A Naturally Occurring Mutation in the SLC21A6 Gene Causing Impaired Membrane Localization of the Hepatocyte Uptake Transporter*

The organic anion transporter SLC21A6 (also known as OATP2, OATP-C, or LST-1) is involved in the hepatocellular uptake of a variety of endogenous and xenobiotic substances and drugs. We analyzed 81 human liver samples by immunoblotting and found one with a strongly reduced amount of SLC21A6 protein suggesting mutations in the SLC21A6 gene. The SLC21A6 cDNA from this sample contained five base pair changes in one allele; three of the mutations resulted in amino acid substitutions designated SLC21A6-N130D, SLC21A6-P155T, and SLC21A6-L193R. The former two were polymorphisms (SLC21A6*1b and SLC21A6*4), whereas SLC21A6-L193R represents the first naturally occurring mutation identified in one allele of the SLC21A6 gene, which affects protein maturation and organic anion transport. We introduced each of the mutations into the SLC21A6 cDNA and established stably transfected MDCKII cells expressing the respective mutant SLC21A6 protein. Immunofluorescence microscopy and uptake measurements were used to study localization and transport properties of the mutated proteins. Both proteins carrying the polymorphisms were sorted to the lateral membrane like wild-type SLC21A6, but their transport properties of the substrates cholate and 17β-glucuronosyl estradiol were altered. Importantly, most of the mutant protein SLC21A6-L193R was retained intracellularly, and this single amino acid exchange abolished transport function.

The different membrane domains of the hepatocyte contain uptake transporters and export pumps, which are responsible for the uptake of substances from the blood into hepatocytes and for the excretion into the extracellular space, respectively. Whereas the apical, i.e. canalicular, membrane of the hepatocyte contains predominantly members of the ABC1 transporter family of export pumps (1, 2), the basolateral membrane contains a variety of uptake transporters and export pumps belonging to different families (3, 4). One large family of uptake transporters with members expressed in hepatocytes is the OATP family of organic anion transporters (solute carrier family SLC21A). At least three members of this family are expressed in human hepatocytes: SLC21A6, also known as OATP2 (5, 6), OATP-C (7, 8), or LST-1 (9), SLC21A9 or OATP-B (7, 8), and SLC21A8 or OATP8 (10). SLC21A6 is located in the basolateral membrane of hepatocytes (6) and is involved in the uptake of different endogenous organic anions including conjugated and unconjugated bilirubin (6, 11), cholate, urate, uric acid, and 17β-glucuronosyl estradiol (6, 9–11). In addition, this uptake transporter is important for the removal of a variety of xenobiotics and drugs from the blood including the organic anion bromosulfophthalein (BSP) (11), the HMG-CoA-reductase inhibitor pravastatin (5), and the endothelin A antagonist BQ-123 (8).

Variations in genes encoding proteins involved in hepatobiliary elimination have long been recognized as important determinants of drug response and disposition. This may be exemplified by single nucleotide polymorphisms in genes encoding members of the cytochrome P450 family, mediating phase I of drug metabolism. Such variations have been described to alter drug responsiveness and drug toxicity and may be associated with a loss of therapeutic efficacy (12–14). In addition, polymorphisms in conjugation enzymes of the phase II reactions have been discussed as reasons for interindividual variability in drug metabolism (15). More recently, polymorphisms in genes encoding transport proteins involved in detoxification have become of interest for studies on drug metabolism. Polymorphisms in the human ABC B1 (MDR1) gene encoding the drug resistance-related MDR1 P-glycoprotein have been linked to alterations in fexofenadine (16) and digoxin (17) disposition. So far, little is known about polymorphisms in genes encoding uptake transporters. Tirona et al. (18) described the identification of polymorphisms in the SLC21A6 gene, their prevalence in different populations, and their consequences for substrate transport (18). Most recently, Nozawa et al., (19) analyzed the allelic frequency of polymorphisms in the SLC21A6 and the SLC21A9 genes in the Japanese population. Up to now, no information had been available on mutations in uptake transporter genes causing a loss of transport function.

In this work, we identified and characterized a haplotype of the SLC21A6 gene encoding a mutant protein of the SLC21A6...
transporter. This haplotype contains five base pair exchanges resulting in three amino acid substitutions. One of these substitutions is the first naturally occurring mutation in the SLC21A6 gene leading to impaired protein maturation and insertion into the plasma membrane. We examined the consequences of each of the three amino acid substitutions with respect to cellular localization of the protein and to the transport characteristics in comparison with wild-type SLC21A6.

**EXPERIMENTAL PROCEDURES**

**Liver Samples**—Human liver tissue was obtained from patients of Caucasian origin undergoing liver resection at the Department of Surgery, Campus Virchow-Clinic, Humboldt University, Berlin, Germany (20). Liver pieces were immediately placed into RNAlater (Ambion, Hunttington, UK) for DNA and RNA preparation, incubated for 24 h at 4 °C, and then stored frozen at −80 °C until DNA or RNA preparation. In addition, a liver wedge biopsy was obtained from a subset of these patients and frozen in liquid nitrogen. The study was approved by the local ethics committee and conducted according to the ethical guidelines of the Declaration of Helsinki. All patients gave written informed consent.

**Materials**—Pepstatin, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, agar, fetal calf serum, and the protein standard mixture (relatively molecular weight of 26,600–180,000) for the SDS-PAGE were from Sigma. RNase inhibitor (RNasin), Stratascript Moloney murine leukemia virus reverse transcriptase, TaqDNA polymerase, and restriction enzymes were from Stratagene (La Jolla, CA). Lysozyme and amplification were from Roche Molecular Biochemicals; agarose was from Roth (Karlsruhe, Germany).

**Antibodies**—The EAG5 antibody was raised in rabbits against the human apical conjugate export pump MRP2 (ABCC2) (21). The polyclonal antibody ESL was raised against the C terminus, and the monoclonal antibodies mMDQ and mESL against the N-terminal and C-terminal end of the human SLC21A6 protein, respectively (6). The mouse monoclonal antibody against desmoplakin (mixture) was purchased from Progen (Heidelberg, Germany). Alexa Fluor 546-conjugated goat anti-rabbit antibodies for1ha troom temperature were from Molecular Probes (Eugene, Oregon) and Cy3-conjugated goat anti-mouse antibody was from Dianova (Hamburg, Germany).

**Amplification of SLC21A6-M5 cDNA**—A cDNA fragment covering the entire coding region of the human SLC21A6 gene was amplified using single-stranded cDNA synthesized with SLC21A6-S2 total RNA as template (20). In addition, 2 μg of total RNA were reverse-transcribed using 2.5 μg of oligo(dT)18 primer as described (22). For PCR amplification the following primer pair was used: oOATP2RT (5′-GGCTTTCTACATTGATC-3′) and the reverse primer omutL193R (5′-CAATTGACAGCAGGACG-3′). Successful mutagenesis was verified by restriction analysis and sequencing.

**Semi-quantification of SLC21A6 mRNA Expression**—Semi-quantitative analysis of SLC21A6 mRNA expression in stably transfected MCKII cells was performed using the LightCycler SystemTM (Roche Molecular Biochemicals) as described (24) with the primer pair oOATP2RT (5′-TGCACTTGGAGGACGCTGAT-3′) and oOATP2RT. rev (5′-CTTCATGGATCATGCTATTTT-3′). The mRNA values in transfected cells expressing the mutant SLC21A6 cDNAs are given as percent of the mRNA value in wild-type SLC21A6-transfected cells.

**Cell Culture and Transfection**—Madin-Darby canine kidney cells strain II (MCKII) were cultured in minimum essential medium (Sigma) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO2. MCKII cells were transfected with the respective plasmids using the polybrenne (hexa-dimethrin bromide) method as described earlier (27). After 48 h of G418 selection (800 μg/ml), single colonies were screened for SLC21A6 expression by immunoblot analysis and immunofluorescence microscopy. For immunofluorescence analysis and transport assays, MCKII cells well grown on polycarbonate membrane inserts inserts (0.5-μm diameter for immunofluorescence analysis and 24-mm diameter for transport assays, pore size 0.4 μm, Costar, Cambridge, MA) to confluence for three days and induced with 10 μM sodium butyrate for 24 h to obtain higher levels of the recombinant protein (27).

**Immunoblot Analysis**—Membrane proteins were diluted with sample buffer and incubated at 37 °C for 30 min before their separation on 4% stacking and 10% resolving SDS-polyacrylamide gels. Immunoblotting was performed using a tank blotting system from Bio-Rad (Munich, Germany) and enhanced chemiluminescence detection (PerkinElmer Life Sciences). The primary antibody (pESL, mESL, or mMDQ) was diluted 1:5000 in TTBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The secondary antibody was either a horseradish peroxidase-conjugated goat anti-rabbit IgG or a goat anti-mouse IgG (Bio-Rad), both used at a 1:10,000 dilution.

**Immunofluorescence Microscopy**—MCKII cells were grown and induced as described above. Cells were then fixed for 20 min with 4% paraformaldehyde in phosphate-buffered saline and permeabilized for 20 min in 1% Triton X-100 in PBS. Subsequently, cells were incubated with the primary antibodies for 1 h at room temperature and then washed three times with PBS and incubated with the secondary antibodies for 1 h at room temperature. All antibodies were diluted in PBS at the following dilutions: pESL at 1:100, anti-desmoplakin at 1:20; Alexa Fluor 488-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG at 1:200. Membranes were cut from the insert and mounted onto glass-slides with 50% glycerol in PBS. Confocal laser-scanning immunofluorescence microscopy was performed on a LSM-510 apparatus (Carl Zeiss, Jena, Germany).

**Cyrosections of human liver samples**—Thick (5-μm thickness) were prepared for immunofluorescence microscopy as described (10, 28). After simultaneous incubation with the primary antibodies for 1 h at room temperature sections were washed three times with PBS and then incubated with the secondary antibodies. Finally, sections were washed three times with PBS, rinsed with distilled water, and then mounted in Moviol ( Hoechst, Frankfurt, Germany).

The antibodies were used as follows: the mouse mESL antibody as an undiluted hybridoma supernatant, the EAG5 antiseraum at a 1:50 dilution in hybridoma supernatant, and Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated anti-rabbit IgG both at a dilution of 1:300 in PBS. Fluorescence microscopy was performed on an Axiovert S100TV microscope (Carl Zeiss) as described (10).

**Transport Assays**—[3H]Bromosulfophthalein (0.5 TBq/ml) was obtained by custom synthesis (Hartmann Analytic, Kiel, Germany), [3H]cholesterol (73 MBq/ml), and [3H]E17βG (1.5 TBq/ml) were from PerkinElmer Life Sciences. Cells were grown on Transwell membrane inserts and induced with butyrate (27) as described. For transport assays, MCKII cells were washed with uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 1.2 mM MgSO4, 1.5 mM CaCl2, 5 mM glucose, and 12.5 mM Hepes, pH 7.3). Subsequently, 1 ml of uptake buffer without substrate was added to the apical compartment and 1 ml of uptake buffer containing the 3H-labeled substrate was added to the basolateral compartment. After 10 min (for BSP and E17βG) or 20 min (for choleylaurine), the cells were washed three times with cold uptake buffer and lysed with 2 ml of 0.2% SDS in water.
The radioactivity in the lysate was determined by liquid scintillation counting.

**RESULTS**

**Identification of Mutant SLC21A6 Protein and Analysis of the Haplotype SLC21A6-M5 in Human Liver**—Eighty-one samples from human livers were analyzed by immunoblotting using an antibody directed against the N-terminal end of the SLC21A6 protein. This antibody detects simultaneously SLC21A6 (OATP2, OATP-C, or LST-1) and SLC21A8 (OATP8) (Fig. 1). In this screening procedure we identified one liver sample, termed SLC21A6-S2, with a strong reduction in the amount of SLC21A6 protein relative to SLC21A8 and compared with the SLC21A6 protein amount in other samples (Fig. 1). The ratio of band intensities of SLC21A6 protein relative to SLC21A8 was determined densitometrically to be 1.6 (median, range 1.2 to 3.6, n = 46) for normal liver samples. The ratio in sample SLC21A6-S2 was 0.6.

We introduced each single base pair exchange identified in sample SLC21A6-S2 leading to the mutant SLC21A6 protein into the cDNA resulting in three amino acid substitutions and two silent mutations. The following nucleotide exchanges were found: A-388 \(\rightarrow\) G resulting in N130D (green); G-411 \(\rightarrow\) A resulting in the silent mutation S137S (gray); C-463 \(\rightarrow\) A resulting in P155T (red); T-578 \(\rightarrow\) G resulting in L193R (blue); and C-597 \(\rightarrow\) T resulting in the silent mutation F199F (gray). The two amino acid substitutions in the second extracellular loop (loop 2) are described as polymorphisms (18) and therefore designated SLC21A6*1b for N130D and SLC21A6*4 for P155T, whereas the substitution L193R located in the adjacent transmembrane helix was unique in 330 DNA samples analyzed and, therefore, described as mutation. Each stably transfected MDCKII cell line expressing the mutant SLC21A6 protein was designated according to the amino acid substitution: SLC21A6-N130D corresponding to the polymorphism SLC21A6*1b, SLC21A6-P155T corresponding to the polymorphism SLC21A6*4, and SLC21A6-L193R corresponding to the mutation L193R.

Expression and Localization of Recombinant SLC21A6 Proteins Containing the Different Amino Acid Substitutions Encoded in the SLC21A6-M5 Gene—We introduced each single base pair exchange identified in sample SLC21A6-S2 leading to an amino acid substitution in the SLC21A6 protein into the SLC21A6 cDNA and examined the consequences of this alteration. Furthermore, a SLC21A6 cDNA was constructed that carried all five base pair exchanges representing the haplotype SLC21A6-M5. The respective SLC21A6 proteins were designated as SLC21A6-WT representing the wild-type form of the SLC21A6 protein (GenBank/EMBL accession number
Bast staining for SLC21A6 was observed as expected in normal liver (Fig. 5, A) with SLC21A6 staining was weak in the basolateral membrane in addition to some intracellular staining. Basolateral staining for SLC21A6 was observed as expected in normal liver (B). Bar, 10 μm.

AJ132573), SLC21A6-N130D representing the polymorphism SLC21A6*M1 resulting in the substitution of Asn by Asp at position 130, SLC21A6-P155T representing the polymorphism SLC21A6*4 with Pro-155 substituted by Thr, SLC21A6-L193R representing the mutation with Leu-193 substituted by Arg, and SLC21A6-M5 corresponding to the haplotype form carrying the three base pair exchanges encoding the naturally occurring mutant protein. We analyzed the localization of the different recombinant mutant SLC21A6 proteins in stably transfected MDCKII cells. The protein levels in the transfected cell lines were compared by immunoblot analysis (Fig. 4). Wild-type SLC21A6, both polymorphisms, and the single mutant protein SLC21A6-L193R were synthesized in comparable amounts. Interestingly, the protein amount of the haplotype form SLC21A6-M5 was decreased despite the fact that the mRNA encoding recombinant SLC21A6-M5 was not reduced in comparison with the mRNAs from the other transfectants as demonstrated by semiquantitative reverse transcriptase-PCR using the LightCycler system. The mRNA amounts relative to the wild-type SLC21A6 (1.0) were 1.2 for the polymorphism SLC21A6-N130D, 1.7 for polymorphism SLC21A6-P155T, 2.7 for the single mutant SLC21A6-L193R, and 1.7 for the haplotype SLC21A6-M5.

The different SLC21A6 transfectants were used to study the localization of the respective recombinant protein by immunofluorescence microscopy. These images demonstrated the lateral localization of the wild-type SLC21A6 protein (Fig. 5A) as shown by colocalization with desmoplakin (Fig. 5C). Both polymorphisms, SLC21A6*1b (Fig. 5, D and F) and SLC21A6*4 (Fig. 5, G and I), were localized similarly as wild-type SLC21A6. However, more SLC21A6*1b and SLC21A6*4 protein appeared to be retained intracellularly as compared with the wild-type SLC21A6. A pronounced change in protein localization was observed after expression of the mutant protein SLC21A6-L193R, which was hardly detectable in the lateral membrane of MDCKII cells and mainly retained intracellularly (Fig. 5, J and L). The small amount of SLC21A6-L193R protein in the lateral membrane was reflected by the weak yellow color in the merged picture (Fig. 5L). The combination of the polymorphisms SLC21A6*1b and SLC21A6*4 with the mutation SLC21A6-L193R was examined by expression of the haplotype form SLC21A6-M5 (Fig. 5, M and O). Cells synthesizing this mutant protein containing the three amino acid substitutions showed no staining of the lateral membrane of MDCKII cells by the antibody ESL (Fig. 5M) as demonstrated by laser scanning microscopy and colocalization with desmoplakin (Fig 5O).

**DISCUSSION**

Uptake of endogenous substances and xenobiotics from blood into hepatocytes is the first step in hepatobiliary elimination and detoxification. To fulfill this important function, the basolateral membrane of hepatocytes is equipped with a number of different uptake transporters. In this study we describe the first naturally occurring mutation in the SLC21A6 gene, which encodes a major uptake transporter for organic anions and bile salts into human liver. In addition, two frequent polymorphisms were detected in the same mutant SLC21A6 allele. Stably transfected MDCKII cells expressing wild-type or mutant SLC21A6 proteins were used to study the effect of the polymorphisms and of the mutation in the localization of the protein and on the transport kinetics. To date, only limited information is available on polymorphisms in genes encoding transporter proteins. So far, mostly polymorphisms in ATP-dependent export pumps have been described and partially characterized. For instance, polymorphisms in the human MDR1 (symbol ABCB1) gene encoding the drug resistance mediating MDR1 P-glycoprotein (17, 31) and in the human ABCC2 gene.

**Fig. 3.** Immunoﬂuorescence localization of SLC21A6 in liver. Cryosections were double-stained with the ESL antibody raised against the SLC21A6 protein (green ﬂuorescence) and the EAGS antibody against the apical conjugate export pump MRP2 (red ﬂuorescence). In the liver SLC21A6-S2 (A) SLC21A6 staining was weak in the basolateral membrane in addition to some intracellular staining. Basolateral staining for SLC21A6 was observed as expected in normal liver (B). Bar, 10 μm.

**Fig. 4.** Immunoblot analysis of normal and mutated SLC21A6 proteins. Crude liver membrane proteins from stably transfected MDCKII cells (40 μg each) and from human liver (4 μg) were separated by SDS-polyacrylamide gel electrophoresis. SLC21A6 was detected by the polyclonal antibody ESL. SLC21A6 proteins expressed in the transfectants SLC21A6-N130D and SLC21A6-P155T correspond to the SLC21A6 polymorphisms SLC21A6*1b and SLC21A6*4, respectively. The SLC21A6 protein in transfectant SLC21A6-L193R corresponds to the single mutation, whereas the SLC21A6 protein in the transfectant SLC21A6-M5 corresponds to the protein encoded by the SLC21A6 haplotype SLC21A6-M5.
encoding the canalicular export pump MRP2 (31, 32) have been reported. Furthermore, mutations in the ABCC2 gene resulting in the absence of the protein from the canalicular membrane have been described (33–35). Tirona et al. have shown that polymorphisms in the SLC21A6 gene vary with respect to their frequency between the European-American and African-American population (18). Furthermore, the authors used biotinylation experiments to study surface expression of several SLC21A6 allelic variants. Most recently, the allelic frequencies of polymorphisms in the SLC21A6 and SLC21A9 genes in the Japanese population were analyzed (19).

In this study, we investigated the localization and functional consequences of two polymorphisms and one mutation in the SLC21A6 protein in stably transfected MDCKII cells. These experiments demonstrated that both polymorphisms, SLC21A6*1b (SLC21A6-N130D) and SLC21A6*4 (SLC21A6-P155T), had only a minor, if any, effect on the localization of the protein as reflected by intracellular retention (Fig. 5). This could be due to an altered interaction with proteins involved in sorting. In addition, the extent of glycosylation of the mutant protein SLC21A6-P155T differs and may affect the quantity detected by immunofluorescence microscopy. A pronounced change in localization was demonstrated for the mutated SLC21A6-L193R (Fig. 5). Most of the SLC21A6-L193R protein was retained intracellularly suggesting an impaired maturation of the protein. Furthermore, as demonstrated in the uptake experiments, MDCKII cells expressing the mutant SLC21A6 protein did not show transport activity with any of the substances tested (Fig. 6). The impaired maturation indicates that the SLC21A6-L193R protein was recognized by the cellular quality control machinery. Studying the localization of the protein SLC21A6-M5 corresponding to the haplotype form no membrane localization of the protein could be detected. In addition to the intracellular retention, the protein amount in transfected MDCKII cells was reduced, despite the fact that the SLC21A6-M5 mRNA was synthesized in a similar amount.
as in the other transfectants. The altered localization of the haplotype protein observed in vitro after transfection was also detected in the liver in vivo as seen in the localization of SLC21A6 in liver sample SLC21A6-S2 (Fig. 3). Despite the fact that both alleles were transcribed, a pronounced change in the localization of the protein was detected (Fig. 3), in addition to the reduced protein amount (Fig. 1). Most of the liver protein was retained intracellularly and only a weak membrane staining could be detected, possibly resulting from wild-type SLC21A6 (Fig. 3). The donor of liver sample SLC21A6-S2 was not available for detailed studies on hepatobiliary elimination. Due to the fact that this mutation was found in a heterozygous form in only one of 330 DNA samples, one may conclude that a homozygous appearance of this mutation in adults is unlikely or may be lethal to the embryo.

The stably transfected cell lines also served to study the transport characteristics of the mutated SLC21A6 proteins. The polymorphisms SLC21A6*1b and SLC21A6*4 differ in amino acids in the predicted second extracellular loop (Fig. 2). These proteins differed in their transport kinetics with all three substrates. Whereas BSP was transported by the SLC21A6*4 protein similar to the wild-type SLC21A6, transport of BSP mediated by the SLC21A6*1b protein was significantly enhanced. In contrast, the transport of E217G mediated by SLC21A6*1b was comparable to the other transfectants. The altered localization of the protein was detected (Fig. 3), in addition to the predicted second extracellular loop (Fig. 2). These proteins or may be lethal to the embryo.

The hypothesis is supported by the fact that serine at position 155 in the human SLC21A family members. Single amino acid substitutions leading to an altered substrate specificity have been described for the conjugate export pump Mrp2 (36). A single amino acid substitution in the rat Mrp2 protein resulted in acquired transport capacity for cholyltaurine, which normally is not a substrate for this transporter (36).

In conclusion we have identified a naturally occurring mutation in SLC21A6 gene leading to an impaired maturation of the protein with reduced membrane localization and abolished transport function. Two frequent polymorphisms located within the second extracellular loop of the SLC21A6 protein were associated with an altered substrate specificity. These results suggest that extracellular loop 2 is involved in substrate recognition. Thus, the detailed analyses of polymorphisms and mutations in the gene encoding the SLC21A6 protein contributes to our understanding of the hepatobiliary elimination of organic anions and possibly to an impairment of the hepatobiliary elimination of drugs and drug candidates.

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