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

Yingjian Wang and Jon S. Morrow‡

From the Department of Pathology Yale University School of Medicine, New Haven, Connecticut 06510

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 34,500 M_. 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.

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 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).

Portions of this work have been presented in abstract form (41). The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF190167.

‡ To whom correspondence should be addressed. Tel.: 203-785-3624; Fax: 203-785-7037; E-mail: jon.morrow@yale.edu.

* This work was supported by National Research Award F32-HL09977 (to Y. W.) and by grants from the National Institutes of Health (to J. S. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.

http://www.jbc.org/
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. RX(X/L)679(R/K)680X681(T/G)682-X683(L/I/V)684-X685(R/K)686X687(L/I/V)688E(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 PCR1 kit was used to perform 5’- and 3’-RACE amplifications, following the instructions of the manufacturer (CLONTECH). For 5’- RACE, the primer used was GTCGCCAGACTCCTGGCC; the primer for the 3’-RACE was GCCCGGGTGAGAATTGCTGGCCGC. 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 7010558 (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 analyzed 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.2

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 (w/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 glycerol and viewed by epifluorescence or by confocal microscopy using an Olympus AX-70 inverted confocal microscope.

Cell Preparations and Extraction—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 cold PBS, followed 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 1 × 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 alkalinized by incubation in −10x 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 M KCl 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.

---

1 The abbreviations used are: PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; PBS, phosphate-buffered saline; nt, nucleotide; EST, expressed sequence tag.

2 P. Stabach and J. Sinard, unpublished observations.
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 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 RX{L/I/V/S/A/N}X{L/I/V}DX{S/T,X}WG{L/I/V}{K/R/H}{L/I/V}{K/R}{L/I/V}. These are marked by an asterisk above the consensus strength.
FIG. 1—continued

| B(continued) |
|----------------|
| **SLP-2** |
| **SLP1** |
| **Stom** |
| **mec-2** |
| **unc-24** |
| **unc-1** |

**SLP2**

**SLP1**

**Stom**

**mec-2**

**unc-24**

**unc-1**

**consensus**

**consensus**

**consensus**

**consensus**

**consensus**
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™ accession no. AC004472). Analysis of this sequence using the program GeneTool™ (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™ accession no. NM004809; Ref. 10); human stomatin (GenBank™ accession no. M81635; Ref. 1); mec-2 (GenBank™ accession no. U26735; Ref. 9); unc-24 (GenBank™ accession no. U42013; Ref. 11), and unc-1 (GenBank™ accession no. U55375; Ref. 30). All members of this family including SLP-2 share the cognate consensus sequence RX\_{L/V}{\text{S/A/NX}}_{5}\text{X}_{6}L/V/DX_{2}\text{WX}_{4}\text{K/R}(X/IV)/L/V/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\_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.

SLP-2 Is Present in Most Tissues, Including Mature Human Erythrocytes—To examine the tissue distributions of the major stomatin gene superfamily members, antibodies were prepared to recombinant human stomatin, human SLP-1, and human SLP-2. After affinity purification and absorption against GST, these antibodies distinguished stomatin from SLP-1 and SLP-2 with high fidelity and little residual activity against GST (Fig. 5). These antibodies and the SLP-2 and SLP-1 cDNAs were then used to examine the distribution of these proteins in a variety of tissues by Western and Northern blotting (Fig. 6). Previous studies have established the distribution of stomatin (1, 2). Although SLP-2 was initially derived from a heart library, its ~1.5-kilobase mRNA was readily detected in all tissues examined (Fig. 6A). These included heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. When normalized to the corresponding actin mRNA signal, the tissues with the highest SLP-2 mRNA levels were heart, liver, and pancreas. This distribution was distinct from the pattern of SLP-1 expression, in which the highest mRNA levels were found in brain and heart, with lesser but still detectable amounts in the other tissues.

A range of tissues and cell lines were examined with the antibodies to SLP-1 and SLP-2. The SLP-1 antibody did not
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
M_r 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.
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 product as its major product, along with a doublet at $M_r$ 34,300, 45,500 and a smaller product with $M_r$ 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 $M_r$ 34,500 product was generated as a major band, with two faint bands detected near $M_r$ 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 for three standard proteins (ovalbumin, 47,500; carbonic anhydrase, 35,300; and soybean trypsin inhibitor, 28,200). All protein bands were visualized by ECL autofluorography. 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 ($M_r$) 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 autofluorography.
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 heretofore 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 do not 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), 106,800 (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 (Mr 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 (left) and was compared with the distribution of alpha 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 alpha spectrin. It also appears to concentrate under points of membrane deformation or intercellular contact.

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.

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
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, several 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. Stomatatin 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 levels of this stomatin. Recent data indicate that both dehydrated hereditary stomatocytosis and familial pseudohyperkalemia are linked to a gene at locus 16q23-qter (4), excluding disorders that might involve SLP-2, only speculation is possible at this point. Review of the human genome data base for other disorders that might involve SLP-2, only speculation is possible at this point. Review of the human genome data base for other disorders that might involve SLP-2, and the potential role in organizing lipid rafts or monovalent cation permeability control.

Acknowledgments—The expert assistance of Paul Stabach and Amy Chang along with Drs. John Sinard, Deepthi Pradhan, and Carol D. Cianci is gratefully acknowledged.

REFERENCES

1. Stewart, G. W., Hepworth-Jones, B. E., Keen, J. N., Dash, B. C., Argent, A. C., and Nielsen, S. (1995) Blood 80, 1568–1575
2. Hiebl-Dirschmied, C. M., Entler, B., Glotzmann, C., Maurer-Fogy, I., Stradwitz, C., and Prohaska, R. (1991) Biochim. Biophys. Acta 1096, 123–124
3. Stewart, G. W., Argent, A. C., and Dash, B. C. (1993) Biochim. Biophys. Acta 1225, 15–25
4. Delaunay, J., Stewart, G., and Iolascon, A. (1999) Curr. Opin. Hematol. 6, 110–114
5. Stewart, G. W. (1993) in Red Cell Membrane Antigens (Tanner, M. J. A., and Anstee, D. J., eds.) pp. 167–175, Balliere-Tindall, London.
6. Innes, D. S., Sinard, J. H., Gilligan, D. M., Snyder, L. M., Gallagher, P. G., and Morrow, J. S. (1999) Am. J. Hematol. 60, 72–74
7. Zhu, Y., Gao, X., Turetsky, T., Tsai, S., Huppert, F. A., Lee, J. G., Cooper, P., Gallagher, P. G., Stevens, M. E., Rubin, E., Mohandas, N., and Mentzer, W. C. (1993) Blood 99, 2404–2410
8. Nuyens, L., Thines-Sempoux, D., and Prohaska, R. (1997) Eur. J. Cell Biol. 73, 261–265
9. Huang, M., Gu, G., Ferguson, E. L., and Chaloff, M. (1995) Nature 378, 292–295
10. Segel, G., and Prohaska, R. (1998) Gene (Amst.) 225, 23–29
11. Barnes, T. M., Jia, Y., Horvitz, H. R., Rovkun, G., and Hekimi, S. (1996) J. Neurochem. 67, 46–57
12. Rajaram, S., Sedensky, M. M., and Morgan, P. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7861–7866
13. You, Z., Gao, X., Mo, M. M., and Barthok, D. (1996) Microbiology 142, 2619–2627
14. Moore, B. R., and Shriver, S. K. (1997) Biochem. Biophys. Res. Commun. 232, 294–297
15. Desseves, J., Berman, A., Dynon, K., La Greca, N., Foley, M., and Tilley, L. (1996) Biochem. Biophys. Res. Commun. 224, 108–114
16. Ho, M. M., Nicolaou, A., Argent, A. C., and Stewart, G. W. (1997) Biochem. Soc. Trans. 25, 4929
17. Nuyens, L., Umlauf, E., and Prohaska, R. (1999) FEBS Lett. 449, 101–104
18. Hiebl-Dirschmied, C. M., Adel, G. F., and Prohaska, R. (1991) Biochim. Biophys. Acta 1065, 195–202
19. Salzer, U., Ahorn, H., and Prohaska, R. (1993) Biochim. Biophys. Acta 1151, 149–152
20. Nuyens, L., Umlauf, E., and Prohaska, R. (1998) J. Biol. Chem. 273, 17221–17226
21. Gallagher, P. G. and Forget, B. G. (1995) J. Biol. Chem. 270, 26358–26363
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Kodak International Biotechnologies, Ind. (1992) Technical Bulletin 1, Rochester, NY
24. Devarajan, P., Stabach, P. R., Mann, A. S., Arditto, T., Kashgarian, M., and Morrow, J. S. (1996) J. Cell Biol. 133, 819–830
25. Kennedy, S. P., Warren, S. L., Forget, B. G., and Morrow, J. S. (1991) J. Cell Biol. 115, 267–277
26. Laemmli, U. K. (1970) Nature 227, 680–685
27. Pierce (1994) Pierce Catalog and Handbook, p. 116, Pierce, Rockford, IL
28. Arghur, B. S., Mladen, T. L., Schaffner, A. A., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
29. Kozak, M. (1984) Nucleic Acids Res. 12, 857–872
30. Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J. (1994) Nature 368, 32–38
31. Brown, D. A., and Rose, J. K. (1992) Cell 68, 533–544
32. Bormann, B. J., Knowles, W. J., and Marchesi, V. T. (1989) J. Biol. Chem. 264, 4033–4037
33. Westberg, J. A., Entler, B., Prohaska, R., and Schroder, J. P. (1993) Cytogenet. Cell Genet. 63, 241–243
34. Schrier, S. L., and Mohandas, N. (1992) Blood 79, 1586–1592
35. Hebbel, R. P., and Mohandas, N. (1991) Blood J. 60, 712–715
36. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572
37. McKusick, V. A. (1997) Online Mendelian Inheritance in Man (OMIM™), Johns Hopkins University and the National Center for BioTechnology Information, National Library of Medicine, Baltimore and Bethesda, MD
38. Argur, P., Brown, D., and Nielsen, S. (1995) Curr. Opin. Cell Biol. 7, 472–483
39. Higgins, D. G., and Sharp, P. M. (1988) Gene (Amst.) 73, 237–244
40. Chou, P. Y., and Fasman, G. D. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. 47, 45–148
41. Wang, Y., Chang, A., and Morrow, J. S. (1999) Blood 94, 189a
Identification and Characterization of Human SLP-2, a Novel Homologue of Stomatin (Band 7.2b) Present in Erythrocytes and Other Tissues
Yingjian Wang and Jon S. Morrow

J. Biol. Chem. 2000, 275:8062-8071.
doi: 10.1074/jbc.275.11.8062

Access the most updated version of this article at http://www.jbc.org/content/275/11/8062

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 10 of which can be accessed free at http://www.jbc.org/content/275/11/8062.full.html#ref-list-1