Molecular Cloning and Characterization of Multispecific Organic Anion Transporter 4 Expressed in the Placenta*

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A cDNA encoding a novel multispecific organic anion transporter, OAT4, was isolated from a human kidney cDNA library. The OAT4 cDNA consisted of 2210 base pairs that encoded a 550-amino acid residue protein with 12 putative membrane-spanning domains. The amino acid sequence of OAT4 showed 38 to 44% identity to those of other members of the OAT family. Northern blot analysis revealed that OAT4 mRNA is abundantly expressed in the placenta as well as in the kidney. When expressed in Xenopus oocytes, OAT4 mediated the high affinity transport of estrone sulfate (Km = 1.01 μM) and dehydroepiandrosterone sulfate (Km = 0.63 μM) in a sodium-independent manner. OAT4 also mediated the transport of oyclinophilic drugs, diuretics, sulfobromophthalein, penicillin G, and bile salts, whereas tetraethylammonium, an organic cation, did not. OAT4 is the first member of the multispecific organic anion transporter family, which is expressed abundantly in the placenta. OAT4 might be responsible for the elimination and detoxification of harmful anionic substances from the fetus.

Organic anions include a variety of drugs and xenobiotics, many of which are harmful to the body. In addition, most lipophilic compounds of both endogenous and exogenous origin are metabolized to organic anions, e.g., sulfate and glucuronide conjugates. The kidney and the liver play a central role in the elimination of these toxic anionic compounds from the body (1–5). In the kidney, proximal tubular cells take up organic anions from the blood via multispecific organic anion transport pathway(s) in the basolateral membrane (1–3). In 1997, the multispecific organic anion transporter, organic anion transporter 1 (OAT1), was first isolated from rat kidney cDNA library by the expression cloning method (6, 7). Human (8, 9) and mouse (10, 11) homolog have been also cloned and characterized. Rat OAT1 is localized at the basolateral membrane of the middle proximal tubule (S2) (12) and mediates the uptake of a variety of organic anions into the proximal tubular cells (6, 13–15). To date, two other isoforms, i.e., OAT2 (16) and OAT3 (17, 36), have been identified.

The transcellular transport of organic anions has been demonstrated in other tissues where the tissue-specific barrier system exists. In the brain, blood-brain barrier and blood-cerebrospinal fluid barrier protect the brain from the invasion of xenobiotics (18–20). Recently, two isoforms of the oatp (organic anion-transporting polypeptide) family, i.e., oatp1 (21) and oatp2 (22), were shown to be expressed in the brain, and oatp1 was localized to blood-cerebrospinal fluid barrier (23). We also suggested the existence of OAT3 in blood-cerebrospinal fluid barrier (17). P-glycoprotein, a member of the ABC (ATP binding cassette) family of transporters, was shown to be expressed in blood-brain barrier (24, 25). Thus, the transporter molecules, which are likely to act as efflux system of xenobiotics in blood-brain barrier and blood-cerebrospinal fluid barrier, have just begun to be identified.

In the placenta, the presence of a tissue-barrier system has also been predicted. However, little is known about the mechanisms by which the placenta carries out this barrier function. The fetus is very vulnerable to foreign substances that enter the fetal circulation, and numerous drugs have been known to cause developmental defects in the fetus. Moreover, the fetus generates harmful metabolites, such as organic anions. Since the fetal kidney and liver possess limited capacity for the secretion, it is probable that the placenta plays the primary role in the excretion of these toxic compounds from the fetus.

In the present study, we report the isolation of a novel member of the multispecific organic anion transporter family, OAT4. A high level of mRNA expression of OAT4 was detected in the placenta as well as in the kidney.

**EXPERIMENTAL PROCEDURES**

Isolation of OAT4—Est (expressed sequence tag) data base were searched for “query OAT1,” and an EST clone (H12876) was identified. The [3P]dCTP-labeled probe was synthesized from the clone H12876 and used for the screening of a human kidney cDNA library. A non-directional cDNA library was prepared from human kidney poly(A)+ RNA (CLONTECH) using the Superscript Choice system (Life Technologies, 1

The abbreviations used are: OAT, organic anion transporter; oatp, organic anion-transporting polypeptide; PAH, p-aminophenolic acid; DHEA-s, dehydroepiandrosterone sulfate;
Inc.). The screening of the cDNA library by H12876 was performed as described elsewhere (17).

Sequence Determination—Specially synthesized oligonucleotide primers were used for the sequencing of the OAT4 cDNA by the dye-termination method using ABI Prism™ 310.

cRNA Synthesis and Uptake Experiments Using Xenopus laevis Oocytes—cRNA synthesis and uptake measurements were performed as described previously (17). The capped cRNA was synthesized in vitro using T7 RNA polymerase from the plasmid DNA linearized with HindIII. Defolliculated oocytes were injected with 10 ng of the capped OAT4 cRNA and incubated in Barth’s solution (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 0.4 mM CaCl₂, 0.8 mM MgSO₄, 2.4 mM NaHCO₃, and 10 mM HEPES) containing 50 µg/ml gentamicin at 18 °C. After 2 to 3 days of incubation, uptake and efflux experiments were performed at room temperature in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4) as described elsewhere (17). We repeated each experiment more than two times to confirm the results. The representative results are shown in Figs. 3 to 7. The kinetic parameters were obtained by an iterative nonlinear least squares method using a MULTI program (26).

Northern Blot Analysis—A commercially available hybridization blot containing poly(A)⁺ RNA from various human tissues (human 12-lane multiple tissue Northern (MTN)™ blot, CLONTECH) was used for the Northern blot analysis for OAT4. We used an OAT4 cDNA fragment (position numbers 1355–1758) as a probe, whose nucleotide sequence showed 60% identity to the corresponding region of human OAT1 and less than 60% identity to those of rat OAT1, rat OAT2, and rat OAT3. The master blot filter was hybridized with the probe overnight at 42 °C according to the manufacturer’s instructions. The filter was washed finally in a high stringency condition (0.1×SSC (1×SSC = 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 65 °C).

RESULTS

An EST data base search identified an EST clone, H12876, which showed significant identity to OAT1, OAT2, and OAT3. A human kidney cDNA library was screened using H12876 as a probe, and a cDNA encoding a novel membrane protein (OAT4) was isolated. OAT4 cDNA consisted of 2210 base pairs encoding a 550-amino acid residue protein. Fig. 1 shows the deduced amino acid sequence of human OAT4 in the alignment with those of human OAT1, rat OAT2, and rat OAT3. The amino acid sequence of OAT4 showed 44, 43, 38, and 43% identity to those of human OAT1, rat OAT1, rat OAT2, and rat OAT3, respectively. OAT4 also showed significant identity to rat OCT1 (35%) (27), rat OCT2 (33%) (28), and rat OCT3 (36%) (29). Kyte-Doolittle hydropathy plot analysis (30) predicted 12 membrane-spanning domains in OAT4 (hydropathy plot not shown). As is the case in the members of the OAT and OCT families, N-linked glycosylation sites (residues 39, 56, 99, 310, 353) and protein kinase C-dependent phosphorylation sites (residue 65, 164, 224, 225, 279, 319, 326, 428, 529) were predicted in the sequence of OAT4 (Fig. 1).

The expression of OAT4 mRNA in human tissues was investigated (Fig. 2). A strong mRNA band was detected only in the kidney (2.7 kilobases) and placenta (2.4 kilobases). No hybridization signals were detected with mRNAs isolated from other tissues, including the brain, heart, skeletal muscle, thymus,
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spleen, liver, small intestine, lung, and peripheral blood leukocytes. Since the size of mRNA bands detected in the kidney and the placenta were different, we screened a human placenta cDNA library using H12876 as a probe and isolated a positive full-length cDNA clone. The sequencing of this clone revealed that the cDNAs isolated from the kidney (the present OAT4 cDNA clone) and the placenta were identical, except that the placenta clone possessed a shorter untranslatable 5' region.

Using the Xenopus oocytes expression system, we investigated transport of organic anions by OAT4 (Fig. 3). The uptake rates of [3H]estrone sulfate, [3H]dehydroepiandrosterone sulfate (DHEA-s), and [3H]ochratoxin A in oocytes expressing OAT4 were much higher than those of control oocytes. A very low rate of uptake of p-aminohippurate (a prototypical substrate of OAT1) via OAT4 was also demonstrated.

Fig. 4 shows the transport properties of estrone sulfate via OAT4. The cell-associated count of [3H]estrone sulfate increased linearly until 2.5 h of incubation in OAT4-expressing oocytes. This result indicates that OAT4 not only binds but also translocates estrone sulfate into cytoplasm (Fig. 4A). The uptake rate of estrone sulfate via OAT4 was not affected by replacement of the extracellular sodium with lithium or choline (Fig. 4B). In the experiment shown in Fig. 4C, the trans-stimulatory effect of estrone sulfate on OAT4-mediated efflux of estrone sulfate was examined. The efflux of estrone sulfate was not trans-stimulated in the presence of extracellular estrone sulfate (0.2 and 2 μM).

The concentration dependence of OAT4-mediated uptake of [3H]estrone sulfate and [3H]DHEA-s was examined (Fig. 5). OAT4-mediated uptake of these two compounds showed saturable kinetics and followed the Michaelis-Menten equation. Nonlinear regression analyses yielded \( K_m \) values of 1.01 ± 0.15 μM and 0.63 ± 0.04 μM and \( V_{max} \) values of 1.78 ± 0.16 pmol/h/oocyte and 1.26 ± 0.04 pmol/h/oocyte for estrone sulfate and DHEA-s, respectively.

To investigate the substrate selectivity of OAT4, inhibition study was performed. cis-Inhibitory effect of 5, 50, and 500 μM concentrations of various compounds on OAT4-mediated [3H]estrone sulfate (50 nM) uptake was investigated (Fig. 6). Because of its cytotoxic effect, 100 μM sulforaphenolethain was used instead of 500 μM. Five μM of unlabeled estrone sulfate and sulforaphenolethain showed definite inhibitory potency. Probenecid, piroxicam, ibuprofen, diclofenac, furosemide, bumetanide, and corticosterone (a neutral steroid hormone) exhibited modest but dose-dependent inhibitory activity at 5 and 50 μM. Penicillin-G, cholic acid, and taurocholic acid showed much weaker inhibition. In contrast, 500 μM p-aminohippuric acid (PAH), glutaric acid, salicylic acid, and azidothymidine did not show inhibitory activity. \( \beta \)-Estradiol, tetraethylammonium (an organic cation), and Na₂SO₄ (an inorganic sulfate) also did not show any inhibitory effect on OAT4-mediated [3H]estrone sulfate uptake. Since in the result shown in Fig. 3, a very low rate of uptake of PAH via OAT4 was demonstrated, we examined the inhibitory potency of PAH at higher concentrations. The result indicated that high concentrations of PAH (more than several mM) inhibited OAT4-mediated uptake of estrone sulfate (data not shown).

Interaction of OAT4 with several sulfate conjugates and glucuronide conjugates was also examined (Fig. 7). Five μM sulfate conjugates (estrone-s, DHEA-s, p-nitrophenyl-s, α-naphthyl-s, 4-methylumbelliferin-s, and β-estradiol-s) showed inhibitory potency on OAT4-mediated uptake of [3H]estrone sulfate. At 100 μM, all of the sulfate conjugates except minoxidil sulfate potentially inhibited OAT4 (data not shown). In contrast, all of the glucuronide conjugates with similar side chains showed no inhibition at 5 μM. Even at 500 μM, only chloramphenicol glucuronide and α-naphthyl-β-glucuronide showed inhibitory potency (data not shown).

**DISCUSSION**

In the present study, we reported the isolation of OAT4. OAT4 mRNA was abundantly expressed in the placenta as well as in the kidney and mediated the transport of anionic compounds, including sulfate conjugates.

Fetal blood is separated from the maternal blood circulation by the polarized cells, i.e. syncytiotrophoblast, which possess carrier-mediated transport pathways similar to those in the renal proximal tubules and intestinal epithelial cells. Substantial knowledge on the placental transfer of essential compounds from the maternal body to the fetus has been gained, and the expressions of nutrient transporters such as glucose transporters (31), amino acid transporters (32), a vitamin transporter (33), and nucleoside transporters (34) have been identified in the placenta. These carrier proteins facilitate the supply of essential compounds to the developing fetus. In contrast, little is known regarding the elimination pathways for toxic compounds from the fetus. Since the fetal kidney and liver possess a limited capacity for excretion (35), the placenta probably plays the primary role in the elimination of the toxic compounds from the fetus. The present study demonstrated a high level of expression of OAT4 in the placenta and the multiple substrate recognition by OAT4. Rat OAT2 (16) and human OAT3 (36)² are not expressed in the placenta. The expression of human OAT1 in the placenta was extremely weak or none. Thus, OAT4 is predominantly expressed in the placenta among

² S. H. Cha, T. Sekine, Y. Kanai, and H. Endou, unpublished observation.
OAT isoforms and is likely to mediate the excretion of toxic anionic substances from the fetal body. Recently, two candidate excretory pathways have been identified in the placenta. ABCP (37), a member of the ABC superfamily, and OCT3 (29) were demonstrated to be expressed in the placenta. Both of the members of the ABC transporter superfamily and OCT family mediate the elimination of xenobiotics from the body. The entity “placental barrier” may be composed of these xenobiotics transporters, including OAT4.

The placenta is a unique endocrine organ producing peptide hormones and steroid hormones. The placenta synthesizes a large amount of steroid hormones, especially estrogens (38). In the placenta, estrogens (estrone, estradiol, and estriol) are synthesized from DHEA-s or 16α-OH DHEA-s, both of which are derived from the fetal adrenal gland. Thus, the fetus and placenta function together as an endocrine unit for the production of estrogen, which is important for continuation of the pregnancy. DHEA-s, however, shows undesirable effects (e.g. intrauterine growth retardation) on the fetus, at high blood concentrations (39). Efficient uptake of DHEA-s by the placenta, therefore, is required not only for the production of estrogen but also for the protection of fetus from the cytotoxicity of DHEA-s. Among OAT isoforms, OAT3 also mediates the high affinity transport of estrone sulfate (17). However, as stated above, the expression of OAT3 in the human placenta is little or none. It is probable that OAT4 is localized in the trophoblast membrane facing the fetal blood and mediates the placental uptake of DHEA-s, although the localization of OAT4 in the placenta has not yet been identified.

OAT1 shows affinity for compounds possessing negative or partial negative charge(s) and an appropriately sized hydrophobic core (3, 6, 13). The finding that estradiol showed no inhibitory effect on the OAT4-mediated uptake of estrone sulfate whereas estradiol sulfate showed a strong inhibitory effect suggests that the anionic moiety is essential for substrate recognition by OAT4. p-Nitrophenyl sulfate and α-naphthyl sulfate were shown to interact with OAT4. Thus, the binding...
The site of OAT4 can accept variable sizes of the hydrophobic side chain (from the phenyl moiety to the steroid core) of the sulfate conjugates. In contrast, glucuronide conjugates with similar hydrophobic side chains showed no or little inhibitory effect on OAT4-mediated transport. The glucuronide moiety might be too large to be accepted by OAT4. Thus, the interaction of OAT4 with the hydrophilic part (negatively charged site) of the substrate seems rather stringent. The molecular mechanism underlying the multispecific substrate recognition by OAT4 is considered to depend largely on the nonspecific hydrophobic interaction between the heterogeneous and variably sized hydrophobic structures of the substrates and OAT4.

Site-directed mutagenesis has shown that specific residues are required for the recognition of organic anions by OAT4 (44). The transport mechanism of OAT4 (in other words, the driving force of OAT4) is an important issue that should be clarified. OAT1 is a tertiary active organic anion/dicarboxylate exchanger in physiological condition, and OAT1-mediated transport of organic anions is indirectly coupled to the Na⁺ ion in the extracellular medium (2, 43). The energetically uphill transport of organic anions via OAT1 is accelerated by outwardly directed dicarboxylate gradient (6, 7), which is maintained by sodium dicarboxylate cotransporter (2, 43). Transport activity via OAT4 did not directly depend on the extracellular sodium ion. Trans-stimulatory effect was not demonstrated in the present study. Thus, the driving force of OAT4-mediated transport still remains to be elucidated. Likewise, the driving forces of OAT2 and OAT3 also have not been clarified yet. Recently, a distinct organic anion transporter, oatp1, was revealed to be an organic anion/glutathione exchanger (44). Unspecified endogenous substances might be the counterion in the transport of OAT4. Further studies are required to address this issue.

In conclusion, we report the molecular cloning of OAT4. OAT4 is exclusively expressed in the placenta and the kidney. OAT4 mediates the high affinity transport of estrone sulfate and DHEA-s and shows affinity for a variety of anionic substances. OAT4 is likely to mediate the placental uptake of DHEA-s, a precursor of estrogen. Furthermore, OAT4 may play
the major role in the excretion of toxic anionic substances from the fetal body.

REFERENCES

1. Moller, J. V., and Sheikh, M. I. (1983) Pharmacol. Rev. 34, 315–358
2. Pritchard, J. B., and Miller, D. S. (1993) Physiol. Rev. 73, 765–796
3. Ulrich, K. J. (1997) J. Membr. Biol. 158, 95–107
4. Ulrich, K. J., and Rumrich, G. (1993) Clin. Investig. 71, 843–848
5. Petzinger, E. (1994) Rev. Physiol. Biochem. Pharmacol. 123, 47–211
6. Sekine, T., Watanabe, N., Hosoyamada, M., Kanai, Y., and Endou, H. (1997) J. Biol. Chem. 272, 18526–18529
7. Sweet, D. H., Wolff, N. A., and Pritchard, J. B. (1997) J. Biol. Chem. 272, 30988–30995
8. Hosoyamada, M., Sekine, T., Kanai, Y., and Endou, H. (1999) Am. J. Physiol. 276, F122–F129
9. Kuze, K., Graves, P., Wilson, E. J. G., Beier, D. R., and You, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 695–698
10. Tojo, A., Sekine, T., Nakajima, N., Hosoyamada, M., Kanai, Y., and Endou, H. (1999) J. Neurosci. 19, 1018–1024
11. Tsuda, M., Sekine, T., Takeda, M., Cha, S. H., Kanai, Y., and Endou, H. (1999) J. Pharmacol. Exp. Ther. 290, 179–182
12. Ullrich, K. J. (1997) J. Clin. Endocrinol. Metab. 83, 761–766
13. Ullrich, K. J., and Rumrich, G. (1993) Clin. Investig. 71, 843–848
14. Griffiths, M., Yao, S. Y. M., Abidi, F., Phillips, S. E. V., Cass, C. E., Young, J. D., and Baldwin, S. A. (1997) Biochem. J. 328, 739–743
15. Morgan, D. J. (1997) Clin. Exp. Pharmacol. Physiol. 24, 869–873
16. Race, J. E., Grassl, S. M., Williams, W. J., and Holtzman, E. J. (1999) Biochem. Biophys. Res. Commun. 255, 508–514
17. Allikmets, R., Shiram, L. M., Hutchinson, A., Romano-Spica, V., and Dean, M. (1998) Cancer Res. 58, 5337–5339
18. Branchaud, C. L., Goodyer, C. G., and Lipowski, L. S. (1983) J. Clin. Endocrinol. Metab. 56, 761–766
19. Schuetz, J. D., Kauma, S., and Guzelian, P. S. (1993) J. Clin. Invest. 92, 1018–1024
20. Simonson, G. D., Vincent, A. C., Seberg, K. J., Huang, Y., and Iwanij, V. (1994) J. Cell Sci. 107, 1065–1072
21. Nee, B., Hagenbuch, B., Stieger, B., and Meier, P. J. (1997) FEBS Lett. 425, 79–86
22. Shimada, H., Inui, K., Gokor LT., and Iwanij, V. (1997) J. Cell Sci. 109, 13675–13680
23. Schomig, E., Spitzenberger, F., Engelhardt, M., Martel, P., Ording, N., and Grundemann, D. (1998) FEBS Lett. 425, 79–86
24. Branchaud, C. L., Goodyer, C. G., and Lipowski, L. S. (1983) J. Clin. Endocrinol. Metab. 56, 761–766
25. Cordon-Cardo, C., O'Brien, J. P., Casals, D., Rittman-Grauer, L., Biedler, J. L., Melamed, M. R., and Bertino, J. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 695–698
26. Leibach, F. H., Fujita, T., Fei, Y.-J., and Endou, H. (1999) Biochem. Biophys. Res. Commun. 224, 500–507
27. Yamaoka, K., Tanigawara, T., Nakagawa, T., and Noe, B. (1997) J. Clin. Endocrinol. Metab. 83, 786–790