Identification and Characterization of Human SLP-2, a Novel Homologue of Stomatin (Band 7.2b) Present in Erythrocytes and Other Tissues*

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Human stomatin (band 7.2b) is a 31-kDa erythrocyte membrane protein of unknown function but implicated in the control of ion channel permeability, mechanoreception, and lipid domain organization. Although absent in erythrocytes from patients with hereditary stomatocytosis, stomatin is not linked to this disorder. A second stomatin homologue, termed SLP-1, has been identified in nonerythroid tissues, and other stomatin related proteins are found in Drosophila, Caenorhabditis elegans, and plants. We now report the cloning and characterization of a new and unusual stomatin homologue, human SLP-2 (stomatin-like protein 2). SLP-2 is encoded by an ~1.5-kilobase mRNA (GenBank™ accession no. AF190167). The gene for human SLP-2, HUSLP2, is present on chromosome 9p13. Its derived amino acid sequence predicts a 38,537-kDa protein that is overall ~20% similar to human stomatin. Northern and Western blots for SLP-1 and SLP-2 reveal a wide but incompletely overlapping tissue distribution. Unlike SLP-1, SLP-2 is also present in mature human erythrocytes (~4,000 ± 5,600 (± 2 S.D.) copies/cell). SLP-2 lacks a characteristic NH₂-terminal hydrophobic domain found in other stomatin homologues and (unlike stomatin) is fully extractable from erythrocyte membranes by NaOH, pH 11. SLP-2 partitions into both Triton X-100-soluble and -insoluble pools in erythrocyte ghost membranes or when expressed in cultured COS cells and migrates anomalously on SDS-polyacrylamide gel electrophoresis analysis with apparent mobilities of ~45,500, 44,600, and 34,300 M₉. The smallest of these protein bands is believed to represent the product of alternative translation initiated at AUGs beginning with nt 217 or 391, although this point has not been rigorously proven. Collectively, these findings identify a novel and unusual member of the stomatin gene superfamily that interacts with the peripheral erythrocyte cytoskeleton and presumably other integral membrane proteins but not directly with the membrane bilayer. We hypothesize that SLP-2 may link stomatin or other integral membrane proteins to the peripheral cytoskeleton and thereby play a role in regulating ion channel conductances or the organization of sphingolipid and cholesterol-rich lipid rafts.

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Previously known as band 7.2b because of its relative electrophoretic mobility in samples of human red blood cell ghost preparations, stomatin is a less characterized integral erythrocyte membrane protein with a molecular mass of 31 kDa (1, 2). Deficiency of stomatin in red cells is associated with hereditary stomatocytosis, a disease with marked red cell shape abnormalities and increased monovalent cation permeability (for reviews, see Refs. 3 and 4). However, linkage studies and direct sequencing establish that a defect in stomatin is not the cause of this disorder (4–6), and mice lacking stomatin retain normal red cell morphology and apparently normal function (7). The role of stomatin thus remains a mystery. In a human amniotic cell line, stomatin concentrates preferentially in plasma membrane protrusions and appears to co-localize with cortical actin microfilaments (8). In Caenorhabditis elegans, a stomatin homologue (MEC-2) is required for sensory mechanoreception and the gating of an oligomeric sodium channel (9). A second homologue in C. elegans (UNC-24), a protein most similar to a human stomatin homologue termed SLP-1 (10), is required for normal locomotor response to volatile anesthetics and contains a region of sequence homologous to the nonspecific lipid transfer protein (11). A third homologue in C. elegans (UNC-1) also appears to play a central role in the organism’s response to volatile anesthetics (12). In plants, a homologue of stomatin (slp) is required for bean nodulation and growth in media containing hypertonic monovalent cations (13). Together, these data implicate stomatin (or a homologue) as an adapter between ion channels and the cytoskeletal network, perhaps influencing channel stability and organization in the plasma membrane. Other observations suggest that stomatin binds calpromotin (involved with the activation of the charybdotoxin-sensitive calcium-dependent potassium channel of red cells) (14) and participates in the trans-bilayer exchange or reorientation of phospholipids (15, 16).

The structure or disposition of stomatin in the membrane is not well defined; available data suggest an unusual topography. Sequence analysis predicts that stomatin has a single 23-residue hydrophobic domain near its NH₂ terminus, and it is palmitoylated just proximal to this predicted hydrophobic domain on Cys²⁹ (in the mature protein, Cys³⁰ in the derived sequence (17)). Sequences distal to the putative transmembrane segment are hydrophilic and are predicted to form a bipartite β-sheet and α-helical structure (3). The large COOH-terminal domain is cytoplasmic, based on its selective protease sensitivity in leaky ghosts but not intact red cells (18). The protein is phosphorylated on Ser²⁹, and the short NH₂-terminal sequence containing the phosphorylation site (which is proximal to the hydrophobic domain) is also cytoplasmic in its orientation (19). Thus, both ends of the protein must face the cytosol. It is unknown whether other portions of stomatin, such as the predicted β-sheet region, enter the bilayer or whether the hydrophobic region of stomatin enters the bilayer but does...
not span it. In the membrane, stomatin appears as large (n ~ 9–12) homo-oligomers, a property bestowed by sequences near the COOH terminus (20).

The identification of stomatin homologues has provided important insights into the potential functions of this gene superfamily. Three homologues have been identified in C. elegans: MEC-2, UNC-24, and UNC-1. As noted above, MEC-2 appears to link degenerin channels, homologues of mammalian epithelial sodium channels, to a microtubule based cytoskeletal network; UNC-24 and UNC-1 bestow sensitivity to certain volatile anesthetics. The only vertebrate homologue of stomatin identified is human SLP-1, which is most abundant in the brain and shares many similarities with UNC-24 (10). All of these proteins as well as the stomatin from other species (e.g. mouse and zebra fish) share a characteristic NH₂-terminal hydrophobic domain as well as a consensus stomatin signature sequence that defines the stomatin gene family (i.e. RX₉(L/I/V)/K/RH/L/I/V/X/R/K/R/L/I/V/K/R) (as defined by the PROSITE program, using data derived from Ref. 21).

We now report the cloning and characterization of a new member of the vertebrate stomatin gene family. We name this gene, identified in a human heart cDNA library, HUSLP2, and the derived protein SLP-2 (stomatin-like protein 2). Similar to other family members, SLP-2 shares the cognate stomatin signature sequence noted above. However, it is the first member of this family to be recognized that lacks a NH₂-terminal hydrophobic domain. SLP-2 is widely expressed in many tissues, as is SLP-1, but unlike SLP-1 it is also found in the mature human erythrocyte membrane. In the erythrocyte, it associates with the cortical spectrin-actin cytoskeleton and probably with other integral membrane proteins but is not itself integral to the membrane bilayer. In the erythrocyte membrane, it also appears to exist at least partially as an oligomeric protein complex. These features distinguish it from stomatin and SLP-1 and suggest that members of this gene superfamily may function as both integral and peripheral membrane proteins. The identification of SLP-2 as a second stomatin-related protein in red cells and as a new component of the peripheral membrane skeleton also may have implications for understanding the phenotype of certain hemolytic conditions.

**MATERIALS AND METHODS**

**Cloning and Sequencing**—Unless otherwise stated, all molecular biological procedures followed standard methods (22). Candidate sequences were amplified from a Marathon-Ready cDNA library prepared from human heart muscle (CLONTECH, Palo Alto, CA). The Advantage cDNA PCR kit was used to perform 5’ and 3’-RACE amplifications, following the instructions of the manufacturer (CLONTECH). For 5’-RACE, the primer used was GTCCCCAGACTCTCTGCGGCC; the primer for the 3’-RACE was GCGCGTTGGAATGCTGGCGCG. PCR products were purified by agarose gel electrophoresis and cloned into TA cloning vector (Invitrogen). All constructs were amplified, cloned, and sequenced multiple times to verify the fidelity of the cDNA sequences obtained. Automated DNA sequencing was carried out by the Keck Laboratory (Yale University). FLAG-tagged SLP-2 constructs were prepared using a synthetic oligonucleotide representing the coding sequence of the last seven amino acids of stomatin followed by the FLAG sequence and a stop codon (23), paired with a primer corresponding to the desired ATG initiator codon. The PCR product was cloned into the pSG5 expression vector (Stratagene) prior to transfection into either COS or 293T cells.

**Northern Blot**—Northern blot analysis of multiple human tissues was performed according to the instructions of the manufacturer, using their multiple human tissue Northern blot (catalog no. 7760, lot 7010555 (CLONTECH)). Randomly 32P-labeled BamHI fragments of SLP-1 or SLP-2 cDNA were used as hybridization probes. The loading of mRNA was verified by probing β-actin mRNA with the probe provided in the multiple human tissue Northern blot kit.

**Antibody Production**—Antibodies were raised in New Zealand White rabbits as before (24). The cDNAs of SLP-1 and SLP-2 in pcR2.1 (Invitrogen) were digested with BamHI and EcoRI, and the BamHI/EcoRI fragments were cloned into pGEX-3X (Amersham Pharmacia Biotech) to produce recombinant GST fusion proteins representing the approximate C-terminal two-thirds of each protein (corresponding to amino acids 65–290 for SLP-2). These were expressed in the DH5α strain of Escherichia coli to generate the corresponding recombinant fusion proteins. The fusion proteins were purified by SDS-PAGE and stained lightly with Coomassie Blue, and the recombinant proteins were sliced form the gel, emulsified in incomplete Freund’s adjuvant, and used as antigens for immunization. Sera were affinity-purified, and the reactivity to GST was removed by immunosorption with GST immobilized on agarose beads. Antibodies directed against a mouse full-length stomatin GST fusion protein prepared in a similar fashion were kindly provided by Paul Stabach and Dr. John Sinard.

**Immunofluorescence**—Fresh human erythrocytes were washed twice with chilled PBS and then air-dried as smears on glass slides. These were then fixed in methanol on ice for 10 min. The cells were then washed with cold PBS and blocked with 5% BSA (v/v) in PBS for 1 h. Primary antibodies were applied overnight in a humidified chamber, followed by PBS rinse. Cy3-labeled secondary anti-rabbit antibodies were then applied for 2 h. After washing, slides were mounted with glass coverslips and viewed by epifluorescence or by confocal microscopy using an Olympus AX-70 inverted confocal microscope.

**Cell Preparations and Extractions**—Cell lines were from ATCC and were transfected using LipofectAMINE™ (Stratagene), following the manufacturer’s protocol. Fresh human erythrocytes and erythrocyte ghosts were prepared by washing twice with chilled PBS, cold PBS by lysis in a 20-fold volume of 5 mM sodium phosphate, pH 7.5, in the presence of 1 mM EDTA and various protease inhibitors (25). Triton extraction was carried out at 4 °C for 15 min by suspending approximately 10⁶ cells (erythrocyte ghosts or cultured cells) in 1% Triton in PBS. Triton-soluble and-insoluble fractions were separated by centrifugation for 30 min at ~30,000 × g. Packed ghosts were alkaline extracted by incubation in 10× volume of 15 mM NaOH for 15 min at 4 °C; extractable and inextractable fractions were separated by centrifugation as above. Salt extractions were carried out by first incubating freshly prepared erythrocyte ghosts with 0.1 mM EDTA at pH 8–9 at 37 °C for 30 min. The pellet resulting from this extraction (largely consisting of erythrocyte inside-out vesicles) was then incubated with 0.5 μM XcI under the same conditions for an additional 30 min.

**Other Procedures**—For Western blotting, cells or tissues were lysed in lysis buffer (2% SDS in PBS plus protease inhibitors) and separated by SDS-PAGE. After transfer to polyvinylidene difluoride membrane, proteins of interest were detected with affinity-purified antibodies. In vitro translations were performed with the TNT™ coupled reticulocyte lysate system (Promega) following the manufacturer’s instructions. Each reaction used 2 μg of plasmid DNA. SDS-PAGE analysis followed the method of Laemmli (26). Protein determinations were carried out using the Pierce BCA method (product 23225), as described in Ref. 27.
FIG. 1. The complete cDNA sequences of SLP-2 in relation to other members of the stomatin gene family. A, key contigs identified from the EST database, along with the full-length sequence reported here. Color coding and the arrows reflect the directionality of the sequences as they are found in the database. The GenBank™ accession numbers for each of the ESTs are as given; nt positions are given at the top. Additional EST clones identified in a Blast search of the database are given in Table I for both SLP-1 and SLP-2. The full-length cDNA sequence was verified from four independent PCR-amplified clones of human SLP-2 and is available in GenBank™ as accession number AF190167. B, alignment of the derived amino acid sequence of SLP-2 with SLP-1 (gb-NM004809; Ref. 10), stomatin (gb-M81635; Ref. 1), mec-2 (gb-U26735; Ref. 9), unc-24 (gb-U42013; Ref. 11), and unc-1 (gb-U55375; Ref. 30). Also shown is the consensus strength (bars; red represents complete conservation), the putative hydrophobic transmembrane-like segment that is absent in SLP-2 and only partially present in mec-1 (yellow shaded box). The cognate consensus residues shared by all members of the stomatin (band 7.2b) gene family are RX2(L/I/V)(S/A/N)X6(L/I/V)D(R/K)(L/I/V)E(L/I/V)(K/R). These are marked by an asterisk above the consensus strength.
FIG. 1—continued

[Diagram showing sequence alignment and consensuses]

**Legend:**
- SLP2
- SLPI
- Stom
- mec-2
- unc-24
- unc-1

[Sequence alignment details and consensuses provided]

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**Consensus Sequences:**
- SLP2
- SLPI
- Stom
- mec-2
- unc-24
- unc-1

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Fig. 1—continued
SLP-2 sequence further upstream. In no case was additional 5'-sequence identified. From the human genome data base, SLP-2 was identified on chromosome 9p13 (GenBank\textsuperscript{TM} accession no. AC004472). Analysis of this sequence using the program GeneTools\textsuperscript{TM} (BioTools, Inc.), which is designed to find potential exons in genomic sequences, satisfactorily identified most of the exons responsible for the expressed SLP-2 sequence but did not predict any convincing exons upstream of the ATG beginning at nt 64. In vivo and in vitro expression of the SLP-2 cDNA also generated proteins of the correct size (see below).

Based on these criteria, it was concluded that the full-length SLP-2 is as shown in Fig. 1.

**SLP-2 Lacks the Hydrophobic Domain Found in Other Family Members**—The derived amino acid sequence of SLP-2 is compared in Fig. 1B with the sequences of human SLP-1 (GenBank\textsuperscript{TM} accession no. NM004809; Ref. 10); human stomatin (GenBank\textsuperscript{TM} accession no. M81635; Ref. 1); mec-2 (GenBank\textsuperscript{TM} accession no. U26735; Ref. 9); unc-24 (GenBank\textsuperscript{TM} accession no. U42013; Ref. 11), and unc-1 (GenBank\textsuperscript{TM} accession no. U55375; Ref. 30). All members of this family including SLP-2 share the cognate consensus sequence RX\textsubscript{X7/9}\textsubscript{X2/3/I/V/G/L/V/W}X\textsubscript{X3/4/I/V/K/R/H}X\textsubscript{X6/7/I/V}X\textsubscript{X7/9}/KS/K/R) that defines proteins of this gene superfamily. Clustal analysis revealed that SLP-2 defines a new branch of the superfamily (Fig. 2A), approximately equidistant between stomatin and SLP-1 (Fig. 2B). However, when the predicted secondary structure and hydrophobicity of SLP-2 is compared with other stomatin family members, significant differences are apparent (Fig. 3). All previously recognized stomatin family members share a characteristic NH\textsubscript{2}-terminal hydrophobic domain, and most have a consensus sequence for palmitoylation centered on Cys\textsuperscript{29} (17). These properties presumably allow stomatin, SLP-1, and close homologues to intercalate directly into the lipid bilayer. Neither of these features (i.e. a site for palmitoylation or a hydrophobic domain) are present in SLP-2 (Fig. 3).

However, immediately distal to the missing hydrophobic domain (TM in Fig. 3), SLP-2 shares strong sequence homology with both stomatin and SLP-1 in the region predicted to contain \(\beta\)-sheet and \(\alpha\)-helix structure. The overall amino acid composition of SLP-2 is also similar to other stomatins, although it is predicted to be a bit more basic with an anticipated isoelectric point of 7.3. Other predicted biophysical properties, and its composition are shown in Fig. 4.

**RESULTS**

Cloning of cDNA Encoding SLP-2—A BLAST search (28) of the EST data base using human stomatin cDNA sequence (1) identified several human and mouse sequences that encoded proteins with significant homology to stomatin. Sequence analysis revealed that they derived from two distinct genes. The most significant of the dbEST sequences are listed in Table I. One set of sequences belonged to human SLP-1. The others identified a novel gene, SLP-2, as described in this report. These sequences were used to select oligonucleotide primers that enabled the PCR amplification of the relevant sequences. To extend the sequence of SLP-2 at both 5'- and 3'-ends, we performed RACE by PCR using a Marathon-Ready cDNA library from human heart (Clontech, Palo Alto). Several overlapping clones were sequenced, and a 1,303-bp cDNA that included the full-length sequence of SLP-2 was assembled (Fig. 1A and GenBank\textsuperscript{TM} accession no. AF190167). Sequence analysis revealed a coding region of 1071 nt, predicting a 357-amino acid protein (Fig. 1B). The sequence at the 5'-end of the mRNA is interesting for the presence of three potential ATG initiator sites, all sharing the same open reading frame, as discussed below. The presumptive start site begins at nt 64. A satisfactory Kozak initiation sequence is present immediately upstream of this ATG (29), but since the sequence upstream of this ATG is relatively short and in frame, there was concern whether all coding sequence had been identified. To address this issue, 12 additional clones were sequenced from a series of four additional PCRs, and the genomic data base was exhaustively searched for evidence of ESTs that extended the

![Fig. 2](https://example.com/fig2.png)
detect any protein in these blots; the reasons for this are
unknown but may relate to the apparent reduced sensitivity of
this antibody compared with the antibody against SLP-2 (data
not shown). The antibodies to SLP-2 revealed a pattern that
correlated well with its mRNA expression profile (Fig. 6B).
In most tissues, the SLP-2 antibody detected a band at either
M_r; 45,500 or M_r; 44,600. Both of these bands are substantially
larger than the predicted size of SLP-2 (38,537 kDa). In COS
cells, A431 cells, and red blood cells, both the M_r 45,500 and
44,600 bands were evident, although the larger band was most
prominent. These three cell types also displayed a faint immu-
noreactive band at M_r ∼34,300. The origin of the multiple
bands is unknown (see below). SLP-2 thus represents a novel
stomatin gene superfamily member, one with an unusual struc-
ture. It is also a previously unrecognized component of the
mature erythrocyte membrane.

Recombinant SLP-2 Migrates as Multiple Bands Comparable
with Those of Wild Type SLP-2—

The calculated size of SLP-2 is

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<td>38.76%</td>
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A, a-helix; B, b-sheet; T, turns.

Fig. 3. SLP-2 lacks the amino-terminal hydrophobic domain of the other
stomatin family members. The predicted secondary structure of SLP-2,
SLP-1, and stomatin is shown based on the Chou and Fasman algorithm as im-
plemented in the program Protran™(DNASTar, Inc.) (40). Also shown is the
hydrophobicity profile for each protein, and the putative transmembrane
hydrophobic region of stomatin and SLP-1 is shaded yellow. Note that SLP-2 lacks this
region, but beyond this region it shares other features characteristic of stomatin
and SLP-1, including a proximal region predicted to be rich in b structure and a
more distal region rich in a-helix. The overall similarity of SLP-2 and SLP-1 to
stomatin over this region is given, for consensus lengths of 160 and 201 residues,
respectively. A, a-helix; B, b-sheet; T, turns.

Fig. 4. Composition and predicted biophysical features of the SLP-2
protein. The protein is predicted to have an isoelectric point of 7.3 and its compo-
sition is conventional. These analyses were carried out using the program
Protran™.
SLP-2 Associates with the Triton-insoluble Cytoskeleton in Erythrocyte Ghosts—Quantitation of the Amount of SLP-2 Immunoreactive Material in Erythrocytes by Comparative Western Blotting

SLP-2 and stomatin-like proteins (SLP-1 and SLP-2) are widely distributed. A, Northern blot of electrophoretically separated human poly(A)+ mRNAs (∼2 ng) from various adult tissues probed for SLP-1 and SLP-2. The same blot was hybridized for actin as a control for RNA loading. The positions of standards at 1.35 and 2.4 kilobases are as indicated. The SLP-2 mRNA is ∼1.5 kilobases. B, Western blot demonstrating SLP-2 in various cultured cell lines and human tissues. Similar experiments carried out with the available antibodies for SLP-1 did not detect SLP-1 in these blots. Note the presence of strongly SLP-2-immunoreactive bands at Mr ∼45,500 and ∼44,600. Most cells and tissues contained only one of these two bands, although both were present in COS cells, A431 cells, and red blood cell ghosts. Also detected in Madin-Darby canine kidney, COS, and A431 cells and in human heart, skeletal muscle, and red blood cells was a faint immunoreactive band at Mr ∼34,500 (*). Additonal minor bands can also be detected in more heavily loaded samples (e.g. see Fig. 8). The origins of the minor bands and the reasons for the anomalous migration of the major SLP-2 band on SDS-PAGE (calculated mass of 38,537 Da), are unknown (see "Discussion"). The apparent molecular weights (Mr) are shown along the ordinate for three standard proteins (ovalbumin, 47,500; carbonic anhydrase, 35,300; and soybean trypsin inhibitor, 28,200). All protein bands were visualized by ECL autoradiography.

38,537 kDa, substantially smaller than the major bands observed in the tissue Western blots. These observations again raised the question of whether the cDNA that had been characterized represented the full-length product or whether the additional bands arose from post-translational modification, proteolysis, alternative initiation, alternative mRNA splicing, or protein associations not dissociated under the conditions of SDS-PAGE. To address these questions, SLP-2 was expressed either in cell-free lysates or after transfection into COS cells (Fig. 7). In cell-free rabbit reticulocyte lysates, full-length SLP-2 cDNA in the pcR2.1 vector generated a Mr ∼45,500 protein as its major product, along with a doublet at ∼34,500 and a smaller product with Mr ∼26,300. When a second cDNA was used that contained a stop codon downstream of the initiator ATG (the codon beginning at nt 64), only the Mr ∼34,500 product was generated as a major band, with two faint bands detected near Mr ∼40,000. SLP-2 was also expressed in cultured COS and 293T cells, where the Triton X-100 solubility of the transfected products could be evaluated along with their apparent molecular weights. In these experiments, the eight-residue FLAG epitope tag was incorporated onto the COOH terminus of SLP-2 (22). Four SLP-2 bands were evident in the soluble fraction of COS cells: Mr ∼45,500, ∼44,600, ∼34,300, and ∼26,300 (Fig. 7B lane 1). Although differing in their relative abundance, this pattern is similar to that observed for wild type SLP-2 in COS cells (cf. Fig. 6). Only the largest two bands (Mr 45,500 and 44,600) were present in the detergent-insoluble pellet and together constituted about 60% of the total FLAG-SLP-2 in these cells (Fig. 7B, lane 2). When the same full-length construct was expressed in 293T cells, the predominant product generated was at ∼44,600, with only a small amount of the Mr 45,500 product evident. Both of these proteins were fully soluble after detergent extraction (Fig. 7B, lanes 5 and 6). Finally, a FLAG-tagged truncated SLP-2 construct was prepared that deleted the first initiator ATG (Fig. 7B, lanes 3 and 4). Expression of this construct yielded a single soluble product at Mr ∼40,000. The sequences flanking the three potential initiator sites in the SLP-2 gene, along with the calculated Mr values of the resultant products, are given in Fig. 7C.

SLP-2 Associates with the Triton-insoluble Cytoskeleton in Erythrocyte Ghosts—Quantitation of the Amount of SLP-2 Immunoreactive Material in Erythrocytes by Comparative Western Blotting using Recombinant SLP-2 as a Standard Revealed ∼4,000 ± 5,600 (±2 S.D.) copies/red cell (data not shown). The disposition of SLP-2 in mature human erythrocytes was examined based on its resistance to extraction by detergent or salt.

Fig. 5. Affinity-purified polyclonal antibodies discriminate between human SLP-2, SLP-1, and stomatin. Total bacterial lysates containing GST fusion proteins of SLP-2, SLP-1, stomatin (Stom), or GST alone were separated by SDS/PAGE and Western blotted using affinity-purified rabbit polyclonal antibodies against each of these proteins. The proteins loaded in each lane are as designated. The amounts of protein loaded into each lane were as follows: 10 ng (lane 1), 20 ng (lane 2), and 40 ng (lane 3). The antibodies used for blotting each gel, along with the Mr of three molecular weight standards, is shown to the left of each gel.

Fig. 6. Stomatin-like proteins (SLP-1 and SLP-2) are widely distributed. A, Northern blot of electrophoretically separated human poly(A)+ mRNAs (∼2 ng) from various adult tissues probed for SLP-1 and SLP-2. The same blot was hybridized for actin as a control for RNA loading. The positions of standards at 1.35 and 2.4 kilobases are as indicated. The SLP-2 mRNA is ∼1.5 kilobases. B, Western blot demonstrating SLP-2 in various cultured cell lines and human tissues. Similar experiments carried out with the available antibodies for SLP-1 did not detect SLP-1 in these blots. Note the presence of strongly SLP-2-immunoreactive bands at Mr ∼45,500 and ∼44,600. Most cells and tissues contained only one of these two bands, although both were present in COS cells, A431 cells, and red blood cell ghosts. Also detected in Madin-Darby canine kidney, COS, and A431 cells and in human heart, skeletal muscle, and red blood cells was a faint immunoreactive band at Mr ∼34,500 (*). Additonal minor bands can also be detected in more heavily loaded samples (e.g. see Fig. 8). The origins of the minor bands and the reasons for the anomalous migration of the major SLP-2 band on SDS-PAGE (calculated mass of 38,537 Da), are unknown (see "Discussion"). The apparent molecular weights (Mr) are shown along the ordinate for three standard proteins (ovalbumin, 47,500; carbonic anhydrase, 35,300; and soybean trypsin inhibitor, 28,200). All protein bands were visualized by ECL autoradiography.
was used. A full-length SLP-2 cDNA in pCR2.1 was transcribed and expressed in the presence of 35S-labeled methionine using a rabbit reticulocyte lysate. Shown is an autoradiograph of the expressed protein. Note the presence of a single protein product at M₉ 45,500 (calculated M₉ 38,537) when SLP-2 cDNA was used in the reaction (lane 1). When naked DNA was added in lanes 2 and 4 as controls without the vector, no transcription was evident. In lane 3, SLP-2 cDNA in pCR2.1 with a mutation that created a stop codon just downstream of the first ATG generated multiple bands identical to SLP-2 in COS cells. 293T cells, with the former showing bands at 38,537, 33,597, and 26,459 Mr, while SLP-2 in COS and 293T cells. All constructs placed an eight-residue FLAG epitope tag at the COOH terminus of the recombinant protein. Transiently transfected cells were then extracted with Triton X-100, and the soluble and pellet fractions were analyzed by Western blotting with anti-FLAG antibodies. The positions of the COS and 293T samples are as marked.

Lanes 1 and 2, full-length FLAG-SLP-2 (soluble and pellet fractions, respectively). Lanes 3 and 4, truncated FLAG-SLP-2 (soluble and pellet fractions, respectively). Lanes 5 and 6, full-length FLAG-SLP-2 (soluble and pellet fractions, respectively) in 293T cells. Note that the full-length FLAG-SLP-2 generates a pattern identical to that in wild type COS and 293T cells, with the former showing bands at M₉ 45,500 and 44,600 and occasionally at M₉ 34,300, while only the M₉ 44,600 band was present in the 293T cells. Approximately 60% of the FLAG-SLP-2 was in the membrane, while all was extractable in the 293T cells. By comparison, the truncated FLAG-SLP-2 construct appeared as a single band of M₉ 40,500. C, comparison of the three possible initiator sites in the SLP-2 gene, together with the predicted size of the resultant protein. All transcripts are expected to terminate at nt 1134.

In Fig. 6, just three SLP-2 immunoreactive bands were evident in erythrocytes. However, in the more heavily loaded gels shown in Fig. 8, many additional bands are apparent (a-j in Fig. 8A). Four of these bands (the most prominent) corresponded closely to those apparent in the transfected COS cells, at M₉ 45,500, 44,600, 34,300, and 26,300. All other bands were larger than M₉ 45,500. Under conditions of detergent extraction, only the major SLP-2 band at 44,600 was soluble; all other bands (that collectively accounted for about 60% of the total SLP-2), including the major SLP-2 band at 45,500, remained with the detergent-insoluble matrix (Fig. 8B). This insoluble fraction is operationally defined as the cytoskeletal matrix, although GPI-linked proteins may also remain insoluble under these conditions (31).

The presence of immunoreactive SLP-2 bands larger than M₉ 45,500 suggested that some fraction of the protein might exist in covalent oligomeric complexes, or at least in complexes that are incompletely dissociated in SDS (such as occurs with glycoporin dimers (32)). In preliminary experiments (data not shown), it was found that in freshly prepared human red cell ghost two pools of SLP-2 appeared to exist: one strongly associated with the membrane that resisted salt-extraction and a second pool that was fully extracted by 0.5 M KCl and that appeared to be composed of a high molecular weight complex involving still unidentified partners. In future studies, it will be important to identify the nature of the extractable oligomeric complex and its relationship to the more tightly associated membrane pool of SLP-2. To evaluate whether SLP-2 was also found in the cytosol of red cells, the hemolysate and membrane fractions from a fixed amount of red cells were analyzed by Western blotting; no SLP-2 was detected in the hemolysate under conditions in which the SLP-2 band was clearly discernible in the ghost (data not shown). In addition, the cellular distribution of spectrin, stomatin, and SLP-2 was examined in fresh human red cells by indirect immunofluorescent microscopy (Fig. 8C). In all cases, these proteins were present only beneath the plasma membrane, and in noncontacted cells they were approximately evenly distributed about the membrane. When two erythrocytes were in close contact, SLP-2 appeared to concentrate beneath the cell-cell contact sites, a property not shared by spectrin or stomatin.

SLP-2 Is a Peripheral Membrane Protein—While the structure of SLP-2 is consistent with its disposition as a peripheral membrane protein, its inability to be fully extracted by 0.5 M KCl suggested a tighter association with the bilayer than most skeletal proteins. To further explore this issue, the extractability of stomatin and SLP-2 with Triton X-100 or pH 11 NaOH was compared (Fig. 9). Triton extracted a portion of both stomatin and SLP-2. Conversely, stomatin was completely resistant to NaOH extraction (the hallmark of an integral membrane protein), while SLP-2 was completely extracted by such treatment. The NaOH extractability of SLP-2 confirms that it exists in red cells as a peripheral (but well attached) membrane protein.

DISCUSSION

The studies presented here identify SLP-2 as a novel member of the stomatin gene superfamily and reveal several unusual properties of this protein that may offer insights into its function. Distinguishing features include the following: 1) SLP-2 uniquely lacks a hydrophobic domain and functions as a peripheral (versus integral) membrane protein; 2) multiple SLP-2-related protein bands are evident on SDS-PAGE analyses of erythrocytes and other cells (most of which migrate more slowly than expected based on their calculated Mr); 3) SLP-2 is present in mature erythrocytes as well as in many if not all other types of tissues and cells; 4) SLP-2 partitions into a large oligomeric protein complex that is fully salt-extractable; 5) SLP-2 maps to the same chromosome as stomatin, although at a different locus (9p13 versus 9q34.1 for stomatin (33)); and 6) SLP-2 appears to concentrate in regions of erythrocyte membrane deformation or cell-cell contact. Collectively, these observations describe an unusual protein, reveal a novel and here-tofore unrecognized component of the peripheral membrane skeleton of erythrocytes, and suggest novel and testable hypotheses as to its function.

The origin of the multiple sized SLP-2 protein bands evident in Western blots of erythrocytes, COS cells, and A431 cells remains uncertain. The observed bands fall into two categories: set 1, composed of approximately four bands of Mr 45,500, 44,600, 34,300, and 26,000, and set 2, a group of more variable bands above Mr 45,500. Of the first group, the two largest bands (Mr 45,500 and 34,300) are the most abundant. In most cell types, just one of these two bands is present, although both appear in COS cells, A431 cells, and erythrocytes. Similarly, the smaller bands at 34,300 and 26,000 are absent in most tissues and cell types but are expressed (albeit in lesser amounts) in COS, A431, and red cells. The in vitro translation of SLP-2 cDNA also generates this same ensemble of protein.
bands as does FLAG-labeled recombinant SLP-2 when expressed in COS cells (but not 293T cells). While it remains possible that these smaller bands represent proteolytic products generated from the parent band at 45,500 Mr, we favor this interpretation. Instead, taking into consideration the consistency of these bands in three diverse cell types, their complete absence in other cell types, and their appearance after in vitro translation, we propose that these bands represent the products of tissue-specific alternative pre-mRNA splicing, alternative translation initiation at downstream AUGs, or both.

Two candidate AUGs for such alternative initiation would be those at nt 217 and 391, the latter flanked by an excellent Kozak sequence. While future experimental work will be required to prove this conjecture, it is also worth noting that removal of the NH2-terminal portions of SLP-2, as would occur with initiation at either of the downstream AUGs, leads to a SLP-2 in the soluble (S) and insoluble (P) fractions was examined by Western blotting. The Coomassie Blue-stained fractions (left panel) reveal the expected fractionation of proteins between the soluble and insoluble pools, with all of the cytoskeletal proteins remaining in the Triton-insoluble pool. Two prominent SLP-2 immunoreactive bands are present in ghosts (right panel, bands g and h; also the inset in B). The smaller band (Mr ~44,600) is Triton-soluble; the larger band (Mr ~45,500) is Triton-insoluble. At higher loadings (20 mg versus 10 mg), additional SLP-2 immunoreactive bands are observed at Mr values of 110,200 (a), 100,800 (b), 94,300 (c), 84,600 (d), 79,800 (e), 48,200 (f), 45,500 (g), 44,600 (h), 34,300 (i), and 26,300 (j). B, densitometric scans of the Coomassie Blue-stained ghosts and the SLP-2 Western blots of ghosts and the Triton-soluble and -insoluble fractions. Note that only band g (~44,500) is soluble; the rest remain with the Triton-insoluble pellet. Inset, enlarged view of the segregation of SLP-2 bands g and h between the soluble and insoluble Triton fractions. Over multiple determinations, 34 ± 10% (~± 2 S.D.) of SLP-2 was Triton-soluble. By comparison, stomatin was 15 ± 10% (~± 2 S.D.) Triton X-100-extractable in these experiments (Data not shown and Fig. 9). C, the intracellular distribution of SLP-2 in mature human erythrocytes was observed by indirect immunofluorescent microscopy (right) and was compared with the distribution of cFBL spectrin in these cells (left) and the distribution of stomatin (center). Note that substantially all of the detectable SLP-2 is arrayed with the membrane but in a more punctate pattern than is spectrin. It also appears to concentrate under points of membrane deformation or intercellular contact.

FIG. 8. SLP-2 associates with the Triton-insoluble cytoskeleton in erythrocyte ghosts. A, human erythrocyte ghosts (G) were extracted with Triton X-100 in isotonic buffer, and the presence of

FIG. 9. SLP-2 is a peripheral membrane protein in red blood cells. To further explore the disposition of SLP-2 in the red cell membrane, its Triton extractability was compared with its ability to be extracted by NaOH at pH 11. Also compared in this assay was the extractability of stomatin under the same conditions. As before (Fig. 8), the majority of stomatin and SLP-2 was insoluble in Triton X-100 extracts. Conversely, when ghost membranes were extracted with pH 11 NaOH, a condition that removes all peripheral membrane proteins but does not extract the integral membrane proteins, stomatin was found to behave as an integral protein, while SLP-2 partitioned as a peripheral membrane protein. The panel on the left (CB) is Coomassie Blue-stained; the two panels on the right are Western blots with specific antibody to either human erythrocyte stomatin (stomatin) or human SLP-2. The positions of the molecular weight markers are as indicated.

SLP-2 in the soluble (S) and insoluble (P) fractions was examined by Western blotting. The Coomassie Blue-stained fractions (left panel) reveal the expected fractionation of proteins between the soluble and insoluble pools, with all of the cytoskeletal proteins remaining in the Triton-insoluble pool. Two prominent SLP-2 immunoreactive bands are present in ghosts (right panel, bands g and h; also the inset in B). The smaller band (Mr ~44,600) is Triton-soluble; the larger band (Mr ~45,500) is Triton-insoluble. At higher loadings (20 mg versus 10 mg), additional SLP-2 immunoreactive bands are observed at Mr values of 110,200 (a), 100,800 (b), 94,300 (c), 84,600 (d), 79,800 (e), 48,200 (f), 45,500 (g), 44,600 (h), 34,300 (i), and 26,300 (j). B, densitometric scans of the Coomassie Blue-stained ghosts and the SLP-2 Western blots of ghosts and the Triton-soluble and -insoluble fractions. Note that only band g (~44,500) is soluble; the rest remain with the Triton-insoluble pellet. Inset, enlarged view of the segregation of SLP-2 bands g and h between the soluble and insoluble Triton fractions. Over multiple determinations, 34 ± 10% (~± 2 S.D.) of SLP-2 was Triton-soluble. By comparison, stomatin was 15 ± 10% (~± 2 S.D.) Triton X-100-extractable in these experiments (Data not shown and Fig. 9). C, the intracellular distribution of SLP-2 in mature human erythrocytes was observed by indirect immunofluorescent microscopy (right) and was compared with the distribution of cFBL spectrin in these cells (left) and the distribution of stomatin (center). Note that substantially all of the detectable SLP-2 is arrayed with the membrane but in a more punctate pattern than is spectrin. It also appears to concentrate under points of membrane deformation or intercellular contact.
loss of detergent insolubility in COS cells (e.g., see Fig. 7) and thereby presumably altered intracellular function.

The nature of the higher molecular weight SLP-2 reactive bands is also enigmatic. Large complexes involving erythrocyte proteins have previously only been observed in cells that are oxidatively damaged (34, 35). Preliminary studies suggest that the large SLP-2-containing complexes that exist in fresh red cells might involve a covalent linkage (via a disulfide) of SLP-2 to another protein or proteins. The components of such a putative complex remain to be determined.

Given that stomatin’s self-association appears to be mediated by the COOH-terminal portions of its sequence, a region sharing high homology to SLP-2 and predicted to be largely α-helical, an intriguing possibility that may speak to the role of SLP-2 is that SLP-2 forms mixed oligomers with stomatin. Stomatlin is a much more abundant protein (~100,000 copies/cell) that exists by itself as large oligomers (n = 9–12) in the plasma membrane (20). The low stoichiometry of SLP-2 compared with stomatin requires that only a small subset of the total stomatin in the cell could be directly associated with SLP-2. At a measured ratio of about one molecule of SLP-2 for every 10–40 molecules of stomatin (in the red cell), we envision that each oligomeric stomatin complex might include one copy of SLP-2. Since stomatin oligomers might play a role in organizing cholesterol or sphingolipid-rich membrane rafts, along with the acylated and GPI-linked proteins typically associated with such rafts (36), a linkage to SLP-2 would provide a potential mechanism tying lipid rafts with their embedded proteins to the cytoskeleton.

If SLP-2 does interact with stomatin in red cells, several important implications follow. In both the dehydrated and overhydrated forms of hereditary stomatocytosis, there are defects in monovalent cation control and variable deficiencies in the level of stomatin. Recent data indicate that both dehydrated hereditary stomatocytosis and familial pseudohyperkalaemia are linked to a gene at locus 16q23-qter (4), excluding disorders of stomatin or SLP-2 (both present on chromosome 9) as the cause of these disorders. However, the absence of the overhydrated form of stomatocytosis remains unknown. This is the disorder with a most severe deficiency in stomatin, although stomatin mRNA is normal and appears to be made in normal amounts in afflicted individuals (3). While still unappreciated defects in the untranslated portions of the mRNA cannot be definitively ruled out, a more likely explanation of the available data suggests that in such patients, stomatin is made in normal amounts but is lost due to its rapid degradation, perhaps because it is incorrectly or inadequately assembled onto the plasma membrane (20). The low stoichiometry of SLP-2, the fact that SLP-2 forms mixed oligomers with stomatin, and the existence of various reactive bands is also enigmatic. Large complexes involving erythrocyte proteins and play a role in their regulation. In future work, it will thus be important to examine the interaction of SLP-2 with stomatin or aquaporin and its potential role in organizing lipid rafts or monovalent cation permeability control.

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