Ca^{2+} Binding in Transmembrane Deleted Mutant of Human Phospholipid Scramblase 1: A Spectroscopic Study

Abdul Majeed, Vincent Gerard Francis, A. Gopalakrishna, Sathyanarayana N. Gummadi[1]

Department of Biotechnology, Indian Institute of Technology Madras

[1]Correspondence address: gummadi@iitm.ac.in

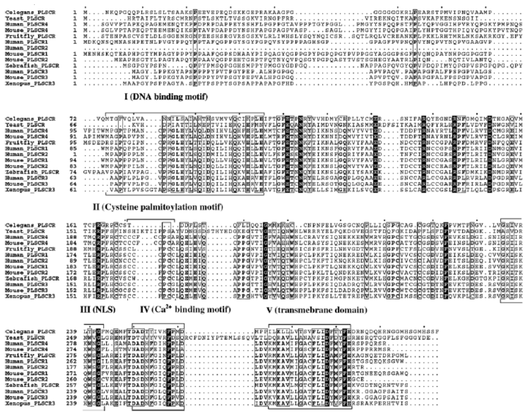
Introduction

Phospholipid scramblases, a group of five homologous proteins are shown to be involved in disrupting plasma membrane phospholipid asymmetry at critical cellular events like cell activation, injury and apoptosis.

The most studied member of this family, human phospholipid scramblase 1 (hPLSCR1), is a single pass transmembrane (TM) protein involved in Ca^{2+} dependent transbilayer redistribution of plasma membrane phospholipids. The predicted TM domain has been shown to be present close to the putative Ca^{2+} binding motif and may function as a site for insertion in the membrane and/or oligomerization of hPLSCR1. However, a detailed mechanism of phospholipid scrambling as well as the role of TM domain in transbilayer movement of PL still requires a proper understanding.

To understand the role of TM domain in transbilayer movement of phospholipid, we constructed a mutant lacking this TM region (ΔTM hPLSCR1) and characterized it's the Ca^{2+} binding properties.

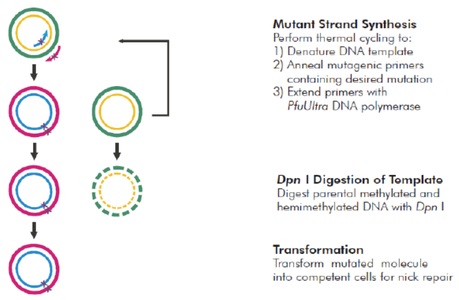
hPLSCR1 – Major Domains



Sahu SK, Gummadi SN, Manoj N, and Aradhyam GK (Archives of Biochemistry and Biophysics 462 (2007) 103–114)

Construction of ΔTM hPLSCR1

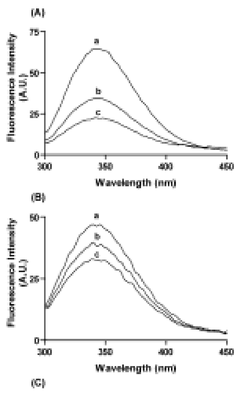
Introduce a stop codon (TAG) just before the Transmembrane domain by site-directed mutagenesis.



X – Stop Codon (TAG)

Quick Change II XL site-directed mutagenesis manual

Affinity by Tryptophan Fluorescence

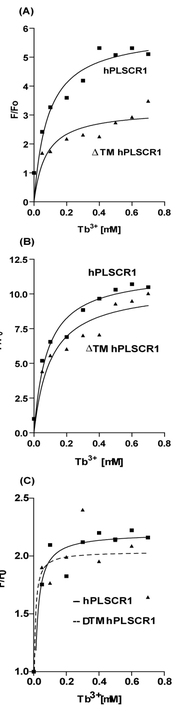


Quenching of intrinsic tryptophan fluorescence due to Ca^{2+} binding. (A) tryptophan fluorescence emission of hPLSCR1 (B) tryptophan fluorescence emission of ΔTM hPLSCR1. Trace a: 1.5 μM protein, b: 1.5 μM protein with 20 mM Ca^{2+}, 1.5 μM protein with 40 mM Ca^{2+}. The buffer used was 20 mM Tris–HCl, pH 7.5 with 200 mM NaCl.

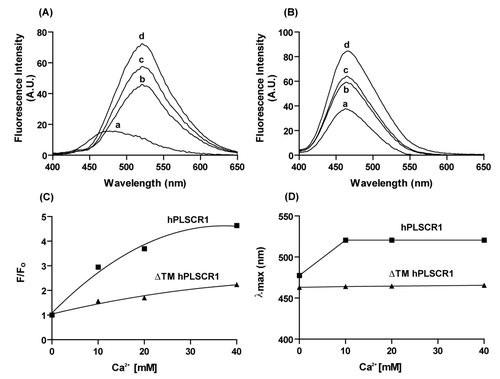
Spectra were recorded with excitation at 280 nm, emission from 300 nm to 450 nm, with scan speed of 200 nm/min at 25 ºC. (C) Curve fitting plot to determine the binding constant of Ca^{2+} to protein. The binding constants of the protein ligand interaction were determined from Scatchard plot using non-linear regression analysis. The binding affinity of Ca^{2+} to hPLSCR1 (25.42 ± 2.6 mM) was found to be two fold greater than that to ΔTM hPLSCR1 (52.86 ± 10.7 mM). The data shown here represent at least three independent sets of experiments.

FRET study using Terbium

Luminescence spectra of Tb^{3+} binding to hPLSCR1 and ΔTM hPLSCR1. (A) Relative fluorescence of luminescence peak at 492 nm of both hPLSCR1 and ΔTM hPLSCR1 (1.5 μM each) with increasing $TbCl_3$. (B) Relative fluorescence of luminescence peak at 547 nm of both hPLSCR1 and ΔTM hPLSCR1 (1.5 μM each) with increasing $TbCl_3$. The buffer used was 20 mM Tris–HCl, pH 7.5 containing 200 mM NaCl. (C) Relative fluorescence of luminescence peak at 590 nm of both hPLSCR1 and ΔTM hPLSCR1(1.5 μM each) with increasing $TbCl_3$. Excitation was done at 295 nm with excitation and emission band widths set at 2 nm and 5 nm. The data shown here represent at least three independent sets of experiments.

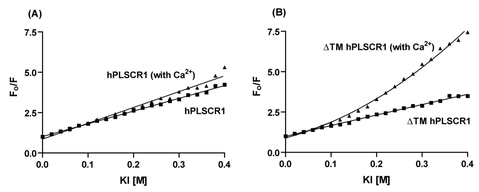


Surface Hydrophobicity Changes



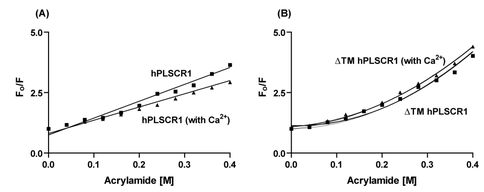
Probing protein surface hydrophobicity changes using ANS. (A) hPLSCR1 (B) ΔTM hPLSCR1. Trace a: apoprotein (1.5 μM), b: protein (1.5 μM) with 10 mM Ca^{2+}, c: protein (1.5 μM) with 20 mM Ca^{2+}, d: protein with 40 mM Ca^{2+}. (C) Relative enhancement of the maximum fluorescence intensity due to Ca^{2+} binding. (D) Shift of the fluorescence emission maximum (λ_{max}) due to Ca^{2+} binding. Fluorescence spectra were recorded with an excitation of 365 nm from 400 to 650 nm, scanning speed of 100 nm/min at 25 °C. The buffer used was 20 mM Tris–HCl, pH 7.5 containing 200 mM NaCl. The results shown are representative of at least three independent sets of experiments. The data shown here represent at least three independent sets of experiments.

Accessibility of trp residues by Iodide



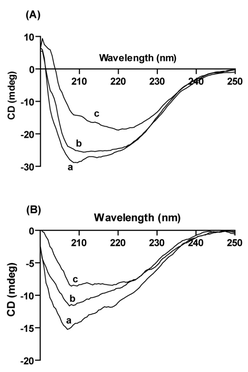
Quenching studies using potassium iodide. (A) Stern-Volmer plots of 1.5 μM hPLSCR1 in the presence and absence of 15 mM Ca^{2+} by I^- quencher. (B) Stern-Volmer plots of 1.5 μM ΔTM hPLSCR1 in the presence and absence of 15 mM Ca^{2+} by I^- quencher. The results shown here represent at least three independent sets of experiments.

Accessibility of trp residues by Acrylamide



Quenching studies using Acrylamide. (A) Stern-Volmer plots of 1.5 μM hPLSCR1 in the presence and absence of 15 mM Ca^{2+} by acrylamide quencher. (B) Stern-Volmer plots of 1.5 μM ΔTM hPLSCR1 in the presence and absence of 15 mM Ca^{2+} by acrylamide quencher. The results shown here represent at least three independent sets of experiments.

Secondary Structure Changes



Changes in the secondary structure of the proteins upon binding to Ca^{2+} as determined by far UV-CD. (A) hPLSCR1, (B) ΔTM hPLSCR1. Trace a: 10 μM protein, b: 10μM protein with 2 mM Ca^{2+}, c: 10μM protein with 5 mM Ca^{2+}, d: 10μM protein with 10 mM Ca^{2+}.

Discussion and Conclusions

- Binding affinities reveal hPLSCR1 has two fold greater affinity to Ca^{2+} than ΔTM hPLSCR1. We hypothesize that TM helix may also be involved in Ca^{2+} coordination, as seen for most Ca^{2+} binding proteins where the helix region of helix-loop-helix motif is involved in Ca^{2+} coordination.
- The relative increment in Tb^{3+} fluorescence of hPLSCR1 was greater than ΔTM hPLSCR1which could be due to (i) one of residue(s) of the TM helix could be involved in coordinating the metal ion, (ii) coordination of metal ion by residues contributed by more than one TM helix which would then require the proteins to self associate.
- Tb^{3+} binding was observed with increased right angle light scattering for both the proteins indicating metal ion induced self association. Right angle light scattering suggests that metal ion induced self association of hPLSCR1 was independent of TM helix.
- Observed increase in ANS fluorescence in presence of Ca^{2+} is presumed to be due protein aggregation which is in accordance with increased right angle light scattering studies.
- As evident from far UV-CD, majority of hPLSCR1 had an α-helical structure and upon Ca^{2+} binding loss of ellipticity suggests that apo-hPLSCR1 is in a well folded conformation in comparison to the Ca^{2+} bound form. In apo-ΔTM hPLSCR1 deletion of TM helix lead to significant loss of secondary structure and the protein adopts an unordered structure in presence of Ca^{2+}.
- Iodide and Acrylamide quenching results support the hypothesis that due to TM helix protein adopts a more compact conformation.
- In conclusion, we propose that the TM helix of hPLSCR1 influences Ca^{2+} binding and the protein adopts a more compact conformation in presence of TM helix.

Acknowledgements

Thanks to Council of Scientific and Industrial Research for making this possible.