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Involvement of 101F6, a Homologue of Cytochrome b_{561}, in the Reduction of Ferric Ions

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We have isolated and characterized a small transmembrane protein, called 101F6, showing high sequence homology to cytochrome b_{561}, a protein containing two binding sites for haem. The newly identified 101F6 contains six membrane spanning domains in which conserved histidine residues are located, and has a molecular mass of 25 kDa. When the haem-binding with bacterial expressed 101F6 was examined, the protein bound haem and the deletion of one histidine residue at 149 caused a loss of the binding. 101F6 mRNA was expressed widely in various tissues, and especially abundant in liver, kidney and lung. It was also expressed in several cultured cell lines. The protein expressed from the 101F6 cDNA in Balb/3T3 cells was about 25 kDa in size and was localized in small vesicles, including endosomes and endoplasmic reticulum of the perinuclear region. Comparison of the location of 101F6 with that of transferrin receptor-1 revealed that the localization of 101F6 in small vesicles was not always the same as the localization of transferrin receptor-1, but was similar to that of haem oxygenase-1. The other homologue to cytochrome b_{561}, SDR-2 was also expressed in the small vesicles similar to the location of 101F6. Finally, reduction of ferric ions as well as of azo-dye increased with 101F6- or SDR-2-expressing cells. Thus, both 101F6 and SDR-2 were localized in small vesicles of cells and played roles in the reduction of ferric ions.

Key words: 101F6, cytochrome b_{561}, ferric reductase, haemoprotein, SDR-2.

Abbreviations: Dcytb, duodenal cytochrome b_{561}; SDR-2, stromal cell-derived receptor-2; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; GST, glutathione S-transferase.

Receptor-mediated endocytosis of diferric-transferrin by various cells is a well-known iron uptake pathway (*1*). The divalent metal transporter I (DMTI), also called Nramp 2, was found to play a role in the absorption of ferrous iron in the small intestine (*2*, *3*). DMTI is also found in the endosomal membrane of most cells (*4*). The uptake of iron can be mediated by a reductase, duodenal cytochrome b (Dcytb) (*4*, *5*) and this protein requires a preceding reduction of the highly insoluble ferric ions to ferrous ions on the outside of cells. Dcytb in mouse duodenal mucosa is increased in mice with transferrin- or iron-deficiency, strongly suggesting that it is involved in iron metabolism (*6*). On the other hand, six transmembrane epithelial antigen of prostate 3 (Steap3) is involved in ferric reduction in erythroid cells (*7*). Steap3 was identified as the mutated gene in mice with heritable anaemia. However, these proteins are not expressed in a variety of tissues, suggesting that there are other yet unidentified ferric reductases. Dcytb is a homologue of cytochrome b_{561}, which is well known to be a transmembrane protein localized to the membranes of catecholamine secretory vesicles of the adrenal medulla, pituitary and other neuroendocrine tissues (*8*). The cytochrome b_{561} family includes several isoforms identified in various genomes and contains four conserved histidine residues that have been proposed to serve as di-haem ligands (*4*, *9*). It is possible that isoforms of cytochrome b_{561} function in the reduction of ferric ions.

Cytochrome b_{561} homologues in mammals are variously designated Dcytb, SDR2, chromaffin granule cytochrome b_{561}, lysosomal cytochrome b_{561} and the gene product 101F6 (*4*, *10*). In addition to these mammalian proteins, many proteins containing cytochrome b_{561}-like domains are known in mammals, insects and plants and some have been demonstrated to be involved in the recycling of ascorbate as an electron donor, which can be required for the generation of noreprinephrine from dopamine by dopamine β-hydroxylase (*8*, *11*).

Although it was reported that Dcytb as well as SDR-2 and cytochrme b_{561} exhibited ferric-reductase activity (*12*), a recent study with Dcytb-knockout mice showed that the loss of Dcytb did not affect the intestinal absorption of iron or the metabolism of iron in the body (*13*). These results gave us the idea that other intestinal proteins that are homologues of Dcytb may be able to reduce ferric ions, or that ascorbate generated by Dcytb may reduce ferric ions. Among the b_{561} family members,

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101F6 has been thought to be a tumour suppressor encoded at the tumour deletion region of chromosome3p21.3 (*14*). The 101F6 contains a transmembrane region, but not dopamine β-hydroxylase domain, suggesting that the protein is not directly involved in catecholamine metabolism, but rather is probably coupled with other types of hydroxylase enzymes. However, the function of 101F6 has remained unknown.

Here, we report the molecular characterization of a member of the cytochrome b_{561} family, 101F6, as a haemoprotein. Expression and functional analyses of 101F6 and SDR-2 as related to iron metabolism in mouse cells are also shown.

MATERIALS AND METHODS

Materials—The [α-^{32}P] dCTP and nylon membranes were purchased from GE Healthcare UK. Ltd, (Buckinghamshire, UK). Restriction endonucleases and DNA modifying enzymes were obtained from Takara Co. (Tokyo, Japan) and Toyobo Co. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from GIBCO-Invitrogen Co. (Carlsbad, CA, USA). Monoclonal (clone 5E10) and polyclonal antibodies for c-myc were products of Sigma Co. (St Louis, MO, USA) and MBL Laboratories (Nagoya, Japan). Monoclonal antibody for human transferrin receptor-1 was a product of Santa Cruz Co. Anti-haem oxygenase-1 was obtained as described (*15*). The mouse adult multiple tissues RNA blot membrane was purchased from BD Biosciences-Clontech (Alto, CA, USA). All other chemicals were of analytical grade.

Plasmids—The full-length cDNAs of mouse 101F6 (*10*) and SDR2 (*16*) were isolated by PCR using a mouse liver cDNA library (*16*). The primers used for 101F6 were AAGAATTCATGGCCCTTTCTGTGGAG and AAGTCGA CCCATGGCTGGATCCTCTT, and those for SDR2 were AAGGATCCATGGCGGCTCCCCAGATC and AAGAATT CCCAATATGTGGTTGATTGC. After a cDNA with the sequence of 101F6 was identified, 5′-RACE with the mouse brain Marathon cDNA library (BD Bioscience-Clontech) was carried out. The DNA fragments were digested with *Bam*HI-*Sal*I or *Bam*HI-*Eco*RI, and ligated into the corresponding restriction enzyme-digested pcDNA3.1(−)/Myc-His B. The resulting plasmids, pcDNA3 (c-myc)-101F6 and pcDNA3 (c-myc)-SDR2, were transformed into *Escherichia coli* XL1-Blue. To construct glutathione S-transferase (GST) fusion protein expression plasmids, portions of mouse 101F6 cDNA were amplified by PCR and the resulting fragments were ligated into pGEX-4T vector (GE Healthcare UK. Ltd). The plasmids thus constructed were pGEX/101F6 (118–781), pGEX/101F6 (118–587) and pGEX/101F6 (118–444) [numbers indicate the base position in mouse 101F6 cDNA (*17*)], which encoded the 101F6 subfragment fused with GST designated as GST-101F6 (2–222), GST-101F6 (2–138) and GST-101F6 (2–110), respectively (numbers indicate the amino acid positions of mouse 101F6 protein). The pGEX-SDR-2 (557–1907) corresponding to GST-SDR-2 (142–592) was also constructed. These proteins expressed in *E. coli* (strain: DH-5α) were induced with 0.3 mM isopropyl-1-thio-β-D-galactoside at 25°C for 12 h. The nucleotide sequence of each construct was verified by DNA sequencing.

Cell Culture and DNA Transfection—Human embryonic kidney HEK 293T cells, HeLa cells and Chinese hamster ovary (CHO) cells were grown in DMEM supplemented with 10% FCS and antibiotics (*15*). The cells were transfected using Lipofectamine (Invitrogen Co. San Jose, CA, USA) or calcium phosphate with pcDNA3-101F6 (myc) or pcDNA3-SDR2 (myc), and were then incubated in the presence of FCS at 37°C for the specified period.

RNA Blots—Total RNA was isolated from mouse erythroleukaemia (MEL) cells, mouse liver BNL-CL2 cells, mouse neuroblastoma Neuro2a cells and mouse fibroblast Balb/3T3 cells that had been subjected to normoxia and hypoxia (2% oxygen) for 6 h, using the guanidium isothiocyanate method as described previously (*15*, *17*). The RNA was loaded on a 1% agarose/formaldehyde gel, electrophoresed and transferred onto a nylon membrane (BD Biosciences) for hybridization with ^{32}P-labelled DNA fragments of mouse 101F6 and β-actin cDNA (*15*, *17*), and then the filter was hybridized and washed as described previously (*17*).

Assay of Ferric Reductase—HEK293T cells transfected with pcDNA3-101F6 or pcDNA3-SDR2 were incubated for 16 h, and washed twice with phosphate-buffered saline (PBS). The reaction mixture (1 ml) containing 100 μM ferric-nitrilotriacetate, 1 mM ferrozine, 2 mM glucose and PBS was added to the cells, and the cells were incubated at 37°C. Aliquots were withdrawn and centrifuged at 12,000*g* for 10 min. The absorbance of the supernatant at 562 nm was measured (*12*). Decolourization of methyl orange (azo-reductase) was also monitored at 480 nm (*18*).

Detection of Haem-binding—The haem-binding of haemoprotein was assayed by the method of Bonfils *et al*. (*19*). Briefly, the GST-fusion protein eluted from glutathione-Sepharose with 50 mM Tris–HCl, pH 6.8, containing 0.5% sodium dodecyl sulfate (SDS), was immediately placed in a slot of a polyacrylamide gel containing 0.1% SDS and separated by electrophoresis. To detect haem, the gel was treated with PBS containing 20 mM luminol, 0.012% hydrogen peroxide and 0.4 mM 4-indophenol and exposed to the X-ray film.

Immunoblotting—Cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Labolatories, Herculus, CA, USA). Conditions for immunoblotting for anti-myc and other antigens, and the detection of cross-reacted antigens were as described (*15*, *17*).

Immunofluorescence Microscopy—Cells were washed with PBS (+) (PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$), fixed with 4% paraformaldehyde for 20 min and permeabilized in 0.1% Triton X-100 with PBS (+) for 1 h. After blocking with 2% FCS in PBS (+), incubation with anti-myc as the primary antibody was carried out, followed by incubation with Cy3-conjugated goat anti-mouse immunoglobulin (BD Biosciences Co.) (*20*). For double staining experiments, the cells were further incubated with anti-human transferrin receptor 1 (Santa Cruz), followed by Cy2-conjugated goat anti-rabbit

immunoglobulin (BD Biosciences Co.). The cells were examined under a Carl Zeiss LSM 510 confocal microscope.

RESULTS

Identification, Cloning and Expression of Analysis of 101F6—A systematic search of the EST databases (*21*) using mouse cytochrome b_{561} (*22*) as a query, resulted in several partial cytochrome b_{561}-like cDNA sequences from human and mouse that did not correspond to the vertebrate cytochrome b_{561}. Specific primers were used to amplify the mouse 101F6 cDNA from a mouse liver cDNA library. This cDNA coded for a novel protein of 222 amino acids (25 kDa, Fig. 1A). After confirmation of the cDNA sequence, 5′-RACE of 101F6 with the mouse brain Marathon cDNA library (BD Bioscience-Clontech) was carried out. Several different cDNAs were isolated, and the sequence of upstream of the putative translation initiation site was examined. The cDNA encodes a protein that fits well into a cytochrome b_{561} family alignment based on the conserved six membrane-spanning domains. The cDNA for another cytochrome b_{561} family member, mouse SDR-2, was also isolated. The protein sequence of mouse 101F6 is clearly shorter than that of mouse SDR-2, which comprises 592 amino acids and longer than those of cytochrome b_{561} (27.8 kDa), dcytb (31.5 kDa) and lysosomal (27 kDa) (Fig. 1B). The most striking homology among the family members is the existence of four conserved histidine residues, which are presumably involved in the binding of haem molecules. Computer predictions using the PSORT II program (*23*) indicated that 101F6 and SDR-2 do not contain any signal peptide. The overall identity in the amino acid sequence with mouse cytochrome b_{561} was 27% for mouse 101F6 and 22% for mouse SDR-2, respectively. The expression of the 101F6 gene was analysed by northern blotting with a filter containing mRNA from various mouse tissues (Fig. 2A). We observed broad expression of ~2.3 kb 101F6 mRNA in various tissues, with relatively high levels in liver and kidney. The 101F6 mRNA was also expressed in several cell lines (Fig. 2B), and any insults of the cells with haemin, hydrogen peroxide, sodium arsenite or hypoxia did not affect the expression (data not shown).

Expression of 101F6 in Cultured Cells—To examine the intracellular localization of 101F6 and SDR-2, plasmids carrying the myc-tagged cDNAs were constructed and transfected into CHO cells. Immunoblotting was done with anti-myc monoclonal antibody. A band corresponding to a molecular mass of 25 kDa was detected in cells expressing myc-tagged 101F6 (Fig. 3A). The molecular mass of this 101F6 was smaller than that of myc-tagged SDR-2 (62 kDa). Indirect immunofluorescence microscopy with the transfected CHO cells revealed that the staining of 101F6 mainly occurred in small vesicles and the endoplasmic reticulum, which is similar to the location of haem oxygenase-1. The location of SDR-2 was similar to that of 101F6 (Fig. 3B and C). Comparison of the staining of 101F6 in HeLa cells with that of transferrin receptor-1 revealed that their locations were different. These results indicated that the location of 101F6 in small vesicles was somewhat different from those containing transferrin receptor-1, corresponding to endosomes.

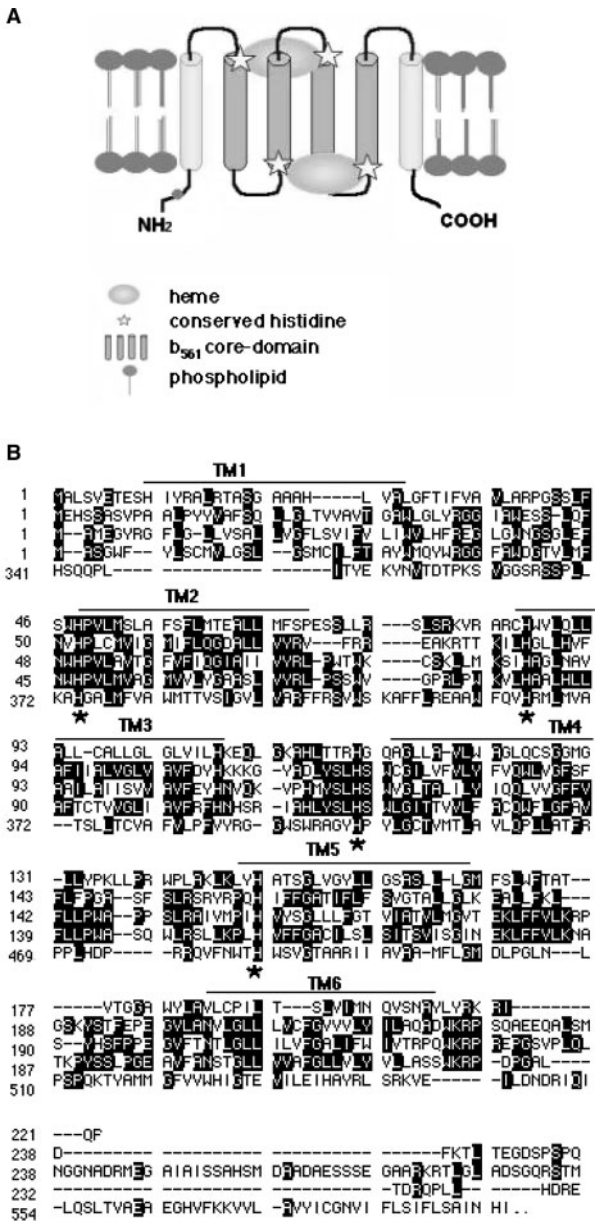

Fig. 1. **(A) Schematic diagrams of 101F6 protein.** (B) Alignment of the amino acid sequences of mouse 101 F6, Dcytb, cytochrome b_{561}, lysosomal b_{561} and SDR-2. The six membrane-spanning domains are underlined. Conserved histidine residues that bind to haem are shown as asterisks.

Haem-binding of 101F6 Expressed in E. coli and the Content of Haem in 101F6- and SDR-2-expressing HEK293-T Cells—To examine if 101F6 contains haem, GST-101F6 (amino acids 2–222) was first expressed in *E. coli* and purified with glutathione-Sepharose. The purified protein was eluted with Laemmli's sample buffer (minus 2-mercaptoethanol) (*24*) and directly analysed by SDS-polyacrylamide gel electrophoresis. The peroxidation of haem was detected at a position

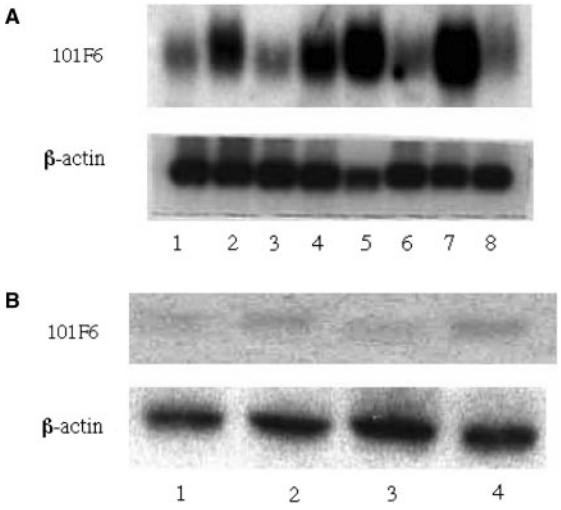

Fig. 2. **Expression of 101F6 mRNA in mouse tissues and cultured cells.** (A) Northern blotting of 101F6 in various tissues. A multiple tissue blot membrane (BD Biosciences-Clontech.) was probed with ^{32}P-labelled DNA fragments of 101F6 (Upper panel), and β-actin (Lower panel). Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney and lane 8, pancreas. (B) Expression of 101F6 mRNA in cultured mouse cells. Balb/3T3 cells (lane 1), MEL cells (lane 2), BNL-CL2 cells (lane 3) and Neuro2a cells (lane 4).

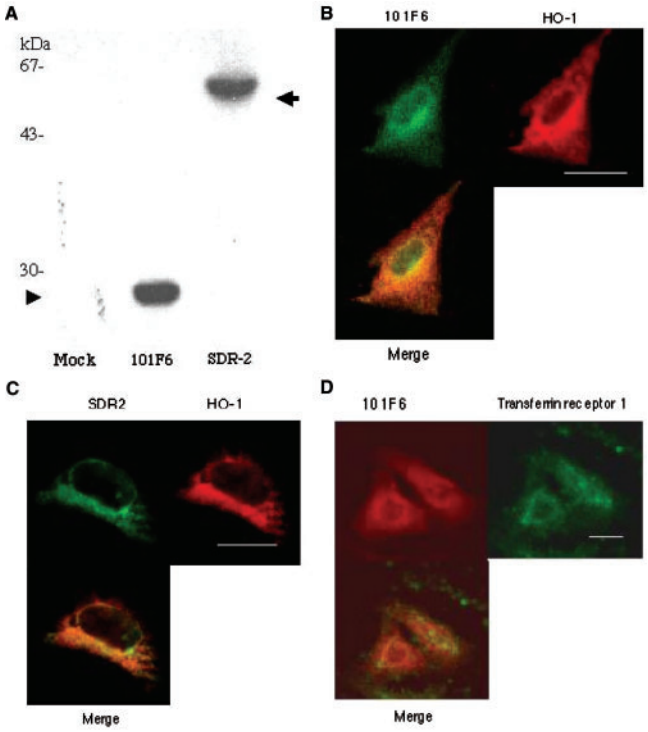

Fig. 3. **Expression of 101F6 and SDR-2 in cultured cells.** (A) Expression of 101F6 and SDR-2 in CHO cells. Cellular proteins from the cells expressing 101F6 and SDR-2 were analysed by SDS-polyacrylamide gel electrophoresis and transferred onto a membrane. Immunoblotting was performed with anti-myc. The arrowhead shows the position of 101F6 and the arrow shows that of SDR-2. (B) Immunofluoresent staining of CHO cells expressing 101F6. Cells transfected with pcDNA-myc-101F6 were plated on glass coverslips and fixed. After blocking, the fixed cells under permeabilized conditions were incubated with anti-myc antibody (green), followed by antibodies for haem oxygenase-1 (red). The merged image confirmed that the dots were co-localized. (C) CHO cells transfected with SDR-2 cDNA were also reacted simultaneously with anti-myc (green) and anti-haem oxygenase-1 (red) to show the localization. (D) Intracellular localization of 101F6 in HeLa cells. The cells transfected with pcDNA3-101F6 were cultured and immunostaining of permeabilized cells was performed with anti-myc antibody (red), followed by monoclonal antibody for human transferrin receptor-1 (green).

corresponding to a molecular mass of 50 kDa in the gel (Fig. 4A). Haem was not detected in gels on which deletion mutants of 101F6 (amino acids 2–138 and 2–110) were electrophoresed, indicating that the histidine residue at position 149 may be essential for haem-binding (Fig. 4C). The GST-SDR-2 (142–592) fusion protein also bound haem (Fig. 4B). In order to examine the haem-binding status of 101F6 and SDR-2 expressed in culture cells, we examined the content of haem in the HEK293-T cells transfected with the corresponding cDNAs. The content of haem in HEK293-T cells expressing 101F6 and SDR-2 was higher than that in the control cells (Fig. 4D).

The Reductase Activity Toward Ferric Ions and Azo-dye in 101F6- and SDR-2-expressing HEK 293T Cells—Finally, to examine the relationship of 101F6 to iron metabolism, we measured the ferric-reductase activity of 101F6. First, NADPH-dependent reduction of ferric ions was measured using cell lysates from HEK293T cells expressing 101F6 or SDR2, but no reductase activity was observed (data not shown). Then, we measured ferric reduction using intact cells expressing 101F6. The ferric-reductase activity of HEK293 T cells expressing 101F6 and SDR-2 was higher that that of control cells (Fig. 5). The iron-dependent decolourization of an azo-dye, methyl orange, as the substrate, with HEK293T cells expressing 101F6 or SDR-2 was higher than that with control cells.

DISCUSSION

In the present study, we characterized a novel type of cytochrome b_{561}, 101F6, which was ubiquitously expressed in mouse cells. The predicted primary structure of this protein showed very high sequence identity in the six membrane-spanning domains and the haem-binding motif with cytochrome b_{561}. The 101F6 was shown to be a membrane protein located in small vesicles, with an undefined role. Another mammalian cytochrome b_{561} family member, SDR-2, contains an obvious dopamine β-hydroxylase domain in the amino-terminal half of the protein, whereas 101F6 and Dcytb do not (*6*, *10*), suggesting that the function of 101F6 may be distinct from that of SDR-2. To clarify the physiological role of 101F6, we also isolated SDR-2 cDNA. The bacteria-expressed GST-SDR-2 fusion protein also contained the haememolecule (Fig. 4C). The contents of haem in Balb/3T3 cells expressing 101F6 and SDR-2 was higher than that in the control cells.

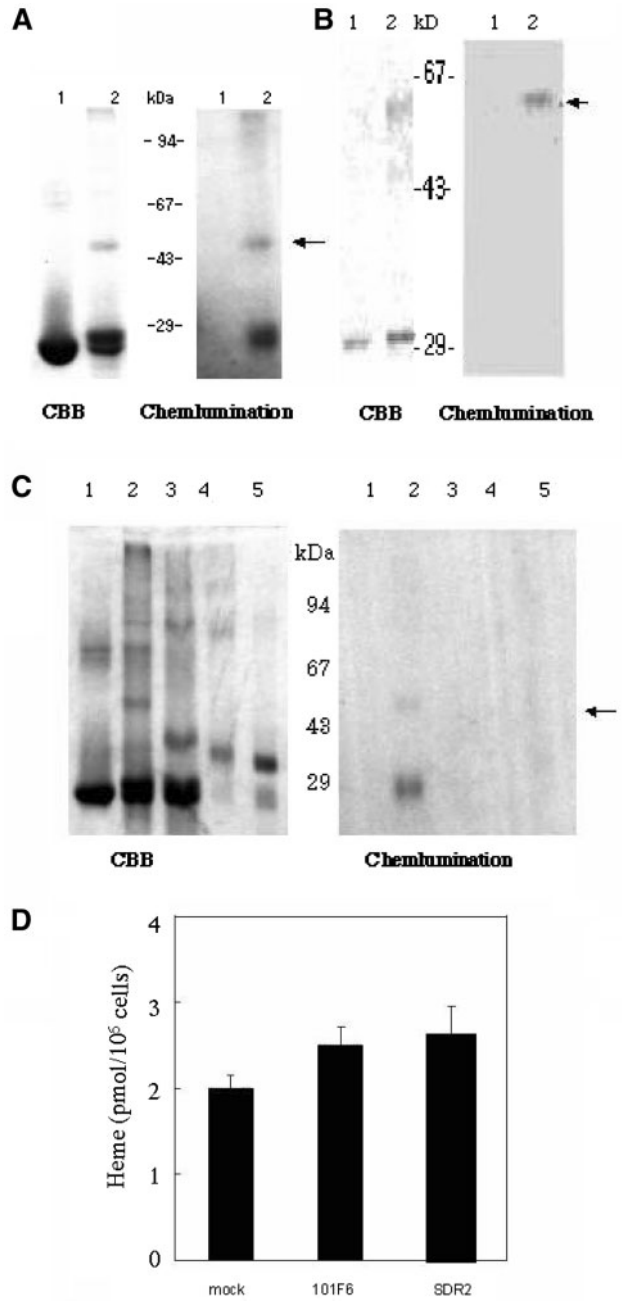

Fig. 4. **The binding of haem by 101F6 and SDR-2.** GST-101F6 (A) and GST-SDR-2 (B) proteins expressed in *E. coli* were purified with glutathione beads. The proteins were eluted with Laemmli's sample buffer minus 2-mercaptoethanol and directly analysed by SDS-PAGE. The gels were treated with luminol/4-indophenyl/hydrogen peroxide solution and exposed to an X-ray film. The gels were also stained with Coommasie brilliant blue (CBB). Arrows show the position of GST-101F6 or -SDR2 fusion protein. (C) GST-fused with deletion mutants of 101F6: GST (lane 1), 101F6 (2–222) (lane 2), 101F6 (2–138) (lane 3) and 101F6 (2–110) (lane 4) were also separated. The peroxidation of haem in the gel was examined. (D) HEK293T cells transfected with 101F6 and SDR-2 cDNA were cultured for 24 h. The content of haem in the cells was then measured. The data are expressed as the average of three independent experiments ± SD.

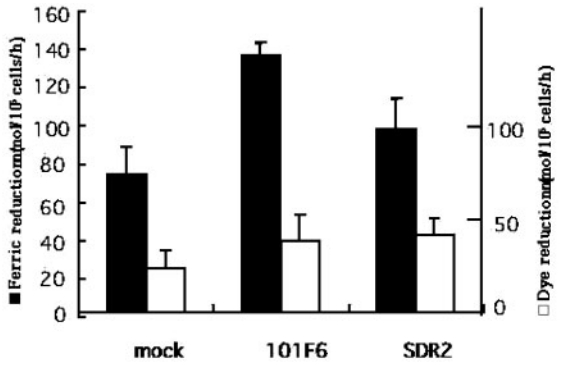

Fig. 5. **The reduction of ferric ions and azo-dye with cells expressing 101F6 and SDR-2.** (A) The ferric-reductase activity (closed columns). HEK 293T cells expressing 101F6 and SDR-2 were cultured for 48 h. The cells were incubated with PBS containing 100 μM ferric–nitrilotriacetate and 5 mM glucose for 2 h. The media were collected and centrifuged at 10,000*g* for 10 min. The reduction of ferric ions was measured by monitoring absorbance at 562 nm. (B) The azo-reductase activity (open columns). The cells were incubated with 20 mM potassium phosphate buffer, pH 6.5, containing 150 mM NaCl, 1 mM methyl orange and 5 mM glucose for 2 h. The decrease of absorbance at 480 nm was measured. The data are expressed as the average of four independent experiments ± SD.

Thus, the bacterial expression of 101F6 or SDR-2 revealed that these proteins are haemoproteins, and the deletion of one at amino acid 149 of the four histidine residues, which is presumably involved in the haem-binding of the 101F6 protein, caused the loss of haem binding. Although cytochrome b_{561} family members are thought to coordinate two binding sites of haem groups to four molecules of histidine, the loss of one histidine caused the complete loss of haem in 101F6. Very recently, when mutants of lysosomal cytochrome b_{561} corresponding to one of the histidine residues putatively bound to the haem moiety were expressed and ferric-reductase activity was examined, none of the mutants exhibited the activity (*11*). Based on these findings, we concluded that changes in the steric structures of the transmembrane regions of these proteins by the deletion or mutation of one histidine resulted in the loss of the potential interaction between the two haem moieties.

Among the cytochrome b_{561} family members, Dcytb and cytochrome b_{561} were expressed in limited tissues (*4*, *25*, *26*). Vargas *et al*. (*12*) reported that SDR-2 is predominantly expressed in liver, kidney and skin and also found that SDR-2 expression in the small intestine was relatively low. The present study showed that 101F6 mRNA is expressed widely in various tissues, including the small intestine, and further found that 101F6 is located in small vesicle-like structures in cells. HO-1, a marker of smooth endoplasmic reticulum, was stained in perinuclear small vesicles, which was almost matched the location of 101F6. On the other hand, the transferrin receptor 1 was stained at the cell surface and endosomes. The intracellular staining of transferrin receptors was similar to, but not the same as that of 101 F6. These results suggest that the function of 101F6 may not be directly associated with the

trnsferrin receptor-1-dependent transport of iron. We showed here that the SDR-2 protein was located in small vesicles, which was similar to the localization seen in the case of the 101F6 protein. The main function of Dcytb was shown to be the reduction of ferric ions in the intestine before the uptake of iron by cells. SDR-2 contains the domain of dopamine-β hydroxylase, whose activity is presumably controlled by binding catecholamine and functions in the ascorbate-dependent reduction of ferric ions. The high expression of SDR-2 mRNA in rat liver and kidney was some somewhat similar to that of 101F6 mRNA (*12*). The present data showed that both 101F6 and SDR-2 exhibited the ferric-reductase activity and had the similar locations in the cells. Therefore, 101F6 protein, in association with other unknown proteins, may function in the reduction of ferric ions.

Yeast is a well studied model organism with respect to iron metabolism and iron transport through the plasma membrane (*27*). Flavin containing transmembrane proteins, Fre1/Fre2, in the plasma membrane of yeast are involved in the reduction of ferric ions prior to uptakes, since Δfre1/Δfre2 double mutant cells show minimal ferric-reductase activity and a severe growth defect (*28*). The expression of Fre1 in Δfre1/Δfre2 mutant cells resulted in the reduction of ferric ions (*29*). Other investigators (*11*) reported that Dcytb and lysosomal cytochrome b_{561} in the Δfre1/Δfre2 mutant cell resulted in the restoration of growth, supporting the notion that replacement of ferric redutase was possible. Otherwise, the ferric-reductase systems including Fre1/Fre2 in yeast, participates in the extracellular reduction of azo-dye (*18*). We then further examined the relationship of the reduction of the azo-dye to the ferric-reductase activity of 101F6 and SDR-2, and found that the decolourization of the azo-dye in the medium of 101F6- or SDR-2-expressing 293T cells was greater than that in control cells. It is unclear how ferric reductase is related to the reduction of azo-dye, but it is possible that some reductant was exported due to the enhancement of the ferric-reductase activity. In this connection, the excretion of anthranilic and 3-hydroxyanthranilic acid was correlated with the surface ferrireductase of yeast (*27*). Based on the observations that ascorbate is the electron donating substrate of another cytochrome b_{561} in chromaffin granules of the adrenal-medulla, we speculate that 101F6 and SDR-2 utilize a reductant, as an electron-donor, to reduce ferric ions.

It was shown *in vivo* and *in vitro* that duodenal ascorbate levels were correlated with the ferric-reductase activity in the duodenum (*4*). Previous studies (*11*) predicted that the arginine residue at 67 (R67) of lysosomal cytochrome b_{561} was essential for ascorbate binding, and the mutation of R67 of lysosomal cytochrome b_{561} abolished the ferric reductase. This arginine residue is conserved in SDR-2, cytochrome b_{561} and Dcytb, which showed possible ascorbate-dependent reduction of ferric ions, but is replaced by serine in 101F6. The conserved Y66 of the lysosomal cytochrome b_{561} proteins also plays a role in ascorbate binding and ferric activity. However, only SDR-2 does not contain tyrosine at the corresponding position, raising the question of whether SDR-2 exhibits the ferric-reducase activity. We found that 101F6 and SDR-2 exhibited ferric as well as azo-dye-reductase activity. We examined the level of ascrobate in 101F6- or SDR-2-expressing cells, but no marked variation of the level was observed upon the expression of these proteins (unpublished observations). Thus, further studies are required to clarify the mechanisms involving electron donors of the reduction of the ferric-reductase activity of cytochrome b_{561} family.

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