PL translocated)} = F_{\text{metal ion}} - F_{\text{control}},$$

where $F_{\text{metal ions}}$ is the relative scramblase activity in the presence of metal ions and F_{control} is the relative scramblase activity in the presence of EGTA (control).

Intrinsic tryptophan fluorescence spectroscopy

Steady-state fluorescence was measured using Perkin Elmer LS-55 fluorescence spectrofluorometer (USA). Fluorescence spectra emission was recorded at unitary wavelengths between 300 and 500 nm, with an excitation wavelength of 295 nm at 25°C and a scanning speed set at 100 nm/min, and the band passes were 5 nm each, unless stated otherwise. Freshly prepared metal ion stock solutions (1 M) prepared in buffer A were used for metal ion titration. The cuvettes were soaked in 10 mM EDTA, rinsed with ion-free distilled water before use. The hPLSCR4 and $Asp^{290}\rightarrow Ala$ mutant was titrated with small aliquots of respective metal ions, and the binding constants (K_a) of metal ions to protein were determined by Scatchard plot using nonlinear curve fitting (Prism 5.0 GraphPad Software Inc., San Diego, USA). Curve fitting was done for a fraction of ligand sites occupied $[(F_o\text{-}F)/(F_o\text{-}F_{sat})]$ vs. ligand concentration using one-site binding nonlinear regression analysis, where F_o is the fluorescence in the absence of a ligand, F_{sat} is the fluorescence at saturated ligand concentration, and F is the fluorescence in the presence of a particular ligand concentration.

Stains-all binding assay

Stains-all, a calcium-mimicking probe, was used to probe the metal ion-binding properties of hPLSCR4 (Caday et al., 1986). The protein was made ion free by dialysis against buffer passed through Chelex-100 resin. Stains-all solutions were freshly prepared by dissolving the dye in ethylene glycol. Briefly, 50 µg of protein was incubated with 20 µM of dye in the dark at room temperature for 1 h. The absorption spectrum was then recorded from 400 to 700 nm using Jasco spectrophotometer with a scan speed of 100 nm/min. Different metal ions were then added to the dye-protein complex, and the spectra was recorded.

Terbium luminescence studies

Tb^{3+}, which possesses a high affinity for Ca^{2+}-binding sites (Brittain et al., 1976), was used to study the Ca^{2+} binding to the hPLSCR4 and $Asp^{290}\rightarrow Ala$ mutant. $TbCl_3$ (50 µM) was added in increments to the protein (1.5 µM each), and the complex was incubated for 5 min before recording the spectra. Samples were excited at 285 nm, and the emission spectrum was recorded between 310 and 560 nm with a scan speed of 100 nm/min and bandwidths of 5 nm each.

Far-UV-CD measurements

The far-UV-CD spectra were recorded using Jasco J-810 spectropolarimeter (Easton, MD, USA) at 25°C with a thermostated cell holder. The spectrophotometer was calibrated with 10-camphor sulfonic acid. The samples were scanned using a 0.1-cm path-length cuvette at a wavelength range of 250–190 nm. The average of three scans was recorded with a bandwidth of 2 nm at a scan speed of 10 nm/min. Protein concentrations of 10 µM were used for far-UV-CD recordings. Buffer blank spectra were collected under similar conditions and were subtracted to obtain the final spectra.

Protein surface hydrophobicity changes by ANS fluorescence

ANS-binding measurements were recorded using Perkin Elmer LS-55 spectrophotometer at 25°C. The binding of ANS to protein was monitored by recording the emission between 400 and 650 nm following excitation at 365 nm with a scan speed of 100 nm/min and a bandwidth of 10 nm each. ANS was added to buffer A to a final concentration of 20 µM and titrated with increasing protein concentration until saturation. To this saturated ANS-protein complex, small increments of Ca^{2+} were added and spectra were recorded. For data evaluation, buffer blanks were subtracted from the spectra.

Acknowledgments

This work was supported by a research grant from the Council of Scientific and Industrial Research, Government of India, New Delhi. V.G.F. was supported by a Junior Research Fellowship from the Council of Scientific and Industrial Research, Government of India, New Delhi.

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Received February 25, 2012; accepted June 4, 2012