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Identification of three new members of the phospholipid scramblase gene family

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Abstract

Phospholipid (PL) scramblase is a 35 kDa protein that is thought to mediate Ca^{2+}-induced bidirectional transbilayer movement of plasma membrane phospholipids in activated, injured, or apoptotic cells. We recently reported the molecular cloning of a PL scramblase of human (HuPLSCR1) and mouse origin, respectively. In the present study, the gene for HuPLSCR1 was cloned from a human genomic library. The gene size is 29.7 kb and includes nine exons. Analysis of the 5′ flanking genomic sequence with luciferase reporter constructs located the promoter to a region spanning from −95 to +60 of the first (untranslated) exon. Furthermore, we report the molecular cloning of three additional novel cDNAs encoding proteins with high homology to HuPLSCR1. The predicted open reading frames encode proteins with 59% (HuPLSCR2; 224 aa), 47% (HuPLSCR3; 295 aa) and 46% (HuPLSCR4; 329 aa) identity, respectively, to HuPLSCR1. All members of the PLSCR gene family conserve those residues contained in the segment of the PLSCR1 polypeptide that was previously shown to bind Ca^{2+}. With the exception of HuPLSCR2, these proteins also each contain multiple PXXP motifs and a PPXY motif located near the N-terminus, implying the potential for interaction with SH3 or WW domain-containing proteins, respectively. HuPLSCR1, 2, and 4 were found to be closely clustered on chromosome 3 (3q23), whereas HuPLSCR3 is located on chromosome 17. Northern blots revealed that the expression of HuPLSCR2 is restricted to testis, whereas HuPLSCR1, 3 and 4 are expressed in most of the 16 tissues examined. Notable exceptions were HuPLSCR4, which was not detected in peripheral blood lymphocytes, and HuPLSCR1 and HuPLSCR3, which were not detected in brain.

Keywords: Phospholipid scramblase; Plasma membrane; Phospholipid asymmetry

1. Introduction

While aminophospholipids are primarily found in the inner leaflet of the plasma membrane of a resting cell, surface exposure of phosphatidylserine (PS) is observed in activated, injured or apoptotic cells [1,2]. Surface-exposed PS plays a central role in promoting blood coagulation, as this lipid serves as assembly site for coagulation factors, including the prothrombinase and tenase enzyme complexes. Aminophospholipids have also been implicated as a signal in the clearance of apoptotic or activated cells through phagocytosis [1,3–7]. This redistribution of

Abbreviations: PL, phospholipid; PS, phosphatidylserine; EST, expressed sequence tag; PCR, polymerase chain reaction

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plasma membrane phospholipids (PLs) appears to be mediated by an increase in intracellular Ca^{2+}, is bidirectional, and is independent of the PL headgroup [1,8–10]. Although the exact mechanisms involved remain to be elucidated, we recently cloned and expressed a protein, termed phospholipid scramblase (HuPLSCR1), which when reconstituted into liposomes exhibits many of the characteristics observed for the Ca^{2+}-induced surface exposure of PS in cells [11,12]. HuPLSCR1 was found to be expressed in a wide range of tissues, and the level of expression appeared to correlate with the observed Ca^{2+}-induced redistribution of PS [12,13]. We subsequently cloned a murine orthologue (herein termed MuPLSCR2), which when reconstituted into proteoliposomes promoted Ca^{2+}-mediated PL scramblase activity [14]. A second murine orthologue, termed MmTRA1b, was recently reported [15]. Whereas PL scramblase activity was not demonstrated for that protein, multiple sequence alignment of the predicted open reading frames revealed that it, rather than MuPLSCR2, may be the true murine orthologue of HuPLSCR1. This prompted a search for additional homologues of HuPLSCR1. Here we report the molecular cloning of cDNAs encoding three novel proteins with high homology to human PLSCR1, suggesting that they belong to the same gene family. The size and tissue distribution of mRNA transcripts for the different HuPLSCR family members are compared. We also describe the gene structure of HuPLSCR1 and analyze its promoter region.

2. Materials and methods

2.1. Cloning of human PL scramblase 1 gene

A BAC human genomic library (Genome System Inc., St. Louis, MO) was screened with a 1.445 kb HuPLSCR1 cDNA probe (GenBank accession number AF098642) by hybridization. A positive clone of approximately 100 kb was obtained, digested with *Eco*RI, and the fragments were cloned into pcDNA3 (Invitrogen). Subclones were identified by hybridization with digoxigenin-labeled HuPLSCR1 cDNA probe, and DNA inserts were sequenced on an ABI DNA Sequencer Model 373 Stretch (Applied Biosystems) using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer).

2.2. Construction of 5′ flanking region deletions of PLSCR1 gene

A 4180 bp DNA fragment consisting of the 5′ flanking region (0 to −4120) and the first 60 bp of the first exon (+1 to +60) of the HuPLSCR1 gene (GenBank accession number AF153715) was cloned into pGL3 basic luciferase reporter vector (Promega). In order to identify the promoter region of the gene, the 5′ flanking DNA was serially deleted both from the 5′ and the 3′ end by PCR-mediated deletion and cloned into pGL3 basic luciferase reporter vector for analysis.

2.3. Cell culture and transfection of Daudi cells

The Burkitt's B cell lymphoma cell line Daudi was cultured in RPMI 1640 complete medium with 20% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml, at 37°C, 5% CO_2. Cells were washed and suspended to 1.35×10^7/ml in Opti-MEM. To 0.8 ml of cell suspension in a 0.4 cm electroporation cuvette, 20 μg of pGL3 5′ flanking region or deletions of HuPLSCR1 and 20 μg of pSV β-galactosidase (Promega) were added. The mixture was incubated for 10 min on ice, and electroporated at 380 V and 500 μF using a Bio-Rad Gene Pulser II (Bio-Rad). After incubation for 10 min at 37°C the cells were plated in 10 ml of RPMI complete medium. Forty-eight hours later, transfected cells were harvested for luciferase and β-galactosidase assay.

2.4. Luciferase and β-galactosidase assay

Luciferase activity was measured with a Luciferase Assay Kit (Promega). In brief, transfected Daudi cells were harvested, washed with phosphate-buffered saline and lysed for 15 min with Reporter lysis buffer. The cell lysate was vortexed for 15 s and centrifuged at $12\,000\times g$ at 4°C for 2 min. In a 96-well plate, 20 μl of lysate was mixed with 100 μl of luciferase assay buffer by automated reagent injection using a MicroLumat*Plus* microplate luminometer (EG and G Berthold), and luminescence was measured for 30 s. β-Galactosidase activity was deter-

mined with *o*-nitrophenyl-β-D-galactopyranoside as substrate. 100 μl of cell lysate was incubated with 100 μl of 4.4 mM *o*-nitrophenyl-β-D-galactopyranoside for 1 h at 37°C, and absorbance was read at 420 nm. Luciferase activity was expressed in arbitrary light units, and corrected for transfection efficiency of β-galactosidase.

2.5. Cloning of PL scramblase family members

BLAST search of the GenBank database of expressed sequence tags (EST) with HuPLSCR1 cDNA identified three distinct clusters of EST clones each displaying overlapping identities. Appropriate EST clones were obtained from American Type Culture Collection, sequenced, and the information was used to design PCR primers specific for the 3′ and 5′ ends of the various homologues. Full-length cDNAs were obtained by PCR using a human erythroleukemia cell (HEL) cDNA library (Clontech; for HuPLSCR2 and HuPLSCR3) or human multiple tissue cDNA (Clontech; for HuPLSCR2 and HuPLSCR4) as template. Each PCR reaction and cloning was performed in triplicate, and Advantage HF_2 DNA polymerase mix (Clontech) was used to decrease PCR-mediated error. PCR products were cloned into pCR2.1 (Invitrogen) for sequencing. Sequence of the coding region was confirmed in at least two independent clones for each of the family members. For HuPLSCR2, identical sequence was found in HEL, and multiple tissue cDNA from heart, pancreas and liver. For HuPLSCR3, the two clones sequenced from HEL were identical, and confirmed in a number of EST clones. For HuPLSCR4, sequence was obtained using multiple tissue cDNA from pancreas, spleen, and heart. Polymorphisms were noted in two positions for HuPLSCR4, as noted in the legend to Fig. 4.

2.6. Chromosomal mapping

The chromosomal location for HuPLSCR2 was determined using the GeneBridge 3 Human Radiation hybrid panel and oligonucleotides 5′-CCTGGTGCTTAGGGTAGACAATATG-3′ and 5′-CTGACGTCCTGGGTAGAAGGCCTGGG-3′ as the forward and reverse primers, respectively (Research Genetics, Huntsville, AL). The primers flank a small intron (88 bp) within the 5′ untranslated region of HuPLSCR2, giving a PCR product of 314 bp. The map position was calculated using the Stanford server (http://www.shgc.stanford.edu).

2.7. Tissue distribution

Human multi-tissue Northern blots (Clontech) were hybridized with random prime labeled cDNA probes of HuPLSCR1, 2, and 4. The HuPLSCR1 probe consisted of nucleotides 1–498 of HuPLSCR1 (GenBank AF098642). The HuPLSCR2 probe (nucleotides 1–1265 of GenBank AF159441) was prepared by digesting EST clone AA813518 with *Not*I and *Xho*I, and the HuPLSCR4 probe (nucleotides 1–851 of GenBank AF199023) by digesting EST clone N78598 with *Not*I and *Xho*I. The cDNA fragments were separated from vector sequences by agarose gel electrophoresis and purified using Wizard columns (Promega). The cDNA probes were labeled with [α-^{32}P]dATP (50 μCi/25 ng cDNA, 3000 Ci/mmol; ICN) using the random prime labeling kit from Boehringer Mannheim to a specific activity $\geq 1\times10^9$ dpm/μg. Due to non-specific hybridization of the cDNA probe, an RNA antisense probe was designed for HuPLSCR3. A PCR product of the 3′ untranslated region of HuPLSCR3 (nucleotides 1044–1279 of GenBank AF159442) was prepared using the forward primer 5′-TGTGAGGAGACCATCACCTCGAC-3′ and reverse primer 5′-AAAGCTGATATGCCTGTGTGCC-3′. The reverse primer contained the T7 promoter sequence (5′-AATTTAATACGACTCACTATAGGG-3′) at the 5′ end. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen). A ^{32}P-labeled antisense RNA probe was prepared using 50 ng of the PCR product as template in T7 transcription reaction with [α-^{32}P]UTP (800 Ci/mmol, 20 μCi/μl; Amersham) following the instructions included in the T7 Strip-EZ RNA kit (Ambion). Multi-tissue Northern blots were prehybridized for 1 h at 68°C in ExpressHyb hybridization buffer (Clontech) followed by hybridization for 18 h at 68°C in the same buffer containing 2×10^6 cpm/ml denatured random prime labeled probe. For HuPLSCR3, the blots were prehybridized in UltraHyb hybridization buffer (Ambion) with 100 μg/ml denatured salmon sperm DNA and 50 μg/ml yeast RNA and hybridized in the same buffer con-

Table 1
DNA sequence at the exon and intron junctions of HuPLSCR1 gene

	Splice donor site		Splice acceptor site	
Exon 1	AGCCAGAG**gt**gcgcgg	Intron 1	ttttt**ag**AACTGTTT	Exon 2
Exon 2	CAAACAAA**gt**aagtaa	Intron 2	aattgc**ag**ACTCACAG	Exon 3
Exon 3	ATTCCAAG**gt**aaagca	Intron 3	tatttc**ag**GACCTCCA	Exon 4
Exon 4	TAAGTCAG**gt**aatttc	Intron 4	tgctat**ag**ATAGATCA	Exon 5
Exon 5	TCTGGAAG**gt**atgtat	Intron 5	gttttt**ag**TTTTAACA	Exon 6
Exon 6	TTCAGGAG**gt**ctgtga	Intron 6	ctttgt**ag**ATAGAAAT	Exon 7
Exon 7	ATTTTGAG**gt**aagaga	Intron 7	caattt**ag**ATTAAATC	Exon 8
Exon 8	TCCTCATT**gt**aagtct	Intron 8	ttatct**ag**GACTTCAT	Exon 9

Exon sequence is shown in uppercase, intron sequence in lowercase letters. The invariant **gt** of intron at the splice donor site and **ag** of intron at the splice acceptor site are shown in bold.

taining ^{32}P-labeled antisense RNA probe (2×10^6 cpm/ml) at 68°C for 18 h. The blots were washed at a final stringency of 0.1×SSC in 0.1% SDS at 50°C (68°C for HuPLSCR3), and exposed to Amersham Hyperfilm MP.

3. Results and discussion

3.1. Human PLSCR1 gene structure

In order to gain insight into the gene organization of HuPLSCR1, a clone of approximately 100 kb of genomic DNA was obtained from a BAC human genomic library by screening with a HuPLSCR1 cDNA probe. *Eco*RI-digested fragments were cloned into pcDNA3, and sequence from six different clones was used to deduce 29.7 kb of HuPLSCR1 genomic DNA. The organization of the gene was deduced by alignment of the genomic sequence with the cDNA sequence for HuPLSCR1 (GenBank accession number AF098642). The HuPLSCR1 gene consists of nine exons, eight introns and 5′ flanking sequence (deposited under GenBank accession numbers AF153715 and AF224492). As shown in Table 1, invariant gt and ag were found at the intron splice donor and acceptor sites. As illustrated in Fig. 1, the first exon is untranslated, with the open reading frame starting in exon 2. Of interest, Kasukabe et al. [16] reported the occurrence of a truncated form of MuPLSCR1 (termed MmTRA1a), the closest murine orthologue of HuPLSCR1, in a mouse monocytic cell line which was highly leukemogenic when injected into syngeneic or athymic mice. In addition, non-leukemogenic sublines became leukemogenic when transfected with MmTRA1a. By contrast, normal macrophages expressed only full-length MuPLSCR1. Comparison of the sequence of MmTRA1a with Fig. 1 reveals that murine MmTRA1a is likely a product of alternative splicing, as the predicted open reading frame reported by Kasukabe et al. [16] starts at a position corresponding to the beginning of exon 6 in HuPLSCR1. It remains to be determined whether the analogous alternatively spliced forms of HuPLSCR1 are similarly associated with leukemias in man.

3.2. Promoter analysis

In order to identify the promoter region for HuPLSCR1, luciferase reporter constructs of 5′ flanking sequence and serial 5′ or 3′ deletions were expressed in Daudi cells, which normally express the HuPLSCR1 gene. As illustrated by the data in Fig. 2, a reporter construct containing 5′ untranslated sequence comprised of −4120 to +60 exhibited strong promoter activity. Deletion of sequence from the 5′ end from −4120 bp to −557 bp did not affect promoter activity. However, deletion from −95 bp of 5′ flanking sequence to +60 bp of the first (untranslated) exon resulted in the loss of more than 97% of promoter activity, locating the promoter of HuPLSCR1 to that region. Computer analysis of HuPLSCR1 5′ flanking sequence using the MatInspector V2.2 program (http://www.gsf.de/biodv/matinspector.html) revealed two GC boxes (TAGGGGAGGGGCCT at −79 to −66 bp, and AGGAGGTGGGCGCA at −59 to −46 bp) and a

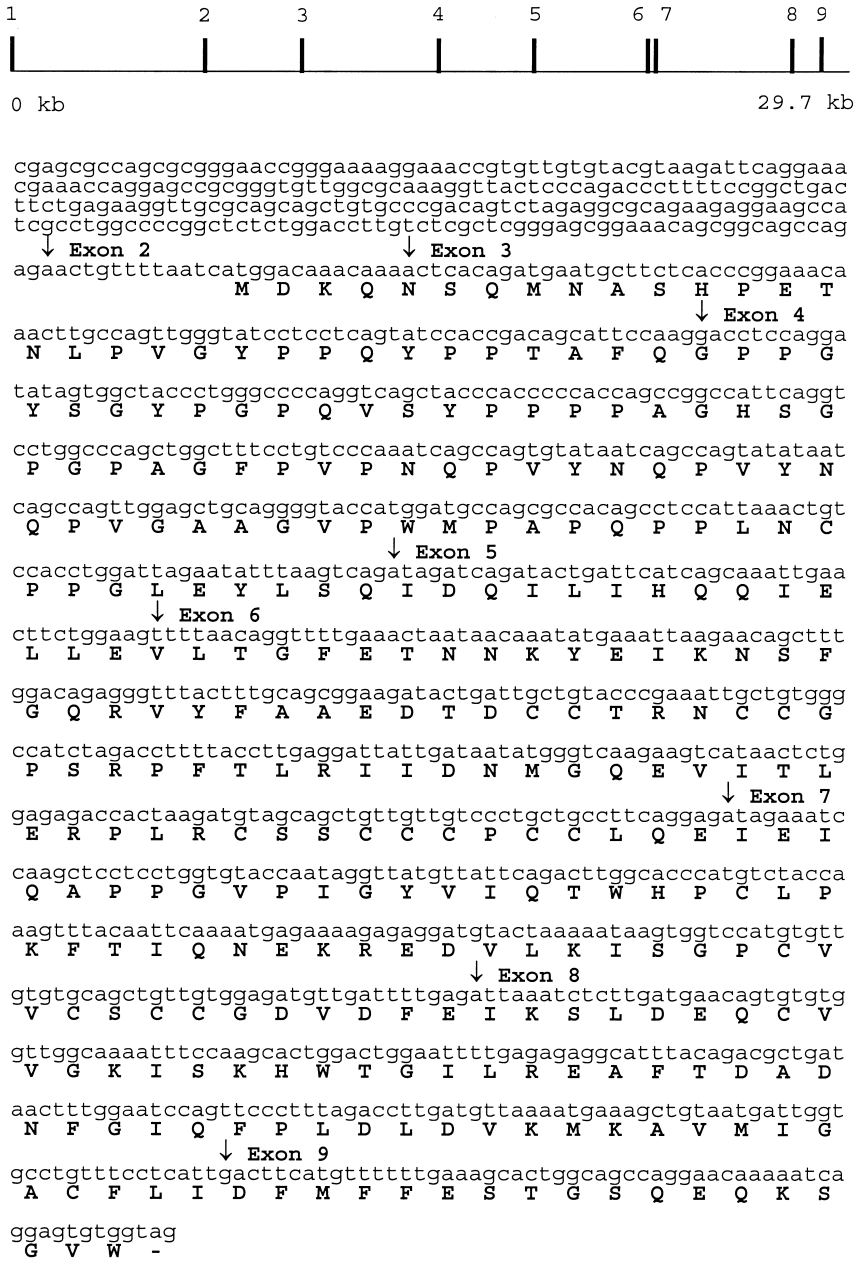

Fig. 1. Gene structure of the HuPLSCR1 gene. (Top) Schematic showing location of intron–exon borders within the HuPLSCR1 genomic sequence of 29.7 kb. Exons are represented by vertical lines. (Below) cDNA sequence of HuPLSCR1 as previously described by Zhou et al. [12] and deposited in GenBank under accession number AF098642. Locations of exons within the cDNA are indicated by arrows. Genomic sequence for HuPLSCR1 deposited in GenBank under accession number AF224492.

CCAAT box (TCTCTCCAATG at −111 to −101 bp) (Fig. 3), consistent with the data in Fig. 2 locating promoter activity to that region. In addition, potential binding sites for transcriptional activators, including activator protein 4 (AP4), upstream stimulating factor (USF), eukaryotic transcriptional regulator 1 (ETS1), interferon-stimulated response element (ISRE), and interferon regulatory factor (IRF), were identified. We had previously identified the single ISRE that is located in the first untranslated exon

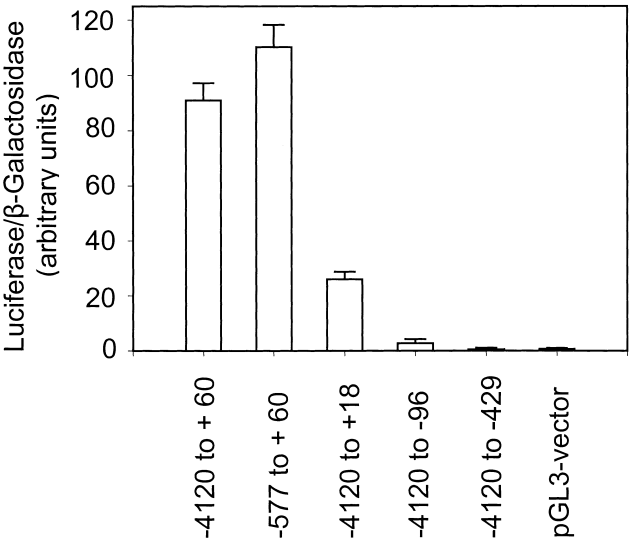

Fig. 2. Effect of 5′ and 3′ deletion of 5′ flanking region of HuPLSCR1 gene on promoter activity. 5′ flanking DNA was serially deleted from either the 5′ end or the 3′ end by PCR, and intact (−4120 bp to +60 bp) or the truncated DNA cloned into pGL3 luciferase reporter vector. Daudi cells were transfected with pGL3 constructs and pSV β-galactosidase, and harvested 48 h later for assay of luciferase and β-galactosidase activity. pGL3 vector (without insert) served as control (see Section 2). Data are expressed as ratio luciferase/β-galactosidase activity. Data of three experiments.

(+21 to +35) as the primary site responsible for the upregulation of HuPLSCR1 by interferon-α in Daudi and a variety of other cells [17].

3.3. Identity of a novel PL scramblase gene family

As previously reported, HuPLSCR1 does not show significant homology to any other known human protein, and no significant consensus to identifiable protein family or domain structures [12]. However, upon performing BLAST searches of the GenBank EST database with HuPLSCR1, we noted three distinct clusters of EST clones that were similar, but distinctly different from the sequence we had originally reported for HuPLSCR1. In order to obtain cDNAs for these putative homologues of HuPLSCR1, sequence derived from relevant EST clones was used to design PCR primers. Full-length cDNAs were obtained by PCR using a cDNA library from HEL cells, and cDNA from multiple human tissues as template. As illustrated in Fig. 4, the cloned cDNAs encode three novel proteins with high homology to HuPLSCR1. The predicted open reading frames encode proteins with 59% (HuPLSCR2; 224 aa; GenBank AF159441), 47% (HuPLSCR3; 295 aa; GenBank AF159442) and 46% (HuPLSCR4; 329 aa; GenBank AF199023) identity, respectively, to HuPLSCR1. Furthermore, cDNAs of novel murine orthologues of HuPLSCR3 (MuPLSCR3; 327 aa; GenBank AF159850) and HuPLSCR4 (MuPLSCR4; partial, in progress) have been cloned and sequenced (data not shown). Closer inspection of the four human PLSCR homologues reveals a low degree of similarity for the proline-rich amino-terminal portion of the proteins (amino acids 1–85 in HuPLSCR1), and highest degree of identity towards the carboxy-terminus, including a region (aa 273–284 in HuPLSCR1) which has been shown for HuPLSCR1 to contain a Ca^{2+} binding site required for the Ca^{2+}-induced transmembrane movement of phospholipids [14]. Of note, the predicted open reading frame for HuPLSCR2, the closest homologue to HuPLSCR1, is missing the proline-rich amino-terminus that is

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-150  AGTTTCCTCTCCTTAACCACCGGACAAACGTCTCTGGAGTCTCTCCAATG   -101
                                                CCAAT box
-100  AGCAAGAAAGCAAGTCGGGGGTAGGGGAGGGGCCTCACACCAGGGGGTGG   -51
                                    GC box          GC box
-50   GCGCAGTCCCTCCTCCAGCTCCTTCACCCTCCAGTAGTCTCGTGGGTCCC   -1
                          AP4                 USF
+1    CGAGCGCCAGCGCGGGAACCGGGAAAAGGAAACCGTGTTGTGTACGTAAG   +50
            AP4   ETS1            ISRE;IRF
+51   ATTCAGGAAA
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Fig. 3. Analysis of genomic 5′ flanking sequence of HuPLSCR1. Shown are consensus promoters and putative binding sites for transcriptional activators in 5′ flanking sequence and first untranslated exon of HuPLSCR1 gene (GenBank accession number AF153715) as predicted by MatInspector V2.2.

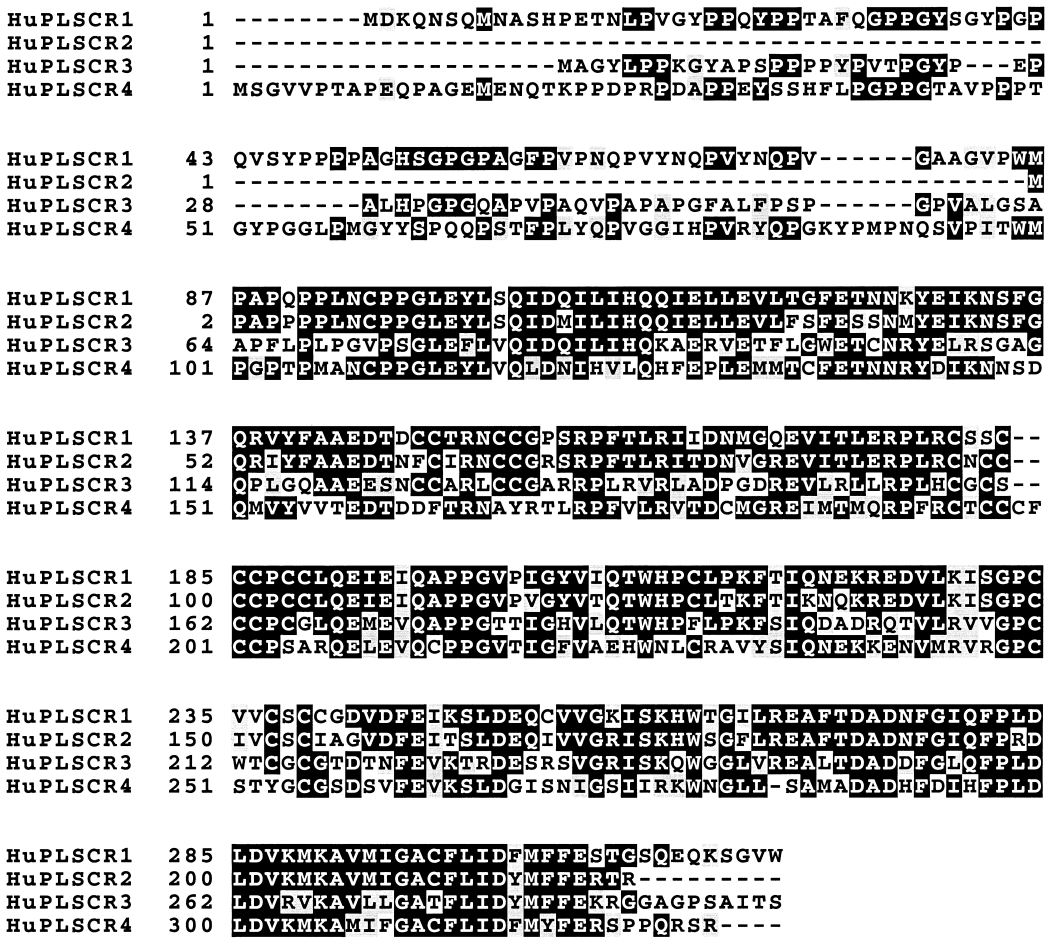

Fig. 4. Alignment of amino acid sequences of four HuPLSCR homologues. Alignment was performed using Clustal W [20]. Identical amino acids are shown on a black, and conservative substitutions on a gray background. cDNA sequences deposited in GenBank under the following accession numbers: HuPLSCR1 (human phospholipid scramblase), AF098642; HuPLSCR2, AF159441; HuPLSCR3, AF159442; HuPLSCR4, AF199023. For HuPLSCR4, sequence obtained for cDNA from pancreas is shown. In sequences from other tissues, nucleotides were A at position 246 and A at position 608 of the cDNA, changing Ser^{34} to Asn, and Val^{155} to Ile, respectively.

characteristic for all other members of this family. As pointed out previously for HuPLSCR1, this region (aa 1–85 in HuPLSCR1) contains a number of PXXP motifs, which may serve as potential binding sites for proteins containing SH3 domains [15,18]. PXXP motifs are also found in the corresponding segments of HuPLSCR3 and 4. In addition, HuPLSCR1, 3, and 4 all contain one or more PPXY motifs in that same region (aa 22–25 and aa 33–36 in HuPLSCR1; aa 15–18 in HuPLSCR3; aa 30–33 in HuPLSCR4), suggesting a potential interaction with proteins containing WW domains [19]. Such domains are primarily found in proteins with signaling or regulatory function. Although binding partners for any of the PLSCR proteins have not been identified to date, it is interesting to note that the functional implication of the missing amino-terminal segment in HuPLSCR2 may be a potential loss of interaction with an adapter or signaling molecule. A similar truncation has previously been noted to confer leukemogenic potential to MuPLSCR1 (MmTRA1a) [15,16].

3.4. Chromosomal assignment of PL scramblase family members

The chromosomal locations of HuPLSCR1, HuPLSCR3 and HuPLSCR4 were determined from

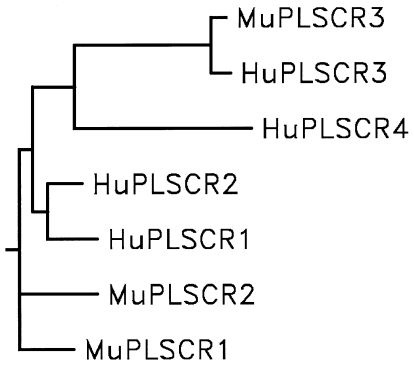

Fig. 5. Phylogenetic tree of human and murine members of the PL scramblase gene family. Multiple sequence alignment for protein sequences of human and murine members of the PL scramblase gene family was performed using Clustal W, and analyzed using the PHYLIP Rooted Phylogenetic Tree program (http://workbench.sdsc.edu/). GenBank accession numbers for HuPLSCR1–4 as indicated in Fig. 4. Accession numbers for murine PLSCR: MuPLSCR1, AF159593; MuPLSCR2, AF015790; MuPLSCR3, AF159850.

nucleotide sequence homologies to STS sequences found on the NCBI Human Gene Map'99 (http://www.ncbi.nlm.nih.gov/genemap/). The genes for HuPLSCR1 (stSG10277) and HuPLSCR4 (GenBank N78598/G37067) are clustered between markers D3S1557 and D3S1306 (164.6–168.3 cM) on chromosome 3 (3q23) at the physical position 537.09 cR_{3000}. HuPLSCR1 has also been independently mapped to chromosome 3 at 3q23 by fluorescence in situ hybridization [15]. A partial sequence for the HuPLSCR3 gene is located between nucleotides 10 501 and 9174 of the gene sequence deposited in GenBank under accession number AF097738, which also encodes a non-receptor tyrosine kinase gene (nucleotides 531–9180). The non-receptor tyrosine kinase gene has been localized to chromosome 17p13.1 between markers D17S1828 and D17S786 (9.8–18.1 cM) at the physical position 53.50 cR_{3000}, thus localizing HuPLSCR3 to that position. The gene for HuPLSCR2 was mapped as described in Section 2, and was found to be located on chromosome 3 (3q23), closely clustered with HuPLSCR1 and HuPLSCR4. The phylogenetic relationship between the various PL scramblase gene family members of both human and murine origin as analyzed by the PHYLIP Rooted Phylogenetic Tree program following multiple sequence alignment using Clustal W is illustrated in Fig. 5.

3.5. Tissue distribution of PL scramblase family members

The tissue distribution for the four members of the PL scramblase family of proteins was evaluated by Northern blotting with ^{32}P-labeled probes specific for HuPLSCR1–4, respectively. The specificity of the probes was ascertained by DNA dot blot (Fig. 6). Whereas amounts of mRNA for HuPLSCR2 in many of these tissues were below the limit of detection, the mRNAs for the other three homologues were expressed in most of the 16 different tissues examined. However, the expression patterns for these three family members show distinct differences. Fig. 7 shows that mRNA for HuPLSCR1 was below the limits of detection in brain and skeletal muscle. As previously reported, two different size transcripts (~2.55 kb and 1.6 kb) were detected for HuPLSCR1

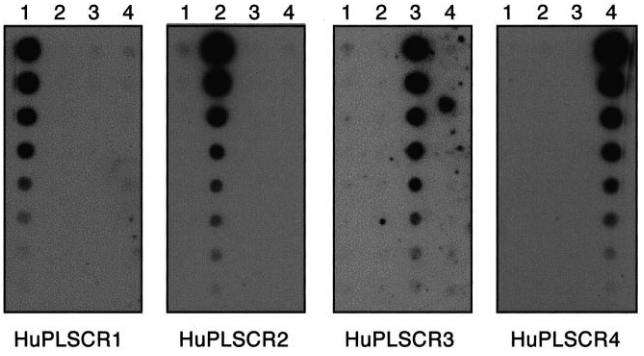

Fig. 6. Probe specificity for different HuPLSCR family members. The specificity of each probe (to be used for Northern blots shown in Fig. 7) was tested by hybridization to dot blots containing two-fold serial dilutions of DNA (0.1 fmol to 1.6 amol) of (1) a pCR2.1 clone containing bases 1–2076 of HuPLSCR1 (GenBank AF098642), (2) HuPLSCR2 EST clone AA813518 containing bases 1–1265 of GenBank AF159441, (3) HuPLSCR3 EST clone AA634048 containing bases 1–1671 of GenBank AF159442, and (4) a pCR2.1 clone containing bases 1–3206 of HuPLSCR4 (GenBank AF199023). The dot blots were hybridized with random prime labeled probes for HuPLSCR1, 2, and 4, and the antisense RNA probe for HuPLSCR3 described in Section 2. Hybridizations with the random prime labeled probes for HuPLSCR1, 2, and 4 (2×10^6 cpm/ml) were performed in ExpressHyb at 68°C for 1 h, and hybridization with the antisense probe for HuPLSCR3 (2×10^6 cpm/ml) was in UltraHyb buffer with 100 μg/ml denatured salmon sperm DNA and 50 μg/ml yeast RNA at 68°C for 18 h. Blots were washed at a final stringency of 0.1×SSC in 0.1% SDS at 50°C, then exposed to film.

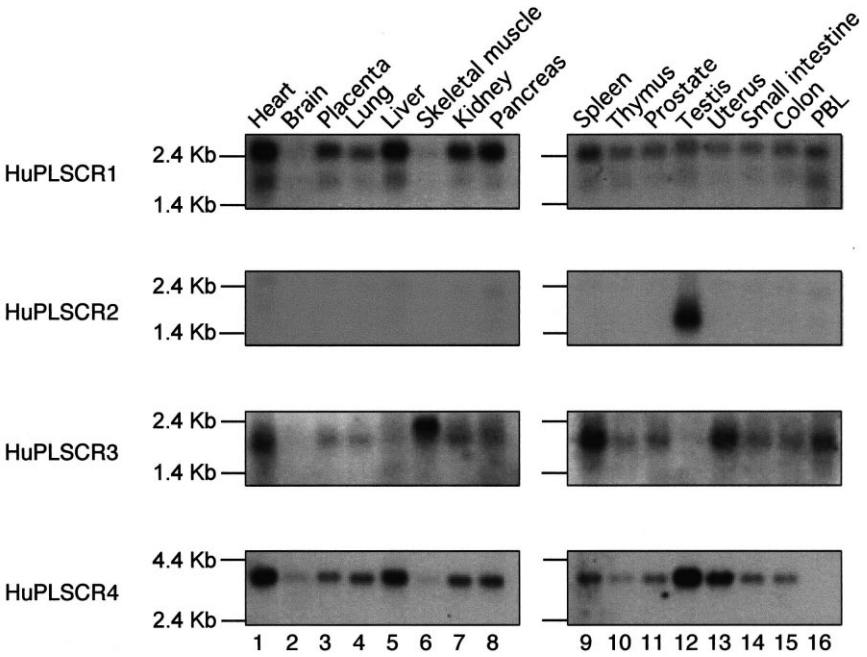

Fig. 7. Differential expression of mRNA from HuPLSCR family members in human tissues. Northern blots containing poly(A)$^+$ RNA from 16 different human tissues were hybridized with probes specific for each HuPLSCR family member as described in Section 2.

in all tissues expressing this gene. Kasukabe et al. have suggested that the different size transcripts arise from alternative polyadenylation signals within the 3′ untranslated region of the HuPLSCR1 gene [12,15]. Interestingly, the expression of HuPLSCR2 mRNA appears to be highly restricted. Although trace amounts of HuPLSCR2 could be amplified from HEL cells through several rounds of PCR for sequencing purposes (see Section 2), a 1.6 kb message was only detected in testis. This result was confirmed by probing a human Multi Tissue Expression Array (Clontech, Cat. #7775-1), which again yielded a positive blot against mRNA of testis only. This blot also revealed that in addition to the tissues listed in Fig. 7, HuPLSCR2 message was also not detected in any tissues of the gastrointestinal tract, bladder, ovary, lymph node, bone marrow, and adrenal, thyroid, salivary or mammary gland (results not shown). HuPLSCR3 mRNA was below the limit of detection in testis, brain or liver. Two sizes of mRNA were detected with the HuPLSCR3-specific probe: whereas a ~1.8 kb mRNA species was observed for most tissues, a ~2.1 kb mRNA transcript was detected in skeletal muscle. An mRNA transcript of ~4 kb was detected for HuPLSCR4 in all tissues examined except peripheral blood lymphocytes. Importantly, HuPLSCR4 mRNA was the only family member expressed at detectable levels in brain tissue. Whether HuPLSCR1, 3, and 4 have redundant function in a number of tissues, or whether these proteins exhibit activities that are distinct for each family member is the subject of future experimentation.

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References

[1] A.J. Schroit, R.F.A. Zwaal, Transbilayer movement of phospholipids in red cell and platelet membranes, Biochim. Biophys. Acta 1071 (1991) 313–329.

[2] P. Devaux, Static and dynamic lipid asymmetry in cell membranes, Biochemistry 30 (1991) 1163–1173.
[3] E.M. Bevers, P. Comfurius, D.W.C. Dekkers, R.F.A. Zwaal, Lipid translocation across the plasma membrane of mammalian cells, Biochim. Biophys. Acta 1439 (1999) 317–330.
[4] P.J. Sims, E.M. Faioni, T. Wiedmer, S.J. Shattil, Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity, J. Biol. Chem. 263 (1988) 18205–18212.
[5] R.H. Wang, G. Phillips Jr., M.E. Medof, C. Mold, Activation of the alternative complement pathway by exposure of phosphatidylethanolamine and phosphatidylserine on erythrocytes from sickle cell disease patients, J. Clin. Invest. 92 (1993) 1326–1335.
[6] P. Williamson, R.A. Schlegel, Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells, Mol. Membr. Biol. 11 (1994) 199–216.
[7] V.A. Fadok, D.L. Bratton, S.C. Frasch, M.L. Warner, P.M. Henson, The role of phosphatidylserine in recognition of apoptotic cells by phagocytes, Cell Death Differ. 5 (1998) 551–562.
[8] P. Williamson, A. Kulick, A. Zachowski, R.A. Schlegel, P.F. Devaux, Ca^{2+} induces transbilayer redistribution of all major phospholipids in human erythrocytes, Biochemistry 31 (1992) 6355–6360.
[9] C.-P. Chang, J. Zhao, T. Wiedmer, P.J. Sims, Contribution of platelet microparticle formation and granule secretion to the transmembrane migration of phosphatidylserine, J. Biol. Chem. 268 (1993) 7171–7178.
[10] E.F. Smeets, P. Comfurius, E.M. Bevers, R.F.A. Zwaal, Calcium-induced transbilayer scrambling of fluorescent phospholipid analogs in platelets and erythrocytes, Biochim. Biophys. Acta 1195 (1994) 281–286.
[11] F. Bassé, J.G. Stout, P.J. Sims, T. Wiedmer, Isolation of an erythrocyte membrane protein that mediates Ca^{2+}-dependent transbilayer movement of phospholipid, J. Biol. Chem. 271 (1996) 17205–17210.
[12] Q. Zhou, J. Zhao, J.G. Stout, R.A. Luhm, T. Wiedmer, P.J. Sims, Molecular cloning of human plasma membrane phospholipid scramblase – A protein mediating transbilayer movement of plasma membrane phospholipids, J. Biol. Chem. 272 (1997) 18240–18244.
[13] J. Zhao, Q. Zhou, T. Wiedmer, P.J. Sims, Level of expression of phospholipid scramblase regulates induced movement of phosphatidylserine to the cell surface, J. Biol. Chem. 273 (1998) 6603–6606.
[14] Q. Zhou, P.J. Sims, T. Wiedmer, Identity of a conserved motif in phospholipid scramblase that is required for Ca^{2+}-accelerated transbilayer movement of membrane phospholipids, Biochemistry 37 (1998) 2356–2360.
[15] T. Kasukabe, H. Kobayashi, Y. Kaneko, J. Okabe-Kado, Y. Honma, Identity of human normal counterpart (MmTRA1b) of mouse leukemogenesis-associated gene (MmTRA1a) product as plasma membrane phospholipid scramblase and chromosome mapping of the human MmTRA1b phospholipid scramblase gene, Biochem. Biophys. Res. Commun. 249 (1998) 449–455.
[16] T. Kasukabe, J. Okabe-Kado, Y. Honma, TRA1, a novel mRNA highly expressed in leukemogenic mouse monocytic sublines but not in nonleukemogenic sublines, Blood 89 (1997) 2975–2985.
[17] Q. Zhou, J. Zhao, F. Al-Zoghaibi, A. Zhou, T. Wiedmer, R.H. Silverman, P.J. Sims, Transcriptional control of the human plasma membrane phospholipid scramblase 1 gene is mediated by interferon α, Blood 95 (2000) 2593–2599.
[18] R.J. Rickles, M.C. Botfield, Z. Weng, J.A. Taylor, O.M. Green, J.S. Brugge, M.J. Zoller, Identification of Src, Fyn, Lyn, PI3K and Abl SH3 domain ligands using phage display libraries, EMBO J. 13 (1994) 5598–5604.
[19] P. Bork, J. Schultz, C.P. Ponting, Cytoplasmic signalling domains: the next generation, Trends Biochem. Sci. 22 (1997) 296–298.
[20] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.