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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 01 September 2014
Final form accepted: 27 January 2015
Published online: 28 February 2015

Volume 20 (2015) pp 177-195
DOI: 10.1515/cmble-2015-0017

Research article

BIOCHEMICAL EVIDENCE FOR Ca^{2+}-INDEPENDENT FUNCTIONAL ACTIVATION OF hPLSCR1 AT LOW pH

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Abstract: Human phospholipid scramblase 1 (hPLSCR1) is a Ca^{2+}-dependent protein known to scramble phospholipids in the plasma membrane resulting in loss of membrane asymmetry. It has been reported that hPLSCR1 exhibits Ca^{2+}-independent activity at low pH. However, the conformational changes induced at low pH leading to functional activation are not known. Our results showed that recombinant hPLSCR1 was functionally activated at low pH, which is similar to the behavior of natively extracted hPLSCR1. Tryptophan fluorescence measurements showed a decrease in Ca^{2+}-binding affinity at low pH, although not at pH 5.5. Far and near UV-CD revealed that low pH induced structural changes, with a significant increase in the β-sheet content of the protein. At the physiological level, decreased hPLSCR1 expression was observed after a period of exposure to low pH. The effect occurred at the promoter level. The expression levels of hPLSCR1 directly correlated with the sensitivity of HEK293 to apoptosis. Based on these results, we conclude that the mechanisms of Ca^{2+}- and pH-induced functional activation of hPLSCR1 are different and that hPLSCR1 expression regulated by low pH could provide insights into the role of hPLSCR1 in cancer progression.

Keywords: Human phospholipid scramblase, Low pH, Tryptophan fluorescence, Promoter, Gene expression, CD spectroscopy, Apoptosis assay, c-FOS, Phosphatidylcholine, Phosphatidylserine, Calcium binding

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Abbreviations used: ECM1 – extracellular matrix protein 1; EGTA – ethylene glycol tetra acetic; HEK – human embryonic kidney cell line; hPLSCR1 – human phospholipid scramblase 1; K_d – equilibrium dissociation constant; NBD-PL - 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labelled phospholipid; PBS – phosphate-buffered saline; PC – phosphatidylcholine; p-hPLSCR1 – promoter of hPLSCR1; PL – phospholipid; PM – plasma membrane; PS – phosphatidylserine; TF – transcription factor; Trp – tryptophan

INTRODUCTION

Human phospholipid scramblase 1 (hPLSCR1) is a type II integral membrane protein known to mediate Ca^{2+}-dependent bidirectional scrambling of phospholipids (PLs) [1, 2]. PLSCRs are multi-domain proteins that mediate a wide variety of cellular functions, such as apoptosis, blood coagulation, immune responses and cell signaling [2]. Despite the demonstration of PL flip-flop in synthetic vesicles, blood coagulation was normal in hPLSCR1-/- null mice, indicating that there are other dedicated scramblases [3].

hPLSCR1 is known to translocate between the nucleus and plasma membrane (PM) via the nuclear import pathway using importin α/β proteins [4]. In the nucleus, hPLSCR1 has been shown to carry out two major functions: binding to the inositol 1,4,5 triphosphate receptor type 1 (IP3R1) promoter; and interacting with topoisomerase IIα to enhance its decatenation activity [5–6]. hPLSCR1 is a substrate for other kinases, such as Src kinase, IgE receptor tyrosine kinase and c-Abl kinase, underlying its importance in various signaling pathways. PLSCR1 enhances the antiviral response of interferon and STAT1 via JNK and protein kinase C delta [7, 8]. hPLSCR1 has also been demonstrated to interact with multiple other proteins, such as onzin, ECM1, the CD1 and 4 cellular receptors, and the HCV proteins EI and E2, modulating their functions [9–12].

The diverse functions mediated by hPLSCR1 indicate that it is a multi-functional protein with distinct regulatory pathways. Scramblases have also been implicated in a wide variety of disorders. For example, in Scott syndrome patients, red blood cells were seen to have defective PS exposure, even in the presence of increased intracellular Ca^{2+}, implying defective scramblase [13]. It was recently reported that a mutation in the splice-acceptor site of the *TMEM-16F* gene resulted in a truncated protein that was responsible for the Scott phenotype [14].

Acid–base homeostasis is a crucial requirement for normal cellular events, with many physiological responses being pH dependent, including enzyme activities, DNA synthesis and intracellular Ca^{2+} level maintenance [15–18]. The intracellular resting pH within the cell (6.8–7.3) is maintained by the Na^{+}–H^{+} exchanger NHE-1 [19, 20]. A wide variety of factors modulate the activity of NHE-1, including hormones and external stimuli [21].

Extracellular acidification (5.5–7.0) is routinely observed under various physiological conditions, such as immune responses, inflammatory acidosis and tumor progression [22, 23]. pH changes (5.5–7.5) mainly occur due to the production of lactic acid and hydrolysis of ATP in the microenvironment of tumors [24, 25]. Autoimmune disorders like rheumatoid arthritis and asthma are also associated with a low pH microenvironment [26].

Acidic microenvironments enhance invasiveness in a variety of cancer cells, both in vitro and in vivo [27]. Such acidity is toxic to normal cells, promoting apoptosis associated with extracellular matrix degradation and VEGF release [28]. However, cancer cells thrive in such environments through upregulation of the NHE-1 transporter and the expression of a variety of tumor growth-

associated proteins, such as IL-8, VEGF, cathepsin B and MMP-9 [29, 30]. Similarly, the expression of stabilin-1, a phagocytic receptor for PS-exposing cells, was upregulated at low pH [31]. TRAIL, which promotes apoptosis in cancer cells, was found to be insensitive at pH 7.5, whereas it was sensitized at low pH [32]. Despite the presence of significant literature on the functional role of PLSCRs, the molecular action of PLSCR mediated flip-flop remains unknown. It is reported that hPLSCR1 purified from Scott red blood cells was functionally activated in the presence of low pH and/or Ca^{2+} [13]. In this study, we provide biochemical evidence for low pH-induced Ca^{2+}-independent activity of recombinant hPLSCR1 and elucidate the physiological effect of low pH on hPLSCR1.

MATERIALS AND METHODS

Materials

N-lauroyl sarcosine (NLS; cat. no. L5125), egg yolk phosphatidylcholine (ePC; cat. no. P3356), phosphatidylserine (PS; cat. no. P5560), phenyl methane sulfonyl fluoride (PMSF; cat. no. P7626), bicinchoninic acid (BCA; cat. no. B9643) and molecular biology grade $CaCl_2$ (cat. no. 3306) were obtained from Sigma. *E. coli* DH5α and *E. coli* BL21 (DE3) strains were obtained from ATCC. cDNA of *hPLSCR1* (cat. no. SC321738) was obtained from OriGene. Luciferin potassium salt (cat. no. L2916) was obtained from Invitrogen. pET-28a (+) vector was obtained from Novagen. pGL3 basic vector was purchased from Promega (cat. no. E1751). Isopropyl β-D-1-thiogalactopyranoside (IPTG; cat. no. MB072), dithiothreitol (DTT; cat. no. MB070), ethylene glycol tetra acetic acid (EGTA; cat. no. RM1530) and other routine chemicals were molecular biology grade and purchased from Himedia Laboratories. 7-nitrobenz-2-oxa-1,3-diazol-4-yl-phosphatidylcholine (NBD-PC; cat. no. 810310) was obtained from Avanti Polar Lipids. Chelex-100 resin (cat. no. 142-1253), SM2 Bio-Beads (cat. no. 152-3920) and protein molecular weight markers were obtained from Bio-Rad. The Ni-NTA (Ni^{2+}-nitrilotriacetate) matrix used for the purification of hPLSCR1 was purchased from Qiagen (cat. no. 30210). Anti-PLSCR1 (cat. no. sc-59645), anti-β-actin (cat. no. sc-1616) and anti-mouse secondary antibodies (cat. no. sc-2005) were purchased from Santa Cruz Biotechnology.

Plasmid constructs

Expression of hPLSCR1 as (His_6) fusion protein (hexa-histidine tag) was done by cloning the ORF into pET-28a (+) vector (Novagen). The open reading frame of *hPLSCR1* (954 base pairs) was amplified from mammalian expression vector pCMV-SPORT6 vector via PCR using the forward primer 5'-GTCCATATGGC ACGGCG-3' and reverse primer 5'-GCTCGAGTGCGGGCCGCC-3' with *NdeI* and *Not1* sites, respectively. The amplicon was then digested and ligated into the *Nde1* and *Not1* sites of pET-28a (+). For promoter studies, a ~1.5 kb fragment of hPLSCR1 promoter ranging from +25 to –1500 bp was PCR amplified from

human genomic DNA using Phusion high fidelity polymerase (Finnzymes) with the forward primer 5'-ATATATGGTACCCAGTCACCATTGGCGGCACA TTTCAG-3' and reverse primer 5'-ACATCTCTCGAGGAGACTCCAGAGAC GTTTGTCCGGTG-3'. It was cloned directionally between the *Kpn1* and *Xho1* sites of the pGL3 basic vector (Promega) and the construct was verified by sequencing. The c-FOS TF expression plasmid was donated by Professor James A. Goodrich.

Expression and purification of (His_6) fusion protein

Overexpression and purification of hPLSCR1 was done as described previously [33]. hPLSCR1-pET-28a (+) plasmids were transformed into *E. coli* BL-21 (DE3) and grown in a selective medium containing kanamycin (50 μg/ml). Induction was done using 0.1 mM IPTG and growth was carried out for 3 h at 37ºC and 180 rpm. Cells were disrupted using probe tip sonicator (Vibrocell ultrasonicator) in buffer A, which contains 20 mM Tris–HCl (pH 7.5) and 200 mM NaCl, with 1 mM PMSF, 1 mM EDTA and 1 mM DTT for 2 min at 4ºC with 1 s on and 2 s off cycles with 30% amplitude. Inclusion bodies (IBs) were collected and resuspended in buffer A containing 0.3% (w/v) N-lauroyl sarcosine and agitated for 24 h at 18ºC and 130 rpm.

For purification, the suspension was centrifuged at 4,400 x g for 15 min at 4ºC and then subjected to dialysis against buffer A with buffer changes three times (after 12 h each time). For spectroscopic analysis His_6-hPLSCR1 was affinity purified using Ni-NTA matrix and the bound proteins were eluted using 250 mM imidazole. No aggregation of protein was seen in the absence of detergent and the purified protein remained soluble in buffer A. Protein concentrations were determined using a BCA kit (Sigma) and the proteins were separated on a 12% SDS-PAGE gel and visualized using Coomassie Brilliant Blue R-250. In all of our biophysical experiments involving purified protein, the protein was dissolved in buffer containing 20 mM Tris (pH 7.5), 200 mM NaCl and 0.025% (w/v) Brij-35. Citrate buffer was used for the experiments at various low pH levels.

Reconstitution into proteoliposomes and the scramblase assay

Proteoliposomes reconstituted with hPLSCR1 were prepared as described earlier [34]. Egg PC (4.5 μmol) PS (9:1) were briefly dried under a stream of N_2 gas and solubilised in buffer B, which consisted of 10 mM HEPES/NaOH (pH 7.5) and 100 mM NaCl, with 1% Triton X-100. For the preparation of proteoliposomes, 0.1 mg of protein was added to the solubilised lipids followed by the addition of SM2 Bio-Beads to remove Triton X-100 completely. Outside-labeled proteoliposomes were prepared via incubation with 0.3 mol% NBD-PLs for 5 min at 37ºC, followed by centrifugation in an MLA30 rotor at 230,000 x g for 45 min at 4ºC to remove traces of unbound NBD-PLs.

For the scramblase assay, outside-labeled proteoliposomes were incubated for 3 h at 37ºC in buffer B in the presence of 2 mM Ca^{2+}/4 mM EGTA or in the relevant buffers. Sample fluorescence (excitation at 470 nm and emission at 532 nm) was monitored continuously at 25ºC 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 to stabilize the fluorescence, and 20 mM sodium dithionite (freshly prepared in 1 M Tris base) was added. The fluorescence was monitored for the next 400 s. The difference between the residual fluorescence in the presence and absence of Ca^{2+} or at various low pH levels after dithionite addition is attributed to Ca^{2+}- or low pH-induced scrambling of NBD-PLs located in the outer leaflet of the proteoliposomes. The calculation was

Scramblase activity [% NBD-PL translocated] = $F_{\text{metal ion/low pH}} - F_{\text{control}}$

where $F_{\text{metal ion/low pH}}$ is the relative scramblase activity in the presence of a metal ion or in a low pH buffer and F_{control} is the relative scramblase activity in the presence of EGTA (control).

Intrinsic tryptophan studies

Steady-state fluorescence measurements were recorded using a Perkin Elmer LS-55 fluorescence spectrophotometer. Fluorescence spectrum emissions were recorded at unitary wavelengths between 300 and 500 nm with an excitation wavelength of 280 nm at 25ºC and a scanning speed of 100 nm/min. The band passes were 10 nm each unless stated otherwise. Freshly prepared stock of 1 M $CaCl_2$ in buffer A was used for Ca^{2+} titration. The cuvettes were soaked in 10 mM EDTA and rinsed with ion-free distilled water prior to use. hPLSCR1 (2 μM) was titrated with small aliquots of $CaCl_2$ (1 mM increments until saturation) and intrinsic tryptophan (Trp) fluorescence changes were monitored. The equilibrium dissociation constant [K_d] was determined via Scatchard plot using nonlinear curve fitting (Prism 3.0 GraphPad Software). Curve fitting was done for the fraction of ligand sites occupied [$(F_o\text{-}F)/(F_o\text{-}F_{sat})$] versus the ligand concentration using one-site binding nonlinear regression analysis, where F_o is the fluorescence in the absence of ligand; F_{sat} is the fluorescence at the saturation of ligand concentration; and F is the fluorescence in the presence of a particular ligand concentration. The determination of the [K_d] at low pH was performed similarly, except that hPLSCR1 was dialyzed against various low pH buffers and the Trp fluorescence was monitored.

Circular dichroism (CD)

CD spectra were recorded using a JASCO J-810 spectropolarimeter (Easton MD) at 25ºC with a thermostated cell holder. The spectrophotometer was calibrated with 10-camphor sulphonic acid. The samples were scanned using a 1- and 10-mm path length cuvette at a wavelength range of 250–190 nm and 250–350 nm. An average of three scans was performed with a bandwidth of 2 nm and the scan speed was set at 10 nm/min. The amounts of protein taken for far UV-CD and near UV-CD were 10 μM and 50 μM, respectively. Background (buffer blank) spectra were collected under similar conditions and were subtracted to obtain the final spectra.

Terbium luminescence studies

Tb^{3+}, a fluorescent metal ion, was used to study the effect of pH on the conformation of hPLSCR1. A freshly prepared stock solution of $TbCl_3$ (0.1M) was used. $TbCl_3$ (100 μM) was added to the protein at various pH and the complex was incubated for 5 min before the spectra were recorded. Samples were excited at 285 nm and the emission spectrum was recorded between 300 nm and 560 nm with the scan speed set at 100 nm/min and bandwidths of 5 nm each at 25ºC.

Transfection and luciferase assay

Human embryonic kidney cell lines (HEK293; 60–70% confluent) were transfected with appropriate constructs using Transpass D2 transfection reagent (NEB, UK) as per the manufacturer's instructions. Cells were washed with phosphate buffered saline (PBS) 24 h post transfection and lysed in assay buffer for the luciferase assay. The *β-galactosidase* gene was co-transfected as an internal control to normalize errors due to differences in transfection efficiency. To study the effect of low pH, the medium was replaced post-transfection with fresh medium with a pH of 7.4 and 6.5 respectively. Changes in the medium pH were achieved through variations in the sodium bicarbonate concentration [35].

Western blot

Cells (~70%) were grown in DMEM medium at various pH levels by varying the sodium bicarbonate ($NaHCO_3$) concentration. Cells were lysed in lysis buffer consisting of 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF and protease inhibitors. The total protein was estimated using the BCA method with BSA as the standard. 50 μg of total protein was loaded onto 12% SDS-PAGE and was transferred onto nitrocellulose membrane. The membrane was blocked using blocking buffer consisting of 10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween-20 and 3% BSA for 1 h at room temperature. Immunoblotting was done using anti-hPLSCR1 (Santacruz) and anti-β-actin (Sigma).

Apoptosis assay

The ability of HEK-293 to undergo apoptosis in an acidic medium was assessed by quantifying the percentage exposure of phosphatidylserine (PS). Annexin V-APC and propidium iodide (PI) were used to stain early and late apoptotic cells. Cells were grown overnight, then incubated at various pH levels with 1% fetal bovine serum (FBS) and grown for 24 h. The cells were then washed twice with PBS and suspended in 1x binding buffer containing annexin V-APC (0.5 μg/ml) and PI (0.6 ug/ml), and incubated in the dark for 20 min. Cytometric measurements were performed using a FACSCalibur flow cytometer (BD Biosciences) and spectral overlap was adjusted using fluorescence compensation.

RESULTS

Principle of scramblase assay

Fig. 1A represents the schematic of the scramblase assay used in this study. Briefly, the outside-labeled proteoliposomes were incubated with EGTA (control) and at low pH (test) for 3 h and the initial fluorescence was measured. Later, the vesicles were treated with sodium dithionite, a membrane-impermeable reagent that irreversibly modifies NBD, thereby quenching the

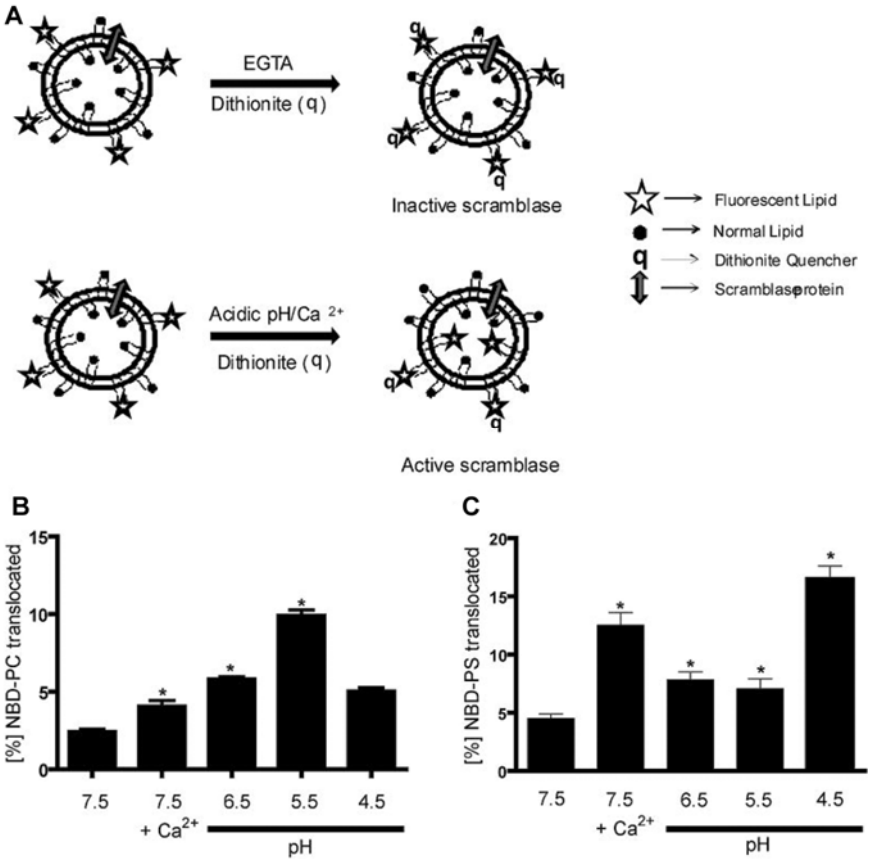

Fig. 1. Calcium-independent bidirectional scrambling of NBD-PLs across proteoliposomes reconstituted with hPLSCR1 at low pH. Proteoliposomes were generated with a mixture of PC:PS (9:1) in the presence of hPLSCR1. The outer membrane was labeled with 0.3% NBD-PC/PS PLs. Proteoliposomes were incubated at 37ºC in the presence of Ca^{2+} or low pH buffers or EGTA. Scrambling of NBD-PLs was assessed via quenching with sodium dithionite. A – Schematic representation of the scramblase assay performed with outside-labeled proteoliposomes. B – The scramblase assay was performed using NBD-PC-labeled proetoliposomes in the presence of Ca^{2+} and various low pH buffers. C – The scramblase assay was performed using NBD-PS-labeled proteoliposomes in the presence of Ca^{2+} and various low pH buffers. The results are representative of at least three sets of experiments (*$p < 0.05$).

fluorescence on the outer leaflet. The vesicles incubated at low pH or at pH 7.5 in the presence of Ca^{2+} sequester labeled lipids to the inner leaflet, so they exhibited higher fluorescence than the control (in the presence of EGTA). The difference in fluorescence intensity measured between the proteoliposomes at low pH and the EGTA-treated sample gives the measure of scramblase activity as a percentage of NBD-PL sequestered.

Scramblase activity at low pH

Overexpression of hPLSCR1 was achieved by cloning the gene into pET-28 a(+) vector and overexpressing in the BL21-DE3 strain of *E. coli* (data not shown). Since hPLSCR1 is a membrane protein, the functional activity of the protein would require its reconstitution into lipid vesicles. Purified recombinant hPLSCR1 was reconstituted into synthetic vesicles and the functional activity of the protein was assayed using NBD-labeled PLs as the tracer molecule. To determine whether low pH activates recombinant hPLSCR1, we performed the scramblase assay at various low pH levels. Fig. 1B shows that recombinant hPLSCR1-reconstituted NBD-PC proteoliposomes were functionally activated at low pH, even in the absence of Ca^{2+}, which is a similar result to one reported earlier for natively extracted PLSCR1 vesicles [13].

The rate of scrambling was higher at pH 5.5 than at pH 4.5 or 6.5. Since hPLSCR1 is known to mediate head group-independent PL scrambling, we performed the scramblase assay using NBD-PS as a tracer molecule. Fig. 1C shows that hPLSCR1 mediates the trans-bilayer movement of PLs at low pH irrespective of the head group PLs. However, the rate of PL scrambling for NBD-PS-labeled vesicles was higher at pH 4.5 than at pH 6.5 or 5.5.

Intrinsic tryptophan fluorescence

Since hPLSCR1 is known to be functionally activated at pH 7.5 in the presence of Ca^{2+}, we reasoned whether hPLSCR1 at low pH still retains the ability to bind to Ca^{2+}. Fig. 2A–D show that binding of Ca^{2+} to hPLSCR1 at various low pH levels resulted in a dose-dependent and saturable quenching of intrinsic tryptophan fluorescence without any shift in the emission maxima. [K_d] were determined by nonlinear curve fitting as described in the Materials and Methods section. Fig. 2E shows that at low pH, there was a decrease in the affinity of Ca^{2+} to hPLSCR1. The decrease was more pronounced at pH 4.5 than at pH 5.5 or 6.5. However the [K_d] revealed that although low pH decreases the affinity of Ca^{2+}, the protein was functionally activated at low pH irrespective of Ca^{2+}. The K_d values reveal that hPLSCR1 is a weak Ca^{2+}-binding protein and the K_d values are in the physiological range as it requires 1000-fold increase in intra-cellular Ca^{2+} concentration for functional activation of the protein.

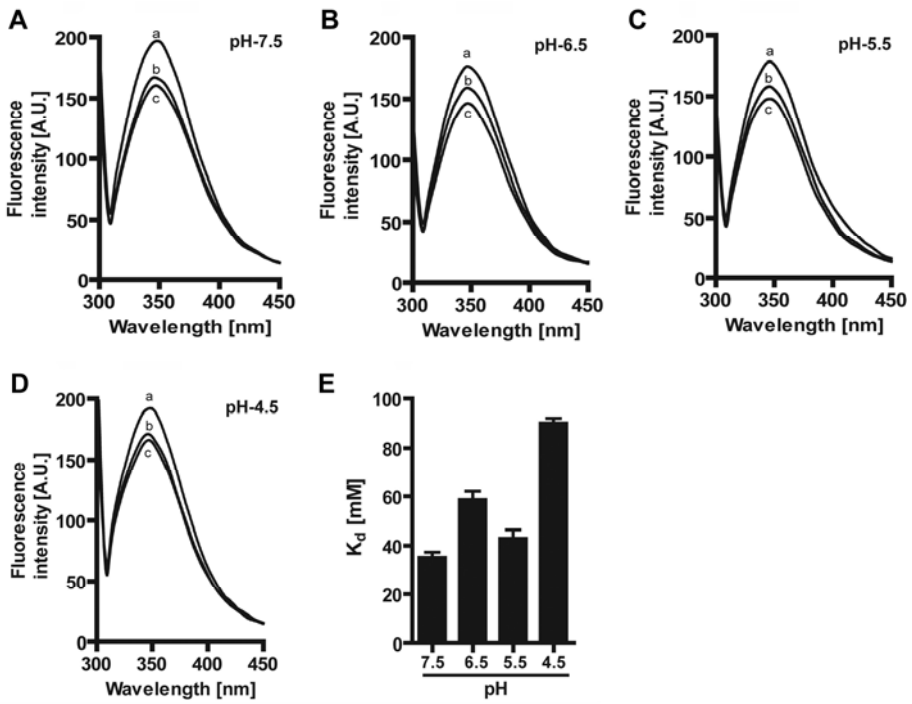

Fig. 2. Determination of Ca^{2+} affinities to hPLSCR1 through monitoring changes in intrinsic tryptophan fluorescence. Protein (2 μM) was dialyzed against various buffers, Ca^{2+} was added in increments and the intrinsic Trp fluorescence was monitored. The protein was excited at 295 nm and emission was monitored in the range 300–450 nm with a slit width of 2 nm and scanning speed of 50 nm/min. [K_d] were determined using a Scatchard plot with a nonlinear regression curve fit. A – hPLSCR1 intrinsic tryptophan fluorescence at pH 7.5. B – hPLSCR1 intrinsic tryptophan fluorescence at pH 6.5. C – hPLSCR1 intrinsic tryptophan fluorescence at pH 5.5. D – hPLSCR1 intrinsic tryptophan fluorescence at pH 4.5. Trace a – apo-protein; trace b – protein in the presence of 0.5 mM Ca^{2+}; trace c – protein in the presence of 1 mM Ca^{2+}. E – [K_d] of Ca^{2+} to hPLSCR1 at various pH levels. The results are representative of at least three sets of experiments ($p < 0.05$).

Far UV-CD spectroscopy

The mechanism of low pH-induced functional activation was studied by monitoring the structural changes induced in the protein. Far UV-CD of hPLSCR1 at pH 7.5 suggests that it is majorly an α-helical protein as evident from the negative minima at 208 nm and 222 nm (Fig. 3A). The observed far UV-CD of hPLSCR1 at pH 7.5 was similar to that in an earlier report [36]. In the presence and absence of Ca^{2+}, no major changes in the secondary structure content of the protein were observed. However, slight changes in the ellipticity pattern of the protein were observed, indicating metal ion-induced conformation changes. The far UV-CD of hPLSCR1 at various low pH levels indicates a secondary structural transition from α-helix to β-sheet (Fig. 3B). At pH 5.5, the far UV spectra of hPLSCR1 show a single negative minimum at 212 nm, which

indicates the presence of an antiparallel β-sheet with very little helical content. At pH 4.5, there was significant loss of helicity along with an increase in the α-sheet/β-turn content of the protein.

The near UV-CD changes reveal significant changes in hPLSCR1 with respect to pH. At pH 7.5, the strong positive minima at 280 nm and 300 nm are drastically reduced in the presence of Ca^{2+} (Fig. 3C). By contrast, at low pH the 280 and 310 nm peaks were significantly lost, but distinct peaks at 265 nm and 285 nm were observed at pH 6.5, indicating change in the environment surrounding the aromatic side chains (Fig. 3D). However, the near UV-CD spectra at pH 5.5 and 4.5 indicate that the aromatic side chains are in a more disordered state than generally seen for proteins that unfold. These structural changes confirm that low pH-induced structural changes were different from Ca^{2+}-induced structural changes at pH 7.5, indicating different functional activation mechanisms.

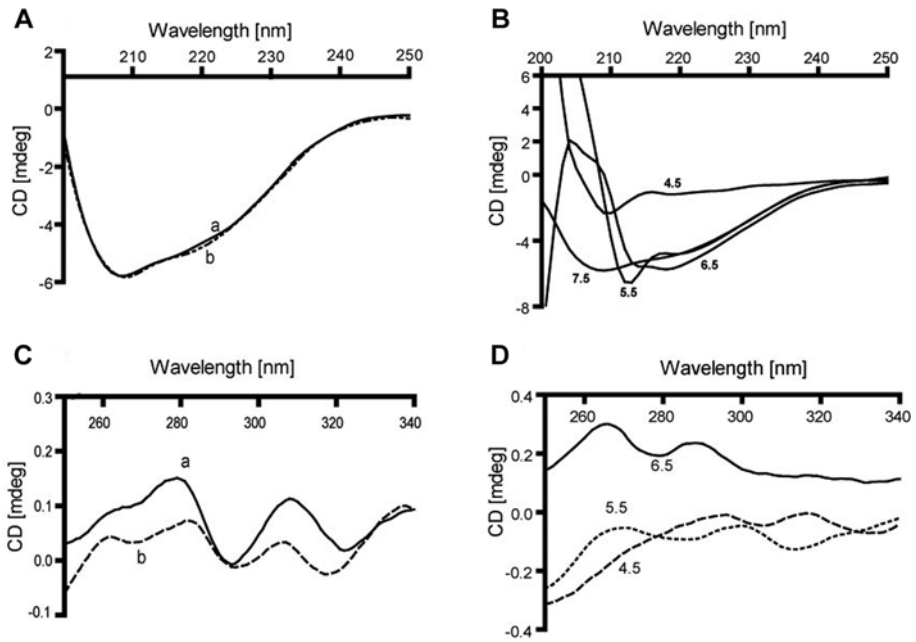

Fig. 3. Monitoring structural changes using CD spectroscopy. Spectra were obtained with 10 μM and 50 μM protein for far UV-CD and near UV-CD respectively. Spectra were subtracted for background contribution and are representative of three scans. A – Far UV-CD spectroscopy of hPLSCR1 at pH 7.5 in the presence (trace a) and absence of Ca^{2+} (trace b). B – Far UV-CD spectroscopy of hPLSCR1 at various low pH levels. C – Near UV-CD spectroscopy of hPLSCR1 at pH 7.5 (trace a) and at pH 7.5 in the presence of 0.1 mM Ca^{2+} (trace b). D – Near UV-CD spectroscopy of hPLSCR1 at pH 6.5 (trace a), pH 5.5 (trace b) and pH 4.5 (trace c).

Tb^{3+} luminescence

To further determine the effect of low pH on the folded state of the protein, Tb^{3+} luminescence was carried out. Since fluorescence resonance energy transfer (FRET) is a distance-dependent phenomenon, it can be used to study the folded state of the protein [37]. Fig. 4 shows that FRET (Tb^{3+} luminescence) decreases with the decrease in pH, indicating that the protein is in a less folded state at low pH. The inset figure shows the decrease in luminescent peaks at 497 and 542 nm due to low pH-induced structural changes in hPLSCR1.

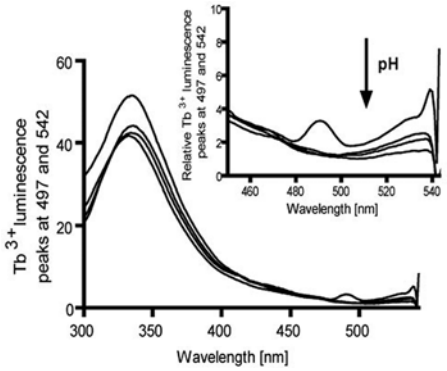

Fig. 4. Terbium luminescence spectroscopy. Tb^{3+}, a luminescent probe, was used to characterize the Ca^{2+}-binding properties of hPLSCR1 at various pH levels. Protein (2 μM) was dialyzed against the appropriate buffers and 100 μM of the Tb^{3+} metal ion was added. The emission was monitored from 300 to 560 nm with an excitation of 280 nm. Inset figure – Luminescent spectra of Tb^{3+} at 497 and 542 nm at various pH levels. The results are representative of three independent sets of experiments.

Effect of low pH on hPLSCR1 expression and its apoptotic sensitivity

Biochemical studies revealed that low pH induces Ca^{2+}-independent scrambling activity. It is also important to understand the physiological relevance of low pH on hPLSCR1 expression and cellular functions. hPLSCR1 is widely expressed in a variety of cells and is known to be constitutively expressed in resting HEK293. Fig. 5A shows that at low pH there was a time-dependent decrease in hPLSCR1 expression in HEK293 cells compared to the state at pH 7.5. Since gene expression is regulated at various stages, such as promoter regulation and mRNA stability, we investigated the regulatory mechanisms that govern hPLSCR1 expression. The effect of low pH on the strength of hPLSCR1 promoter activity was investigated using the luciferase assay. Fig. 5B shows that ~50% decrease in hPLSCR1 promoter activity was observed for HEK293 cells at low pH compared to their state at pH 7.5, indicating regulation at the promoter level.

Additionally, since transcription factors (TFs) are known to regulate promoter activity, we investigated whether redox-dependent TFs such as c-FOS play a role in regulating hPLSCR1 expression. Bioinformatic studies revealed binding sites for c-FOS TF, so we performed co-transfection experiments, which revealed that c-FOS

TF did not alter hPLSCR1 promoter activity. This indicates that other TFs may be involved in regulating hPLSCR1 expression, which needs further study (Fig. 5C).

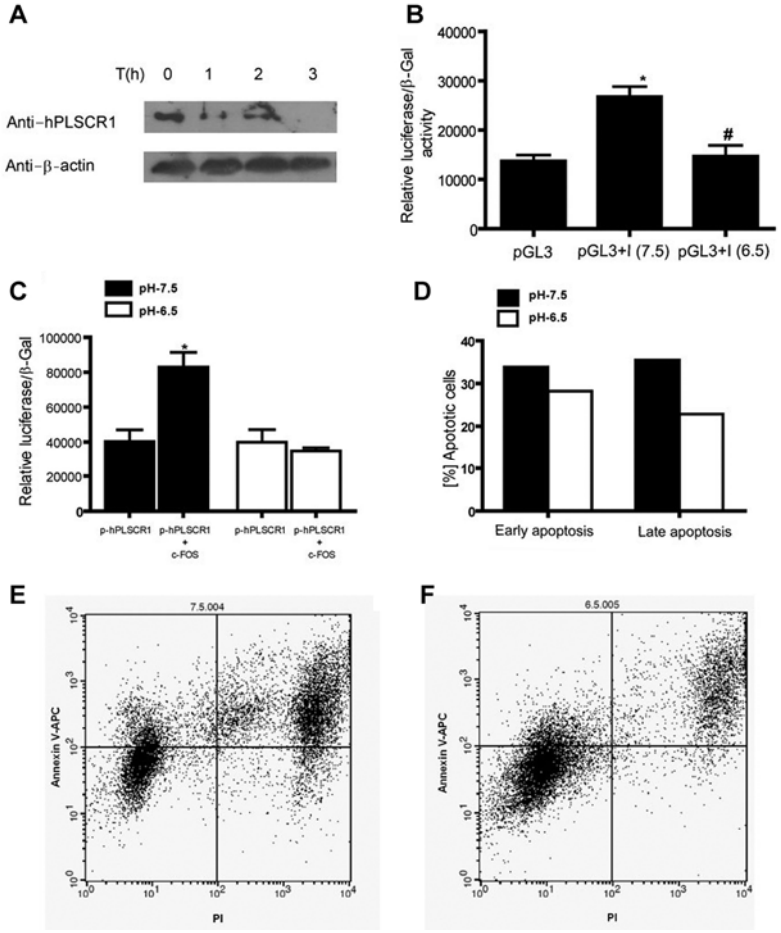

Fig. 5. The expression, promoter regulation and apoptotic sensitivity of hPLSCR1 in HEK293 cells. A – The time course of the change in expression of endogenous hPLSCR1 in HEK293 grown in a medium at pH 6.5. The loading control was probed with β-actin antibody. B – The promoter of hPLSCR1 (–1523 to –1) was cloned into the pGL3 expression system and the strength of promoter expression at various pH levels in HEK-293 cells was quantified using the luciferase assay. The *β-galactosidase* gene was co-transfected as an internal control to normalize errors due to differences in transfection efficiency. C – The effect of c-FOS transcription factor on hPLSCR1 promoter activity in HEK293 cells at various pH levels. D – The percentage of apoptotic HEK293 cells at various pH levels. For the apoptosis assay, apoptosis was induced by serum starvation at the various pH levels and PS exposure was quantified using Annexin V-APC. Propidium iodide (PI) was used to quantify dead cells. E – Dot plot showing HEK293 cells that were apoptotic and dead due to serum starvation at pH 7.5. F – Dot plot showing HEK293 cells that were apoptotic and dead due to serum starvation at pH 6.5. The results are representative of at least three sets of experiments (*, $^{\#}p < 0.05$).

Since hPLSCR1 is an apoptotic protein, we investigated the effect of low pH on the sensitivity of HEK293 undergoing apoptosis. Phosphatidylserine (PS) exposure, a hallmark of apoptosis, was used to differentiate apoptotic cells from normal cells. Fig. 5D shows that at low pH, the apoptotic ability of HEK-293 cells decreased compared to cells at pH 7.5. These results are in agreement with the decreased expression of hPLSCR1 at low pH, underlying the important role of hPLSCR1 in apoptosis. Fig. 5E and F show the apoptosis dot plots for HEK293 cells at pH 7.5 and 6.5 respectively.

DISCUSSION

hPLSCR1 belongs to the ATP-independent class of PL translocators. It was initially identified as a PL transporter but is now considered to be a multi-functional protein. An earlier report showed that native hPLSCR1 from Scott RBCs was functionally inactive. However, detergent-extracted hPLSCR1 showed scramblase activity in vitro in the presence of Ca^{2+} and at low pH [13]. Several reports showed that recombinant hPLSCR1 devoid of any post-translational modifications was also functionally active in reconstituted vesicles in the presence of Ca^{2+}. We reasoned whether low pH also functionally activates recombinant hPLSCR1 and evaluated the structural changes associated with it in comparison to physiological pH. Our results show that recombinant hPLSCR1 was also functionally activated by low pH in the absence of Ca^{2+}.

Since hPLSCR1 mediates head group-independent PL scrambling, we reasoned whether similar head group-independent PL scrambling was also observed at low pH. The results showed that the recombinant hPLSCR1 was functionally activated at low pH irrespective of the PL head group. However, the rate of translocation for various PL head groups at low pH was not similar, and it was higher for NBD-PS-reconstituted proteoliposomes. The higher rate of translocation for NBD-PS by hPLSCR1 could be attributed to the fact that during apoptosis, hPLSCR1 selectively translocates anionic PLs from the inner leaflet to the outer leaflet [38]. A higher translocation rate for anionic PLs was also observed in hPLSCR1 purified from normal RBCs and Scott RBCs. However, the percentage of PLs translocated using hPLSCR1 from native and Scott RBCs was higher than that from recombinant hPLSCR1 [13]. This difference could be explained by the lack of palmitoylation by recombinant hPLSCR1, implying that palmitoylation may be required for the protein to adopt a conformation needed to achieve maximum activity.

Since hPLSCR1 is functionally activated at physiological pH in the presence of Ca^{2+}, we determined the Ca^{2+} affinity of the protein at low pH. Trp is sensitive to changes in the surrounding environment and Ca^{2+} binding is known to induce conformation changes in hPLSCR1 [39, 40]. Therefore, by monitoring the changes in Trp fluorescence in presence of increasing concentrations of Ca^{2+}, the binding affinity of Ca^{2+} to hPLSCR1 at various low pH levels was determined. In case of the Ca^{2+}-binding protein S-100, it was found that alkaline pH

regulated the functional activity of protein in a Ca^{2+}-independent manner [41]. Similarly in gelsolin, an actin-binding protein, low pH was found to override the Ca^{2+} binding and also modulate its activity [42]. Residues in the EF-hand motif are mostly negatively charged at pH 7.5 and interaction of Ca^{2+} with the EF-hand motif is electrostatic in nature [43]. This decrease in affinity of Ca^{2+} to hPLSCR1 at low pH can be due to the protonation of charged residues in the Ca^{2+}-binding motif. For the Ca^{2+}-binding protein calmodulin, pH was found to determine the number of Ca^{2+} ions it can bind with, where three and two Ca^{2+} ions respectively bind at pH 7.5 and 6.5 [44].

Since hPLSCR1 was functionally activated by Ca^{2+} at pH 7.5 through the induction of conformational changes, we questioned whether similar Ca^{2+}-induced conformational changes were also observed at low pH. At low pH, a major structural transition from α-helix to β-sheet was observed, and such structural changes were not observed for hPLSCR1 at pH 7.5 in presence of Ca^{2+}, indicating that the mechanisms of metal ion-induced and low pH-induced functional activation of hPLSCR1 are different. This is in contrast to other Ca^{2+}-binding proteins such as gelsolin and S-100, where pH-induced and Ca^{2+}-induced functional activation and conformation changes were similar [45, 46].

Interestingly, it was found that for the protein cryptolepain retained its activity over a broad range of pH (6.0–12.0). Earlier studies have shown that Ca^{2+}-induced conformational changes in hPLSCR1 involve changes in the surface hydrophobicity pattern. It is hypothesized that oligomerization via self-association of surface-exposed hydrophobic patches could be required for functional activation of the protein [47].

Since hPLSCR1 was found to be functionally activated by low pH, we investigated the physiological relevance of low pH on the transcription and translational levels of hPLSCR1. A variety of factors, including hypoxia, glucose deprivation and pH, have been shown to regulate gene expression [48]. It has been also reported that a 0.5-unit pH drop was observed in the extracellular environment of tumor tissues compared to normal tissue [49]. Additionally, it has been demonstrated that low pH regulates the expression of a variety of genes, including *stabilin-1*, *VEGF* and *EGR1* [50]. Regulation of gene expression by these physiological variables is reflected by decreased transcription, mRNA stability and protein half-life. Therefore, we quantified the effect of low pH on the expression levels of hPLSCR1 in HEK293 cells. Our results clearly showed that hPLSCR1 is an low pH-responsive gene.

It has also been reported that for redox-dependent genes such as *stabilin-1* and *VEGF*, the expression is regulated at the promoter level. We investigated whether similar regulation at the promoter level also exists for hPLSCR1. We cloned the hPLSCR1 promoter and determined the promoter activity at low pH and physiological pH. Interestingly, we found that the hPLSCR1 promoter was downregulated at low pH, which is in contrast to the results of in vitro experiments, which show functional activation at low pH. The relationship between low pH-induced activation and downregulation needs further study.

Gene expression is regulated at the promoter level, so we questioned whether the differential expression of hPLSCR1 is affected by transcription factors (TFs). TF-binding analysis of various acid responsive promoters identified a cluster of TFs that regulate their transcription, including c-FOS, Oct-1, NF-Kb, IKRS and AP-1 [51]. Bioinformatic analysis of hPLSCR1 promoter reveals the presence of a binding site for several TFs, but we focused on the effect of c-Fos, which is a pH-responsive TF [52, 53]. c-Fos TF is in the AP1 class of transcription factors and includes Fra-1, Fra-2 and Fos-B [54]. We assessed whether downregulation of hPLSCR1 promoter could be mediated via c-FOS TF. Luciferase assay results showed no downregulation of hPLSCR1 promoter activity at low pH in the presence of c-FOS TF. We hypothesize that the downregulation of the hPLSCR1 promoter could be mediated via other TFs or it could be the resultant effect of a combination of stimulatory or inhibitory TFs.

hPLSCR1 is an interferon-inducible pro-apoptotic protein that shows a variable expression pattern in a wide variety of cancer cells [8]. Induction of apoptosis by UV radiation in CHO cells has been shown to enhance hPLSCR1 activity and the apoptotic activity was mediated by intrinsic apoptotic pathway. hPLSCR1 over expression also enhanced PS synthesis, with increased incorporation into microvesicles in addition to caspase-3 activation [55]. However, increased expression of hPLSCR1 in cell lines did not result in enhanced exposure of PS at the plasma membrane, indicating that hPLSCR1 may not directly augment apoptosis. In order to determine whether low pH alters the apoptotic sensitivity of HEK293 cells, they were serum starved and PS exposure was quantified. Our results clearly showed that low pH alters the apoptotic sensitivity of HEK293 and that this was directly dependent upon the expression levels of hPLSCR1.

The only limitation of using HEK-293 as a model here is that it is a non-cancerous cell line. In a variety of cancers, hPLSCR1 expression is widely deregulated and the molecular effectors of its variable expression remain largely uncharacterized. Subsequent studies would involve understanding the effect of acidic microenvironment on such cancer cells. Acidic pH plays an important role in several cellular processes, such as cell death, inflammation and cancer, but the molecular mechanisms governing this relationship remain unclear. A recent report also showed that hPLSCR1 is a physiological regulator of onzin, the expression levels of which relate to the sensitivity of cell lines to apoptosis [9]. It has been reported that low pH has pleiotropic effects on cancer cell invasion, metastasis and migration, and on the function of immune cells required for apoptotic clearance.

Downregulation of key cell death molecules is important for cancer progression and for immune evasion. We hypothesize that low pH-mediated downregulation of hPLSCR1 could be one of the mechanisms by which cancer cells evade apoptosis. This needs further evaluation.

Acknowledgements. We would like to thank Mr. S. Ulaganathan for his help in formatting the manuscript. Sathyanarayana N. Gummadi would like to acknowledge BRNS and IIT Madras for the funding. Vincent Gerard Francis would like to acknowledge the Council of Scientific and Industrial research for fellowship.

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