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Murine and Human *SDF2L1* Is an Endoplasmic Reticulum Stress-Inducible Gene and Encodes a New Member of the Pmt/rt Protein Family

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We isolated murine and human cDNAs for *SDF2L1* (stromal cell-derived factor 2-like1) and characterized the genomic structures. Northern blot analysis of the gene expression in various tissues revealed that both murine *Sdf2l1* and human *SDF2L1* genes are expressed ubiquitously, with particularly high expression in the testis. The SDF2L1 protein has an endoplasmic reticulum (ER)-retention-like motif, HDEL, at the carboxy (C)-terminus. Interestingly, SDF2L1 protein also shows significant similarity to the central hydrophilic part of protein *O*-mannosyltransferase (Pmt) proteins of *Saccharomyces cerevisiae,* the human homologues of Pmt (POMT1 and POMT2) and *Drosophila melanogaster* rotated abdomen (rt) protein. In a murine hepatocellular carcinoma cell line, *Sdf2l1* was strongly induced by tunicamycin and a calcium ionophore, A23187, and weakly induced by heat stress but was not induced by cycloheximide. In conclusion, SDF2L1 protein is a new member of Pmt/rt protein family and *Sdf2l1* is a new ER stress-inducible gene.

Key Words: **SDF2L1; stress protein; protein *O*-mannosyltransferase; *rt;* SDF2; BiP/GRP78.**

Sequence data from this article have been deposited with the EMBL/GenBank/DDBJ Data Libraries under Accession Nos. AB043006, AB043007, and AB043008.

Abbreviations used: SDF2, stromal cell-derived factor 2; ER, endoplasmic reticulum; Pmt, protein *O*-mannosyltransferase; *rt, rotated abdomen* of *Drosophila melanogaster;* UPR, unfolded protein response; EOR, endoplasmic reticulum overload response; NF-κB, nuclear factor κ B.

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The endoplasmic reticulum (ER) is a site where synthesizing, folding, and assembling of proteins occur, and it is also a sensor that is exquisitely sensitive to alterations in homeostasis caused by a variety of stimuli. Signals from the ER are transduced to the cytoplasm and the nucleus, resulting in adaptation for survival or induction of apoptosis (1). These signals are identified as an unfolded protein response (UPR) and ER overload response (EOR). The UPR is activated by ER stresses, including calcium depletion from the ER lumen, inhibition of asparagine (N)-linked glycosylation, reduction of disulfide bonds, expression of mutant proteins and overexpression of some wild-type proteins (1). Several ER resident proteins are coordinately up-regulated in response to the UPR pathway and act as molecular chaperones for proteins in the ER. These ER resident proteins are characterized by a specific ER targeting motif, KDEL, or a KDEL-like motif (2). The EOR has recently been identified as a UPR-independent signal transduction pathway, which activates NF-κB and induces proinflammatory and immune response gene expression (1).

Protein *O*-mannosylation starts at the ER with the transfer of mannose from dolichyl phosphate D-mannose to Ser/Thr residues of secretory proteins in yeast, *Saccharomyces cerevisiae.* This reaction is catalyzed by a family of protein *O*-mannosyltransferase (Pmt) proteins. In *S. cerevisiae,* it has been shown that the function of protein *O*-mannosylation is essential for cell wall rigidity and cell integrity (3). Proteins with strong sequence homology to Pmt proteins have been reported in *Drosophila melanogaster,* rt protein (4), as well as in higher vertebrates, mouse and human, POMT1 and POMT2 proteins (5). These evolutionarily

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conserved *PMT/rt* genes play an essential role in physiologically important processes.

In this work, we isolated the complete murine and human cDNAs for *SDF2L1*, stromal cell-derived factor 2-like 1, and we investigated the genomic structures and the expressions of murine and human *SDF2L1*. SDF2L1 protein has an ER-retention-like motif (HDEL) at the C-terminus and shows significant similarity to the central hydrophilic part of the Pmt/rt protein family. Furthermore, we found that *Sdf2l1* mRNA is strongly induced in response to activation of the UPR pathway. Here we report that SDF2L1 protein is a new member of Pmt/rt protein family and *Sdf2l1* is a new ER stress-inducible gene.

MATERIALS AND METHODS

Isolation of murine and human SDF2L1 cDNAs. An oligo(dT)-primed cDNA library was made using mRNA (5 μg) of a murine hepatocellular carcinoma cell line with oligo(dT) primers and a cDNA synthesis kit (ZAPIII-Express cDNA Synthesis and Cloning Kit, Stratagene). To isolate full-length cDNAs, 5.0×10^5 plaques of the cDNA library were screened with a ^{32}P-labelled A82-10 cDNA fragment by the random priming method (Megaprime DNA labelling system, Amersham Pharmacia Biotech). Hybridization was carried out under the condition of 15% formamide, 7% SDS, 1 mM EDTA, 0.5 M $NaPO_4$ and 1% BSA at 65°C. Washing was performed three times in 0.1 × SSC and 0.1% SDS at 65°C. Positive clones were isolated, and the nucleotide sequences of the clones were determined using an automated DNA sequencer (373A, Applied Biosystems).

For isolation of the human orthologue, we screened 5.0×10^5 plaques of a human cDNA library (generated from poly(A)$^+$ RNA isolated from human testis, Clontech) with a ^{32}P-labelled 860-bp PCR fragment (forward, 5′-GCCGGCTGGCGGGATGT-3′; reverse, 5′-TGCTTGAGACAGGTGAGATG-3′) containing the open reading frame (ORF) of *Sdf2l1* cDNA as a probe under a low-stringency condition (the same condition with genomic Southern hybridization, see below).

Computer analysis. To search for homologies with nucleotide and protein sequences in the GenBank database, we used the BLAST algorithm (http://blast.genome.ad.jp/). Prediction of protein localization signals was performed using PSORT II (Horton and Nakai, 1997, http://psort.nibb.ac.jp/).

Northern blot analysis. To investigate the murine tissue distribution, we generated a Northern blot filter containing poly(A)$^+$ RNA (2 μg) of mouse tissues and performed hybridization with ^{32}P-labelled *Sdf2l1* and β-actin probes under the condition of 15% formamide, 7% SDS, 1 mM EDTA, 0.5 M $NaPO_4$, 1% BSA at 65°C.

To investigate the human tissue distribution, human multiple-tissue Northern filters (MTN1, 2, Clontech) were used for hybridization with a ^{32}P-labelled 770-bp PCR fragment containing the ORF of the human *SDF2L1* cDNA (forward, 5′-GCCGGCTGGCGGGATGT-3′; reverse, 5′-TGCTTGAGACAGGTGAGATG-3′) and β-actin probes.

For stress response experiments, RNA was isolated from the cultured cells by acid guanidinium thiocyanate-phenol-chloroform extraction (6). Twenty μg of total RNA was subjected to Northern blot analysis. The probe used for *Sdf2l1* was the same as mentioned above. The probe for *BIP/GRP78* was a 1.1-kb *Eco*RI fragment of pBS-mouse *Bip/Grp78* plasmid (7) (kindly provided by Dr. H. Kubota, Institute for Frontier Medical Sciences, Kyoto University). *GAPDH* was used as a loading control. Hybridization signals were visualized by autoradiography and the intensities were measured by the use of a Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film).

Genomic Southern blot analysis. Five micrograms of murine and human genomic DNA were subjected to each of the restriction enzymes (*Bam*HI, *Eco*RI, *Sac*I and *Hin*dIII) digestion, electrophoresed on a 1.0% agarose gel, and transferred to a nylon membrane. The membrane was hybridized with the ^{32}P-labelled 860-bp PCR fragment of *Sdf2l1* cDNA. Hybridization was carried out under a low-stringency condition, 7% SDS, 1 mM EDTA, 0.5 M $NaPO_4$ and 1% BSA at 55°C. Washing was performed three times in 0.1 × SSC and 0.1% SDS at 55°C.

Isolation of murine and human SDF2L1 genomic DNAs. To determine the genomic structure of *Sdf2l1*, 1.0×10^6 plaques of the murine genomic DNA library containing *Sau*3AI partial digests of mouse liver DNA (Stratagene) were screened with the ^{32}P-labelled 860-bp fragment of *Sdf2l1* cDNA as a probe. Three positive plaques were isolated and studied. Based on the results of genomic Southern blot analysis, 2.0-kb and 3.5-kb *Bam*HI-digested fragments containing exon sequences were subcloned into the pBluescript phagemid vector, and nucleotide sequences of those fragments were determined.

Plaques (1×10^6) of the human genomic DNA library containing *Sau*3AI partial digests of human peripheral blood leukocytes (Clontech) were screened with a ^{32}P-labelled 770-bp PCR fragment (coding region of *SDF2L1:* forward, 5′-CTGCTGGCGCTGTTAGT-3′; reverse, 5′-TCTCTGCCAAGAGTGGC-3′) as a probe. Four positive plaques were isolated. Based on the results of the genomic Southern blot analysis, 3.0-kb and 5.4-kb *Sac*I-digested fragments containing exon sequences were subcloned into the pBluescript phagemid vector, and nucleotide sequences of those fragments were determined.

Cell culture and induction of SDF2L1 in response to cellular stress. We established a murine hepatocellular carcinoma cell line, 5-C373-C2, from radiation-induced C57BL/6 × C3H/He ($B6C3F_1$) mouse hepatocellular carcinoma. The cell line was maintained conventionally in William's medium E (Sigma), supplemented with 10% fetal bovine serum (FBS) (Intergen), 8 μg/ml insulin (Takara), 5 ng/ml epidermal growth factor, EGF (Takara), 0.32 μg/ml hydrocortisone (Sigma), 100 IU/ml penicillin G potassium (Irvine Scientific) and 130 μg/ml streptomycin sulfate (Irvine Scientific) at 37°C in a 5% CO_2 incubator. At 60–70% confluency, the medium was exchanged and the cells were incubated with either tunicamycin (Sigma) (dissolved in dimethylsulfoxide, DMSO) or a calcium ionophore, A23187, (Sigma) (in DMSO), or cycloheximide (Wako) (in distilled water), at serial concentrations for indicated times (8, 9). For heat shock treatment, cells were seeded into flasks, incubated in a 43°C water bath for 30 min, and returned to a 37°C CO_2 incubator for indicated times (10).

RESULTS

During our investigation into the differences between gene expressions in radiation-induced $B6C3F_1$ mice hepatocellular carcinoma cell lines, we serendipitously isolated a clone, A82-10, containing a 350-bp cDNA fragment that is similar to *SDF2*. Since it has been suggested that SDF2 is a novel secretory protein produced from stromal cells (11), we were interested in identifying the homologue of the *SDF2* gene.

Cloning of murine and human SDF2L1 cDNAs. We screened a cDNA library from a murine hepatocellular carcinoma cell line using the A82-10 clone as a probe in order to isolate a full-length cDNA. Four positive clones were isolated, and all of them had similarly sized insert cDNAs. The clone carrying the longest cDNA fragment was chosen, and its nucleotide se-

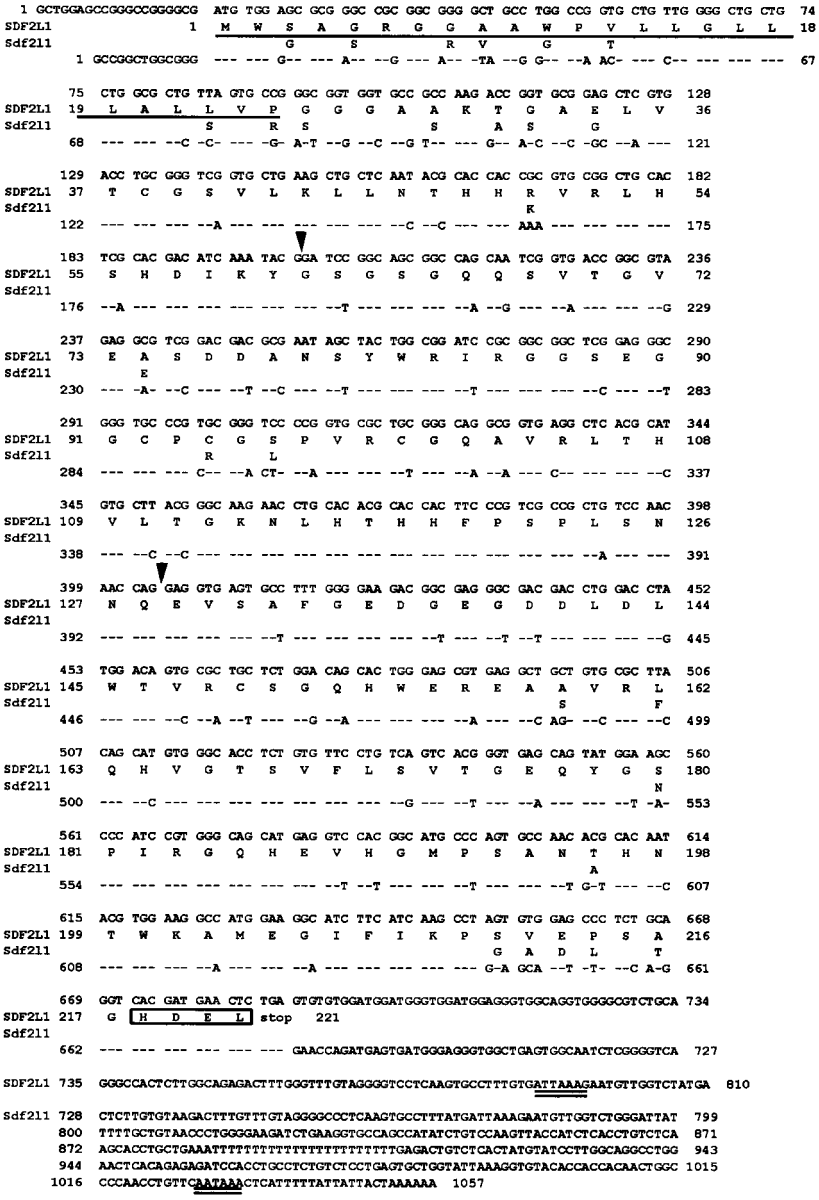

FIG. 1. Nucleotide sequences of murine and human *SDF2L1* cDNAs and predicted amino acid sequences of the encoded proteins. The nucleotide sequences of *SDF2L1* and *Sdf2l1* cDNAs are shown at the top and bottom, respectively. The predicted human and murine SDF2L1 amino acid sequences are given between the nucleotide sequences. Only mouse residues different from the human ones are indicated. Dashes indicate the same bases as above. Junctions of exons are indicated by arrowheads. A single underline indicates the signal peptide, and a double underline indicates the polyadenylation signal. The ER-retention-like motif is boxed. Nucleotides are numbered, beginning with the first nucleotide of the cDNA.

quence was determined. The cDNA, 1057 bp, encodes 221 amino acids. The predicted amino acid sequence was 64% identical to SDF2.

Next, we screened the human testis cDNA library using a PCR fragment containing an ORF of this murine "SDF2-like gene" as a probe. The condition of the screening was determined by genomic Southern hybridization. Under a low-stringency condition, the probe was specifically hybridized to several genomic fragments of humans (see Materials and Methods). We isolated 25 positive clones from the library. We checked the insert sizes of eight of the 25 clones and performed sequencing using the clone carrying the longest insert cDNA. The human cDNA of this gene consists of 810 nucleotides and encodes 221 amino acids. The nucleotide sequence of human cDNA shows 85% identity to that of mouse cDNA in the ORF region (Fig. 1). The predicted proteins of both the human and murine cDNA show 88.2% identity and 90.9% similarity (Fig. 1). Recently, an SDF2-like gene, *SDF2L1*, was identi-

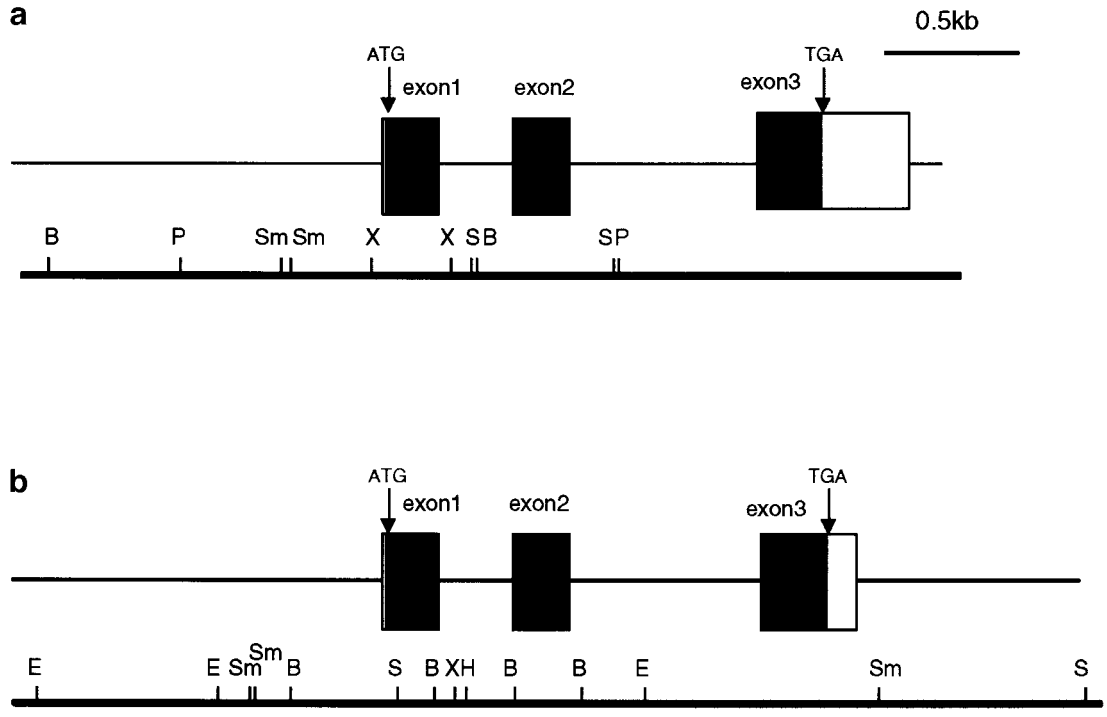

FIG. 2. Genomic structures of the murine (a) and human (b) *SDF2L1* gene. Restriction maps and exon–intron structures are shown. Restriction enzyme site: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; Sm, *Sma*I; X, *Xba*I. Exons are numbered and indicated as vertical open boxes. Coding regions within exons are indicated as filled boxes.

fied as a predicted coding region in a genome project (12). We found that *SDF2L1* is a part of the "SDF2-like gene". Therefore, the names of the genes identified in mouse and human are murine *Sdf2l1* and human *SDF2L1*, respectively.

Genomic structures of murine and human SDF2L1. We isolated each of the mouse and human genomic clones and determined the genomic structures as described under Materials and Methods. *Sdf2l1* consists of three exons, spanning about 2.2 kb of genomic DNA. *SDF2L1* consists of three exons, spanning about 2.0 kb of genomic DNA. The nucleotide sequence is identical to the chromosome 22q.11.2 region (12) (Fig. 2).

Primary structures of SDF2L1 proteins. The hydropathy profiles suggest that murine and human SDF2L1 proteins contain no hydrophobic region other than the N-terminus (data not shown) and possess an ER-retention-like motif, HDEL, at the C-terminus (Fig. 1). Prediction of protein sorting using the PSORT II program suggests that the most likely localization of SDF2L1 is at the ER and the hydrophobic region (amino acids 1–24) is a possible core sequence of signal peptide (Fig. 1). A homology search of proteins in the GenBank database revealed that SDF2 and SDF2L1 proteins have significant similarity to the central hydrophilic part of the protein *O*-mannosyltransferase family of *S. cerevisiae* and *Candida albicans* (3, 13), human homologues of Pmt, POMT1 and POMT2 proteins (5), and rotated abdomen of *Drosophila melanogaster,* rt protein (4) (Fig. 3).

Expression of murine and human SDF2L1 mRNAs. The tissue distribution of *Sdf2l1* mRNA is shown in Fig. 4a. The expression was ubiquitous, being particularly strong in the testis, ovary and uterus and weak in the heart and skeletal muscle. *SDF2L1* mRNA was also expressed ubiquitously in human tissues, with strong expression in the testis, moderate expression in the pancreas, spleen, prostate, small intestine and colon (mucosal lining), and rare expression in the brain and skeletal muscle (Fig. 4b).

Induction of Sdf2l1 mRNA under a cellular stress condition. Several ER resident proteins are stress proteins that undergo increased expression in response to the UPR that is activated by disruption of protein synthesis or calcium homeostasis in the ER (1). Because SDF2L1 protein has the structural feature of an ER-retention-like motif, HDEL, at the C-terminus, we investigated whether cellular stress affects the induction of *Sdf2l1* mRNA using a murine hepatocellular carcinoma cell line, 5-C373-C2.

BiP/GRP78 is one of the major ER resident proteins, and its expression is up-regulated by tunicamycin, an N-linked glycosylation inhibitor, or by A23187, a calcium ionophore (8, 9), but is not affected by heat stress (14, 15) or by cycloheximide (16, 17). We compared the induction of *Sdf2l1* mRNA with that of *Bip/Grp78*

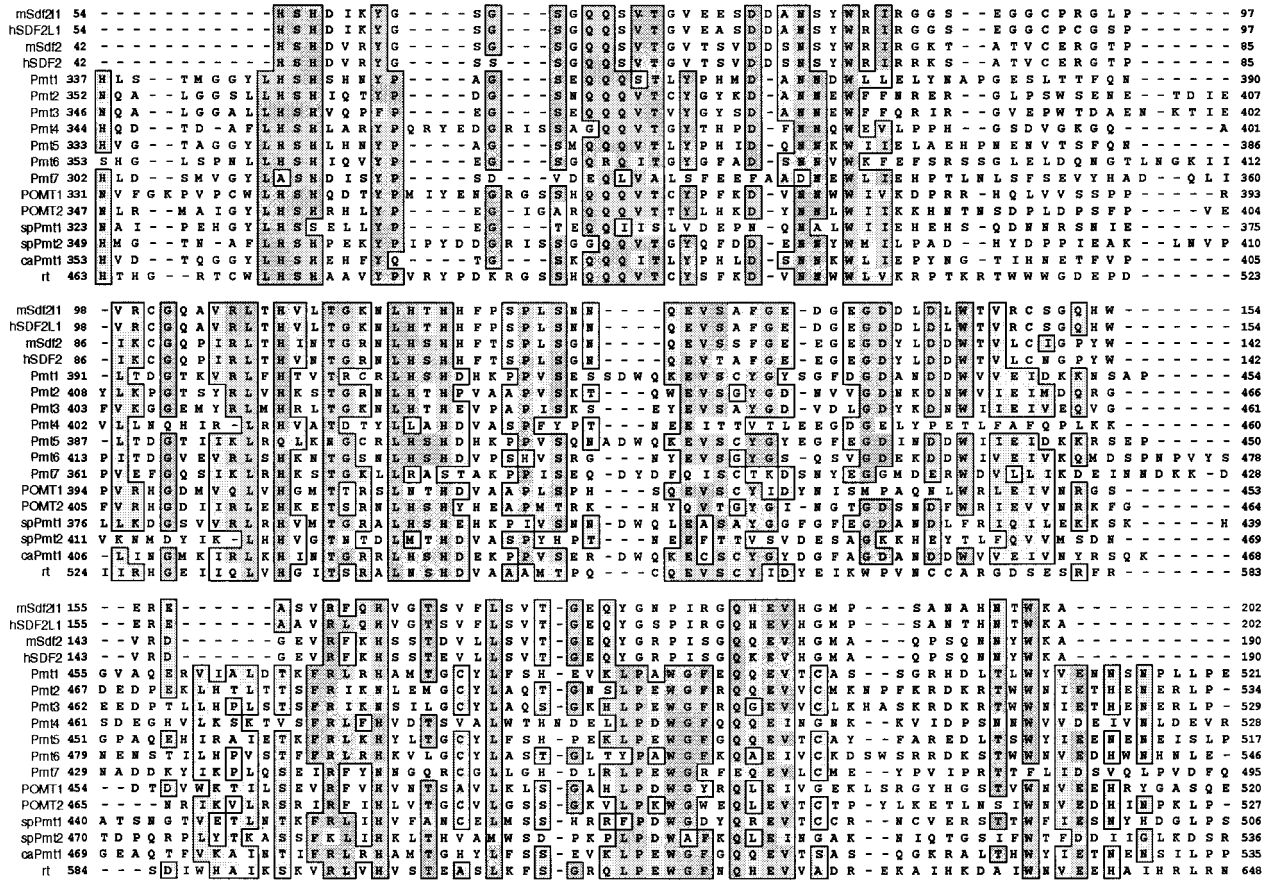

FIG. 3. Amino acid sequence alignment of SDF2L1 and SDF2 proteins with the central part of the Pmt/rt protein family, including *Saccharomyces cerevisiae* (Pmt1–7), *Schizosaccharomyces pompe* (spPmt1–2) (Accession Nos. O13898 and O42933), *Candida albicans* (caPmt1) (Accession No. AF000232), human homologues of Pmt (POMT1–2) and *rotated abdomen* of *Drosophila melanogaster* (rt). Dashes indicate gaps. Numbers on the left and right indicate the position of the first and last amino acid in each lane, respectively. Dark gray-shaded residues are identical and light gray-shaded residues are homologous.

mRNA in 5-C373-C2 cells under various cellular stress conditions. With tunicamycin treatment, *Bip*/*Grp78* expression was up-regulated in a dose-dependent manner up to 0.5 μg/ml and then slightly decreased. The maximal induction was approximately 16-fold compared with untreated cells (Fig. 5a). *Sdf2l1* expression was also up-regulated like the *Bip*/*Grp78* induction until 0.5 μg/ml, then further increased up to 2.0 μg/ml and then slightly decreased. The maximal induction was approximately 26-fold. With A23187 treatment, *Sdf2l1* and *Bip*/*Grp78* were up-regulated in dose-dependent manners up to 5 μM and reached a plateau (Fig. 5b). The maximal inductions were approximately 9-fold for *Sdf2l1* and 8-fold for *Bip*/*Grp78*. The time courses of the inductions of *Sdf2l1* and *Bip*/*Grp78* after treatment with 1 μg/ml of tunicamycin were essentially identical. The expression was gradually increased up to 9 h (Fig. 5c). With 5 μM of A23187 treatment, the expressions of *Sdf2l1* and *Bip*/*Grp78* were gradually increased up to 18 h (Fig. 5d). The induction profile of *Bip*/*Grp78* after treatment with tunicamycin or A23187 is consistent with that in previous reports (8, 9). Heat stress did not affect *Bip*/*Grp78* induction (14, 15), but *Sdf2l1* was weakly induced and the expression was gradually increased up to approximately 4-fold at 24 h (Fig. 5e). Cycloheximide, at the concentration ranging from 1.5 to 100.0 ng/ml, did not affect *Sdf2l1* or *Bip*/*Grp78* induction (data not shown) (16, 17).

DISCUSSION

We have isolated an "SDF2-like gene" from murine and human cDNA libraries, and we have also isolated their genomic DNAs and characterized them. Recently, Dunham *et al.* reported the genomic sequence of chromosome 22 and identified an SDF2-like gene, named *SDF2L1*, as a predicted coding sequence (12) (http://www.sanger.ac.uk/hgp/chr22/1). We noticed that the "SDF2-like gene" is the same as *SDF2L1* because both genes are overlapped, but the predicted *SDF2L1* gene was incomplete. Therefore, this is the first report of the entire human *SDF2L1* gene.

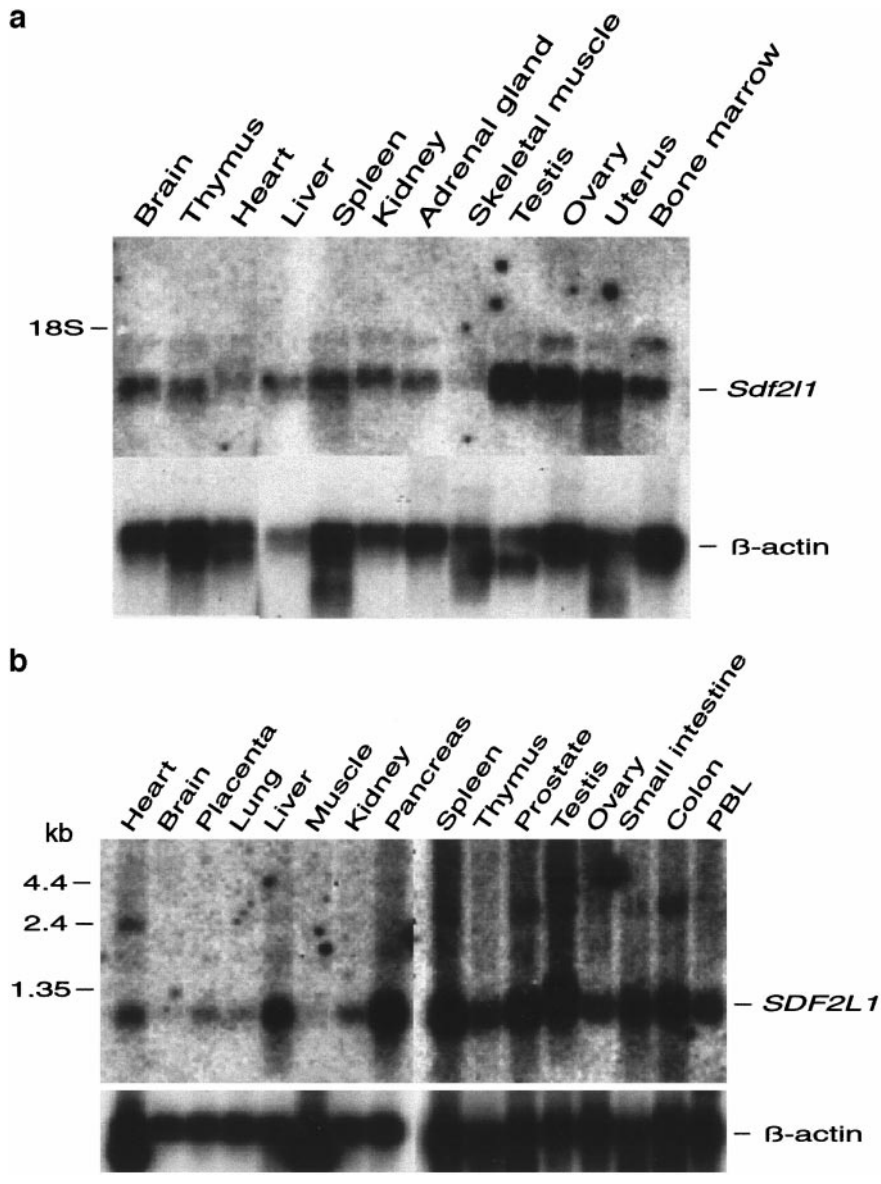

FIG. 4. (a) Expression of the murine *Sdf2l1* mRNA in murine tissues. A Northern blot filter containing 2 μg of poly(A)$^+$ RNA from the brain, thymus, heart, liver, spleen, kidney, adrenal gland, skeletal muscle, testis, ovary, uterus and bone marrow was hybridized with a DNA probe from *Sdf2l1* or β-actin cDNA. (b) Expression of the human *SDF2L1* mRNA in human tissues. Northern blot filters containing 2 μg of poly(A)$^+$ RNA from the heart, brain, placenta, lung, liver, muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes (PBL) were hybridized with a DNA probe from *SDF2L1* or β-actin cDNA.

The amino acid sequences of the SDF2L1 protein possessed an ER-retention-like motif, HDEL, at the C-terminus. Proteins that reside in the ER are usually characterized by tetrapeptide, KDEL, at the C-terminus, and there are some variant ER-retention motifs (2, 18). Interaction of this specific motif with a specific receptor in the Golgi apparatus is thought to be necessary for the retrieval of the ER resident proteins (19). Several ER resident genes are coordinately upregulated in response to the UPR and have the ER stress response element (ERSE), CCAATN$_9$CCACG, in the promoter (7). Tunicamycin and A23187 activate both UPR and EOR (1, 8, 9). Cycloheximide activates only EOR (1). Heat stress induces the cytosolic stress proteins, so-called 'heat shock proteins'. The transcriptional regulation of them is different from ER resident proteins (20). The promoter region of heat shock proteins contains multiple heat shock elements (HSE), which are characterized by inverted repeats of the pentameric sequence nGAAn (20). We investigated what kind of cellular stress promotes *SDF2L1* expression. *Sdf2l1* was strongly induced by tunicamycin and A23187 but was not induced by cycloheximide. These results suggested that *Sdf2l1* is one of the ER-stress

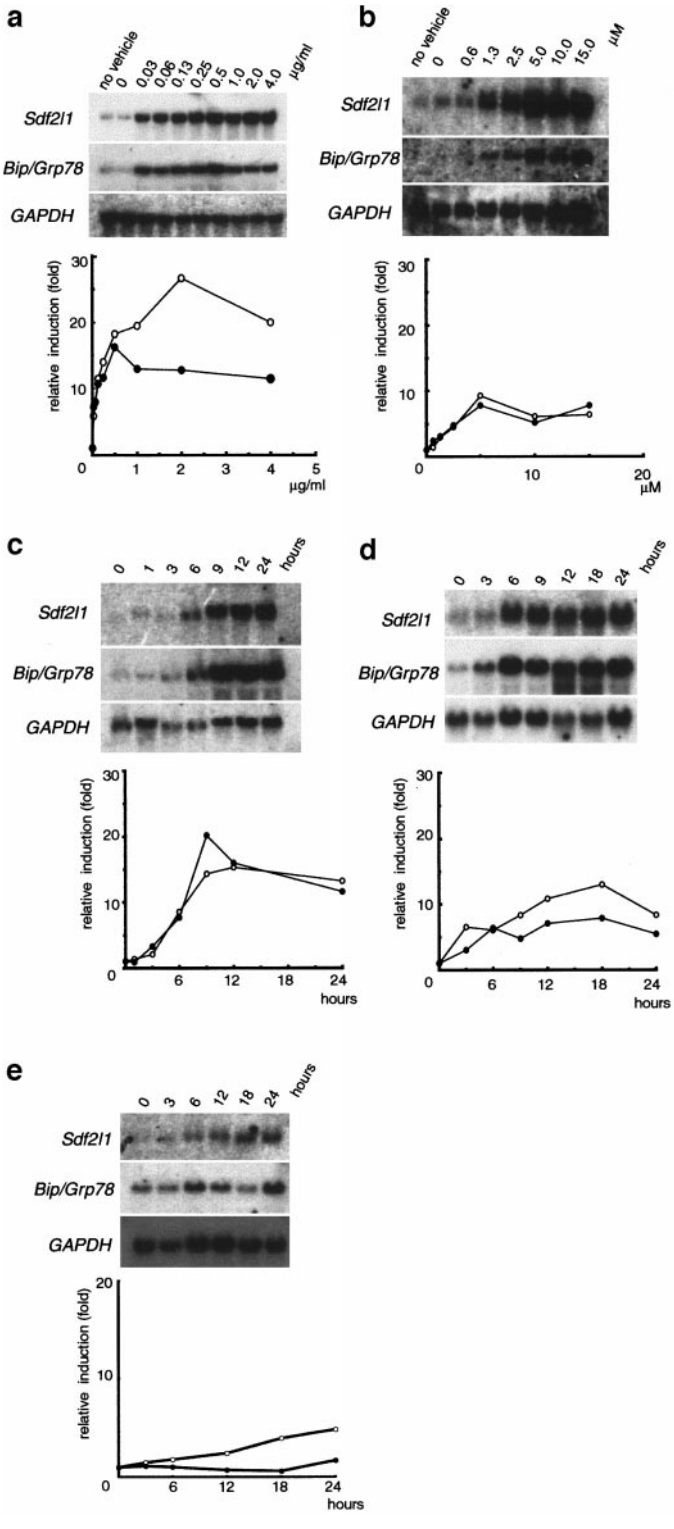

FIG. 5. Northern blot analysis of *Sdf2l1* gene induction. Total RNA was isolated from tunicamycin-treated (a) or A23187-treated (b) cells at indicated concentrations for 14 h. Total RNA was isolated at indicated times from cells treated with 1.0 μg/ml tunicamycin (c) or with 5 μM A23187 (d). For heat stress, the cells were incubated in a 43°C water bath for 30 min and returned to a 37°C incubator for indicated times, and total RNA was isolated (e). The membranes were hybridized with an *Sdf2l1*, *Bip/Grp78* or *GAPDH* probe as indicated. The signal intensity of *Sdf2l1*, *Bip/Grp78* or *GAPDH* was measured using a Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film). The relative induction was normalized by the signal intensity of *GAPDH*. Each relative induction was plotted against the drug concentration (a, b) or the time of treatment (c, d, e). Open circles indicate *Sdf2l1* and closed circles indicate *Bip/Grp78*.

inducible genes and is induced by the UPR pathway. However, the following points are different from the major ER resident proteins. First, the *Sdf2l1* gene does not have an ERSE sequence in the promoter region. Second, the *Sdf2l1* gene, unlike *Bip/Grp78*, is weakly induced by heat stress (14, 15). The *Sdf2l1* gene, however, does not have an HSE sequence in the promoter region. There is no obvious explanation for these points, but it is thought that *Sdf2l1* is under unique transcriptional regulation.

The SDF2L1 protein is similar to SDF2 and has a limited homology with the protein *O*-mannosyltransferase (Pmt) proteins of *S. cerevisiae*. *SDF2* was identified from a cDNA library generated from stromal cell lines using the signal sequence trap method (11). Although SDF2 is reported to be a secretory protein produced from stromal cells, the function of SDF2 is still not known. To determine whether *SDF2L1* is expressed in a specific stromal cell, we performed *in situ* hybridization using a specific probe for *Sdf2l1*. It was found that the *Sdf2l1* gene is not expressed specifically in stromal cells but is expressed nonspecifically in parenchymal cells (S. Fukuda, M. Sumii, K. Kamiya unpublished results). Yeast Pmt proteins reside in the ER membrane and transfer mannose residues from dolichyl phosphate D-mannose to specific Ser/Thr residues (catalyzing protein *O*-mannosylation) (21, 22). The function of Pmt proteins is essential for cell wall rigidity and cell integrity (3). Proteins with strong sequence homology to Pmt proteins have been reported in *Drosophila melanogaster*, rt protein (4), as well as in mouse and human, POMT1 and POMT2 proteins (5). The *rt* gene mutation causes a clockwise twisted abdomen due to defects in muscle structures and alignment of the adult cuticle (4). POMT1 and POMT2 proteins have recently been identified as mammalian homologues of the rt protein (5). Therefore, *POMT genes* are thought to be candidate genes for uncharacterized genetic disorders of the muscular system. Although the function of POMT proteins or the rt protein as mannosyl-transferase has not yet been proven (4, 5, 23), genetic data indicates that the *rt* gene, and maybe *POMT* genes, play an essential role in physiologically important processes. SDF2L1 and SDF2 proteins might also play essential roles in physiological processes because they show significant similarity to the central hydrophilic part of the Pmt/rt protein family.

In conclusion, SDF2L1 protein is a new member of the Pmt/rt protein family and *sdf2l1* is a new ER

stress-inducible gene, but the transcriptional regulation of the *Sdf2l1* gene is slightly different from that of the major ER resident proteins.

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