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Characterization of an Organic Anion-Transporting Polypeptide (OATP-B) in Human Placenta

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Organic anion-transporting polypeptides (OATPs) are a family of multispecific carriers that mediate the sodium-independent transport of steroid hormone and conjugates, drugs, and numerous anionic endogenous substrates. We investigated whether members of the OATP gene family could mediate fetal-maternal transfer of anionic steroid conjugates in the human placenta. OATP-B (gene symbol *SLC21A9*) was isolated from a placenta cDNA library. An antiserum to OATP-B detected an 85-kDa protein in basal but not apical syncytiotrophoblast membranes. Immunohistochemistry of first-, second-, and third-trimester placenta showed staining in the cytotrophoblast membranes and at the basal surface of the syncytiotrophoblast. Trophoblasts that reacted with an antibody to Ki-67, a proliferation-associated antigen, expressed lower levels of OATP-B. OATP-B mRNA levels were measured in isolated trophoblasts under culture conditions that promoted syncytia formation. Real-time quantitative PCR estimated an 8-fold increase in OATP-B expression on differentiation to syncytia. The uptake of [^{3}H]estrone-3-sulfate, a substrate for OATP-B, was measured in basal syncytiotrophoblast membrane vesicles. Transport was saturable and partially inhibited by pregnenolone sulfate, a progesterone precursor. Pregnenolone sulfate also partially inhibited OATP-B-mediated transport of estrone-3-sulfate in an oocyte expression system. These findings suggest a physiological role for OATP-B in the placental uptake of fetal-derived sulfated steroids. (*J Clin Endocrinol Metab* 87: 1856–1863, 2002)

THE PLACENTA IS a major source of the essential steroid hormones that sustain pregnancy. After the first trimester, the placenta becomes the sole biosynthetic source of progesterone (1). As in other steroidogenic tissues, oxidation of cholesterol by cytochrome P450 side chain cleavage (P450scc) generates pregnenolone, which in turn undergoes reduction by 3β-hydroxysteroid dehydrogenase type I in trophoblasts to progesterone (2, 3). Additional sulfated conjugates of pregnenolone can be delivered via both maternal and fetal circulations, thus augmenting the supply of progesterone precursor. After 8 wk gestation, the placenta also serves as the major site of estrogen production, although this does not occur by *de novo* biosynthesis. It relies on the delivery of precursors primarily from the fetal compartment, with some contribution from maternal sources, and on biosynthetic pathways abundant in the trophoblasts (1). The C_{19} steroid, dehydroepiandrosterone (DHEA) and its sulfated conjugate (DHEAS), are synthesized in the fetal adrenal cortex from pregnenolone and 17-OH pregnenolone, reactions mediated by P450c17. DHEAS is in large part converted to 16-hydroxy-DHEAS exclusively in the fetal liver, and these steroid precursor can reach fetal arterial concentrations of 4 μM and 10 μM, respectively (4). In the placenta, DHEAS and 16 hydroxy-DHEAS undergo deconjugation by steroid sulfatase, which is abundant in the syncytial trophoblast (5). Estrone and E2 arise from DHEA, whereas 16-hydroxy-DHEA is converted by P450 aromatase to estriol, the main estrogen excreted in maternal urine (1).

Abbreviations: DHEA, Dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; HCGβ, human CG-β; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide.

Given that the sulfate conjugates of DHEA, 16-hydroxy-DHEA, and pregnenolone are organic anions, their transfer from the fetal and/or maternal circulation across the apical and basal membrane surface of the syncytiotrophoblast to the intracellular milieu for further metabolism is necessarily carrier mediated. A full understanding of the pathways of steroidogenesis within the maternofetal-placental unit requires that such carrier proteins be identified and characterized. The *o*rganic anion transporting polypeptides (OATPs) are members of a multigene superfamily that mediate the Na^+-independent uptake of a host of organic anionic compounds. OATP-A *(SLC21A3)*, which was isolated from human liver, although it is predominantly expressed in the brain (6), is a multispecific transporter whose substrates include estrone-3-sulfate, prostaglandin E2, and anionic cyclic peptides as well as DHEAS (7, 8). OATP-C *(SLC21A6)* is expressed at the basolateral membrane of hepatocytes (9–11) and also transports DHEAS as well as bile salts, eicosanoids, thyroid hormones, and peptides (8, 11). A third member, OATP-B *(SLC21A9)*, has a broader tissue distribution than either OATP-A or OATP-C, as shown by positive signals in heart, brain, lung, liver, kidney, spleen, ovary, small intestine, and placenta on Northern blots (8). To date, however, the elucidation of its transport characteristics has been limited, with only bromosulphothalein and estrone-3-sulfate having been clearly identified as substrates (8, 12). Transport activity toward DHEAS was marginal.

The present work shows that OATP-B, but not the DHEAS transporters OATP-A and OATP-C, is expressed at high levels throughout gestation and represents an uptake mechanism for anionic steroid precursors in human placenta. We demonstrated that OATP-B mRNA is present in freshly iso-

lated cytotrophoblasts and increases during the course of syncytium formation *in vitro*, a cell culture model in which isolated mononuclear cytotrophoblasts aggregate and fuse. This affords a model system with which to further study the mechanisms and regulation of placental organic anion uptake.

Materials and Methods

Materials

Radiolabeled [^{3}H]estrone-3-sulfate (43 Ci/mmol) and [^{3}H]didydroalprenolol (120 Ci/mm) were purchased from NEN Life Science Products (Boston, MA). Unlabeled estrone-3-sulfate and oxprenolol were purchased from Fluka Chemical Co. (Buchs, Switzerland) and pregnenolone sulfate was purchased from Steraloids Inc. (Wilton, NH).

Isolation and expression of OATP-B cDNA

To isolate the full-length cDNA from the placenta, a human placenta rapid-screen cDNA library panel (OriGene Technologies, Rockville, MD) was screened by PCR using specific primers: 5′-CATGGGACCCAGGATAGGGCCAGCG-3′ (forward) and 5′-GGCCTGGCCCCATCATGGTCACTG-3′ (reverse). Several cDNA clones were isolated and the longest, 4.4 kb, was sequenced using cycle sequencing (ALF Express, Amersham Pharmacia Biotech, Dübendorf, Switzerland). The pCMV6-XL4 plasmid-containing OATP-B cDNA was linearized with *Sma*I, and capped cRNA was synthesized using the mMESSAGE mMACHINE T7 kit (Ambion, Inc., Austin, TX). *Xenopus laevis* oocytes were microinjected with 5 ng OATP-B cRNA or 50 nl water as described (13), and uptake studies were performed after 3 d. Accumulation of estrone-3-sulfate was measured at 25 C for 30 min in a medium containing 100 mmol/liter choline chloride, 2 mmol/liter KCl, 1 mmol/liter $CaCl_2$, 1 mmol/liter $MgCl_2$, and 10 mmol/liter HEPES (pH 7.4).

Western blotting and transport assay in membrane preparations

Basal and apical syncytiotrophoblast membranes were isolated from healthy placentae at term, before the onset of labor and within 30 min of scheduled cesarian deliveries, as described (14, 15). The enrichment of the basal membrane preparation was assessed by measuring binding of the β-adrenergic antagonist dihydroalprenolol as described (15, 16). Contamination of the basal vesicles by apical membranes was estimated by assaying for alkaline phosphatase activity (17). Proteins were separated on a 7.5% SDS polyacrylamide gel, transferred to nitrocellulose, and blocked in 5% milk and 0.4% BSA diluted in Tris-buffered saline containing 0.1% Tween 20. The rabbit polyclonal antiserum raised against the 15 C-terminal residues has been described (8). Blots were incubated for 1 h with anti-OATP-B (1:200) and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000). Signals were detected by chemiluminescence (ECL-Plus, Amersham Pharmacia Biotech). To study the uptake of estrone-3-sulfate, rapid filtration studies were performed at 37 C and 4 C in 80 μl medium containing 10 mmol/liter HEPES-Tris (pH 7.2), 250 mmol/liter sucrose, 100 mmol/liter KNO_3, 10 mmol/liter $MgCl_2$, and 0.2 mmol/liter $CaCl_2$, mixed with 20 μl (100 μg) basal membrane vesicles, as described (15). The ice-cold stop solution [250 mmol/liter KCl, 25 mmol/liter $MgSO_4$, 10 mmol/liter HEPES-Tris (pH 7.4)] contained 0.5 mmol/liter unlabeled estrone-3-sulfate.

Microscopy

Paraffin-embedded sections from first-, second-, and third-trimester placentae were processed for immunohistochemistry. Sections were deparaffinized in xylol, rehydrated through a graded series of ethanol, and pretreated in 1 mmol/liter EDTA (pH 8) for 3 min in a pressure cooker. The polyclonal anti-OATP-B antisera was diluted 1:200 and incubated for 1 h. In single-labeling studies, the signal was detected with a biotinylated secondary donkey antirabbit antibody (DAKO Corp., Glostrup, Denmark) and the automated staining system (Ventana, Tucson, AZ) with diaminobenzidine (*brown*) as the chromogen. The MIB-1 monoclonal antibody to the Ki-67 proliferation marker was also used in some sections to assess cell proliferation. In these double-labeled sections, MIB-1 was detected with the automated staining system (Ventana) and diaminobenzidine as the chromogen, whereas OATP-B was detected using an alkaline phosphatase-antialkaline phosphatase complex and fast red as the chromogen. Areas of red OATP-B staining at the basal syncytiotrophoblast were classified as high expression [staining detectable at low-power magnification (×40)] or low expression [staining detectable only at high-power magnification (×250)]. MIB-1-positive nuclei were counted in 10 areas of high and low OATP-B staining. The correlation between the intensity of OATP-B staining and the proliferative index was analyzed nonparametrically using the Mann-Whitney test (Stat View Program, version 5.0.1, SAS Institute, Inc., Cary, NC), with P less than 0.05 regarded as significant. Controls were performed with nonimmune rabbit serum and by preadsorption of the antiserum with the antigenic peptide.

Isolation of cytotrophoblasts

Mononuclear cytotrophoblasts were purified from term placentae as described (18, 19). The purity of the cell preparation was routinely assessed to be 90%, as determined by immunological staining for vimentin and cytokeratin as described (19). Cytotrophoblasts were plated at a density of 1.25×10^5 cells/cm^2 in medium 199 supplemented with 20% FCS. Cells were harvested for RNA isolation after initial plating and after 24, 48, and 96 h. Transport studies were performed as described previously (20) with isolated cytotrophoblasts resuspended in buffer containing 1 μmol/liter [^{3}H]estrone-3-sulfate. Uptake reactions were stopped at 0.25, 0.5, 0.75, 1, 1.5, 2, 5, and 10 min by centrifugation through a mixture of silicon oil; radioactivity in the cell pellet was measured by liquid scintillation counting (20).

Real-time quantitative PCR

Total RNA was reverse transcribed (Superscript II, Life Technologies, Inc., Gaithersburg, MD). As a negative control, template RNA was processed without reverse transcription. PCR was performed on a Taqman ABI PRISM 7700-sequence detector (PE Applied Biosystems, Langen, Germany). The 18S rRNA-labeled VIC dye was used as the internal control. For OATP-B, the forward primer 5′-AGGACGTGCGGCCAAGT-3′ and reverse primer 5′-TCTTTAGGTAGCCGGAGATCATG-3′ were used with a 6-carboxyfluorescein [FAM]/6-carboxy-tetramethylrhodamine-labeled probe, 5′-CATCAAGCTGTTCGTTCTGTGCCACA-3′. For human CG-β (HCGβ), the forward (5′-CAAGGATGGAGATGTTCCAG-3′) and reverse primers (5′-CACGCGGGTCATGGT-3′) as well as the FAM-labeled probe (5′-CTGCTGAGCATGGGCGGGACAT-3′) have been described (21).

Results

A full-length (4.4 kb) OATP-B clone was isolated from a human placenta cDNA library. Sequencing showed that the clone included 258 bases upstream from the ATG translation site and was identical to the cDNA cloned from the human brain (accession no. NM_007256) except for a single nucleotide change (G to A) leading to an Arg to Gln substitution at residue 312. The single amino acid (Ser to Phe) substitution at position 486 previously identified for a cDNA clone isolated from a brain library was not present (8).

The localization and temporal expression of OATP-B was examined on placenta sections representing term (Fig. 1, a through c and e), the second trimester (Fig. 1d), and first trimester (Fig. 1, f and g). At term, OATP-B was abundantly expressed at the basal surface of the syncytiotrophoblast in terminal as well as intermediate villi (Fig. 1a). The specificity of the antibody binding was verified by repeating the staining after preadsorption of the antiserum with 1 μmol/liter antigenic peptide (Fig. 1b). Figure 1c shows background staining in the presence of normal rabbit serum. The basal

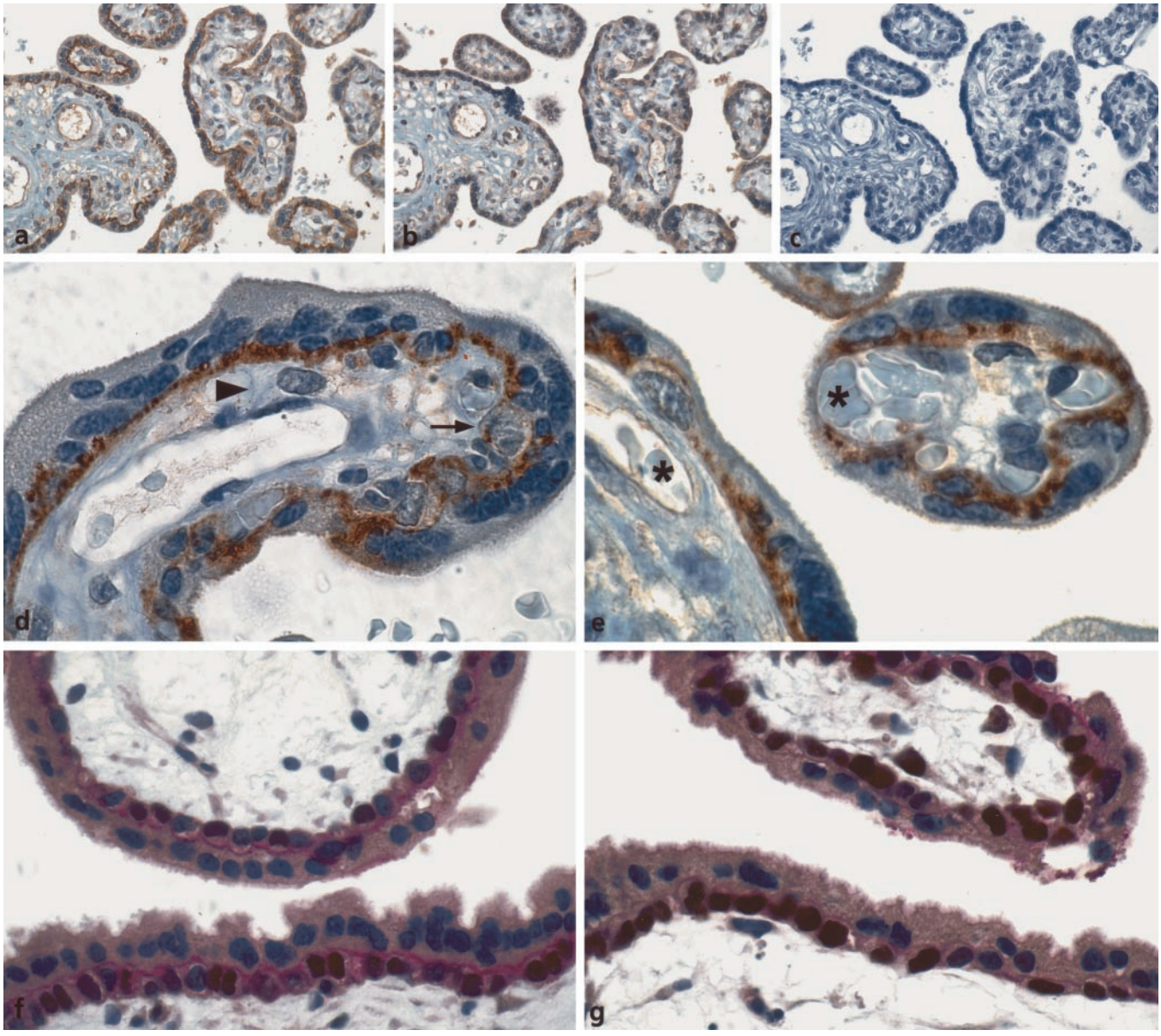

FIG. 1. Immunohistochemical detection of OATP-B in first, second, and third trimester. a–c, Third-trimester placenta. a, Staining for OATP-B was strong at the basal surface of the syncytiotrophoblast. b, Staining decreased to background levels after adsorption of the antibody with the antigenic peptide 1 μM. c, Control section incubated with normal rabbit serum (magnification, ×300). d, Second-trimester placenta, longitudinal section of a terminal villus. The basal surface of the syncytiotrophoblast is strongly reactive. Cytotrophoblasts (*arrow*), which have not yet fused with the syncytia, reveal faint OATP-B staining at their basal surface (magnification, ×1200). e, A terminal villus from the third trimester shows formation of the typical synciocapillary membrane. The OATP-B staining is weaker at the thin membrane stretches in which fetal vessels (*asterisk*) appose the maternal intervillous blood space (magnification, ×1200). f–g, The first-trimester placenta is double labeled with MIB-1, which recognizes the Ki-67 cell proliferation marker (*brown stain*) and OATP-B 1:200 (*red stain*). f, Nonproliferating cytotrophoblast layer shows little nuclear staining but extensive staining for OATP-B at the basal surface of the differentiating syncytiotrophoblast layer. g, Areas with relatively higher proliferation index in the cytotrophoblast cell layer show reduced OATP-B expression at the basal surface of the syncytiotrophoblast layer (magnification, ×600).

surface of the syncytiotrophoblast from second-trimester placentae also stains intensely for OATP-B, whereas staining in the cytotrophoblasts is weaker (Fig. 1d). An uneven subcellular expression of OATP-B within the syncytiotrophoblast was suggested by the obvious staining differences in the morphologically distinct areas of cytoplasm-rich syncytiotrophoblast, compared with the thin stretches of syncytiocapillary membranes (Fig. 1e). The expression appeared weaker at the thin membrane stretches at which fetal vessels appose the maternal intervillous blood space than at the thicker syncytiotrophoblast surface. OATP-B was also abundantly expressed in first-trimester placenta (Fig. 1, f and g). Reactivity was registered with respect to different areas of proliferation, as measured by nuclear staining with a monoclonal antibody to Ki-67 (MIB-1), a nuclear cell proliferation-associated antigen (22, 23). In double-labeled sections, Ki-67

expression could distinguish cytotrophoblasts with a lower (about 50%) proliferation index (Fig. 1f) from those with a higher (approximately 100%) proliferation index (Fig. 1g). Statistical analysis showed an association between areas of lower proliferation and strong OATP-B reactivity (Fig. 1f) and between areas of higher proliferation and weaker OATP-B staining (Fig. 1g). Regions of high OATP-B staining contained 38% (median) MIB-1-positive nuclei (range 7.7 to 64). Regions of low OATP-B staining contained 82% (median) proliferating nuclei (range 42 to 96) (Fig. 2). This difference was significant ($P = 0.0007$).

Basal and apical syncytiotrophoblast membrane vesicles were prepared for immunoblotting with the anti-OATP-B antibody. Measurements of alkaline phosphatase activity showed little cross-contamination of apical membranes in the basal preparation (Table 1). The basal membranes were enriched 16-fold over placental homogenate as determined by dihydroalprenolol-binding assays and contained some contamination (2.4-fold enrichment) with the microsomal enzyme, NADPH cytochrome C reductase (Table 1). On Western blots, a positive signal at approximately 80–85 kDa was evident on membranes isolated from the basal syncytiotrophoblast and not from the apical membranes (Fig. 3A). This signal was displaced after preadsorption of the antibody with 1 μmol/liter of antigenic peptide (Fig. 3B).

To establish a functional correlation between the abundant basal syncytiotrophoblast expression of OATP-B and transport activity, uptake studies were performed with [^{3}H]-estrone-3-sulfate, identified as a substrate in OATP-B cRNA-injected oocytes (8). Accumulation into vesicles isolated from the basal surface was rapid, and the difference between 37 C and 4 C was no longer linear after 20 sec (Fig. 4A). Slow uptake of substrate over time occurred at 4 C and was maximal at 30 min. This presumably corresponded to noncarrier-mediated diffusion or binding of estrone-3-sulfate to the membranes. Figure 4B describes the saturation kinetics of estrone-3-sulfate in basal membranes. Uptake was measured at 20 sec at 37 C and 4 C with varying concentrations of estrone-3-sulfate (1–60 μmol/liter). The temperature-dependent uptake was saturable with an estimated Km of 35 μmol/liter and maximum velocity of 12.3 nmol/min per milligram (Fig. 4B). To test for another relevant steroid substrate for OATP-B in the placenta, competition experiments were performed with pregnenolone sulfate. The temperature-dependent uptake of estrone-3-sulfate (1 μmol/liter) was measured at 20 sec, in the absence and presence of 5, 25, and 100 μmol/liter pregnenolone sulfate (Table 2). Pregnenolone sulfate partially inhibited uptake of estrone-3-sulfate in a dose-dependent manner, from 46% at 5 μmol/liter to 83% at 100 μmol/liter.

The inhibition of OATP-B-mediated transport of [^{3}H]-estrone-3-sulfate by pregnenolone sulfate was also tested in oocytes microinjected with the OATP-B cRNA (Fig. 5). Oocytes microinjected with water served as the control. In the absence of pregnenolone sulfate, there was an accumulation

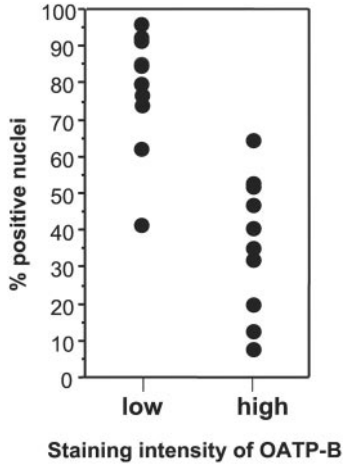

FIG. 2. Correlation between OATP-B staining and proliferative index of trophoblasts in first-trimester placenta. Areas of OATP-B staining (*red chromogen* in Fig. 1, f and g) were classified as high expression or low expression (n = 3 placentae). Nuclei staining positive for the Ki-67 proliferation marker (*brown chromogen*) were counted in 10 areas of high and low OATP-B staining. OATP-B staining and the proliferative index were correlated using the Mann-Whitney test (Stat View Program, SAS Institute, Inc.). Regions of high OATP-B staining contained 38% Ki-67-positive nuclei, whereas areas of low OATP-B staining contained 82% proliferating nuclei. This difference was significant ($P = 0.0007$).

TABLE 1. Characterization of isolated basal syncytiotrophoblast membranes

	Homogenate	Basal	Enrichment
Alkaline phosphatase (U/min·mg protein)	4.3 ± 3.6	3.6 ± 2.5	0.8 fold
Dihydroalprenolol binding (pmol/mg protein)	0.21 ± 0.2	3.5 ± 2.6	16.6 fold
NADPH cytochrome C reductase (mU/min·mg)	0.45 ± 0.16	1.11 ± 0.6	2.4 fold

Values are mean ± SD of triplicate determinations in three placental preparations.

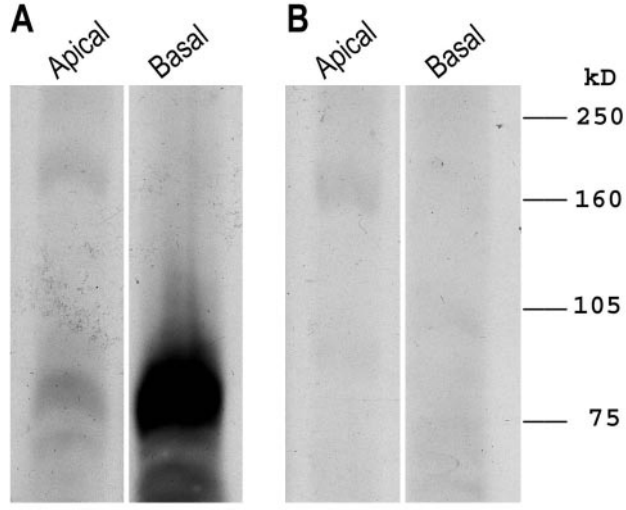

FIG. 3. Western blot of OATP-B in isolated syncytiotrophoblast membranes. Proteins (75 μg) were resolved on a 7.5% SDS PAGE gel, transferred onto a nitrocellulose membrane, and incubated with a polyclonal anti-OATP-B antibody at 1:200. A, A positive signal at 80–85 kDa was present only in basal syncytiotrophoblast membranes isolated from term placentae. B, The antibody was preadsorbed with the antigenic peptide (1 μmol/liter).

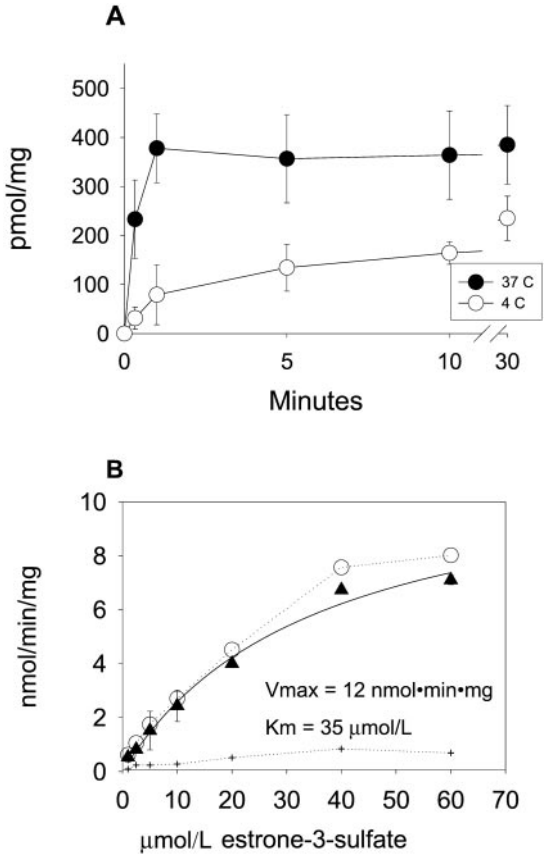

FIG. 4. Uptake of [^{3}H]-estrone-3-sulfate into basal syncytiotrophoblast vesicles isolated from term placentae. A, Time course of accumulation of 1 μM estrone-3-sulfate into vesicles (100 μg) shows a rapid uptake and temperature dependency. The initial rate, calculated as the difference between uptake at 37 C and 4 C, was no longer linear after 20 sec. Data are mean ± SD of triplicate measurements in three placental preparations. B, The saturability of the temperature-dependent component of estrone-3-sulfate transport was studied at 20 sec (○, total uptake at 37 C; +, uptake at 4 C; ▲, difference (37 C minus 4 C). The *solid line* represents the line of best fit, obtained by nonlinear regression analysis of the Michaelis-Menten model to the data.

TABLE 2. Effect of pregnenolone sulfate (Preg SO_4) on uptake of 1 μmol/liter estrone-3-sulfate in basal syncytiotrophoblast membranes

Preg SO_4 (μmol/liter)	Uptake of E-3-S (pmol/min)	% inhibition
0	3.6 ± 0.3[a]	0
5	2.1 ± 0.9	46
25	1.8 ± 0.2	50
100	0.6 ± 0.1	83

Basal syncytiotrophoblast vesicles (100 μg) were incubated with estrone-3-sulfate in the absence and presence of Preg SO_4 at the concentrations indicated. Uptake was measured at 20 sec and was calculated as the difference between the values at 37 C and 4 C.

[a] Mean ± SD of triplicate determinations in two preparations.

of 331 ± 190 fmol/oocyte of estrone-3-sulfate. This uptake was decreased by approximately 80% with the addition of pregnenolone sulfate at 5, 25, and 100 μmol/liter.

To establish an *in vitro* model for further characterization of placental OATP-B within an evolutive milieu that mimics gestation, the mRNA expression was measured in cytotrophoblasts in culture and during their differentiation to syncytiotrophoblast. Freshly isolated cytotrophoblasts expressed OATP-B, as determined by RT-PCR and real-time quantitative PCR. Under the culture conditions described, syncytial formation was maximal at 4 d. This was associated with a greater than 2000-fold increase in the expression of hCGβ mRNA, compared with levels measured after 24 h in culture (Fig. 6). The OATP-B mRNA followed a similar temporal profile and increased 8-fold after 4 d in culture, compared with the values at d 1. Thereafter at 6 d, the expression of both hCGβ and OATP-B declined, which was coincident with the decline in survival of trophoblasts in primary culture. The isolated cytotrophoblasts were also tested functionally for the uptake of estrone-3-sulfate. The linear uptake rate for 1 μmol/liter estrone-3-sulfate, calculated from initial

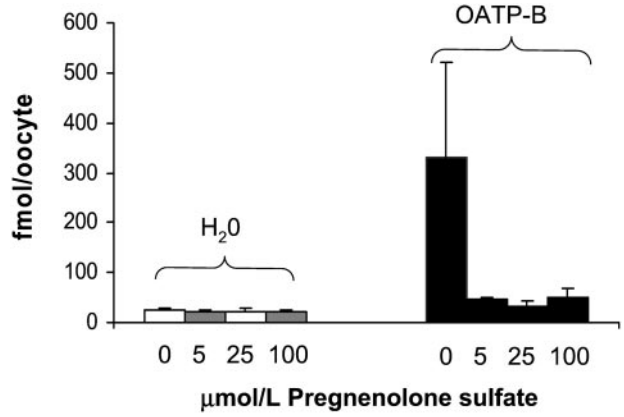

FIG. 5. Uptake of [^{3}H]-estrone-3-sulfate in OATP-B cRNA-injected oocytes. Oocytes were injected with 5 ng cRNA (*black bars*) or water (*gray bars*) and maintained in culture for 3 d. Uptake of estrone-3-sulfate (1 μmol/liter) was measured at 25 C after 30 min in the absence and presence of 5 μmol/liter and 25 μmol/liter pregnenolone sulfate. Data are the mean ± SD of 7–10 oocytes.

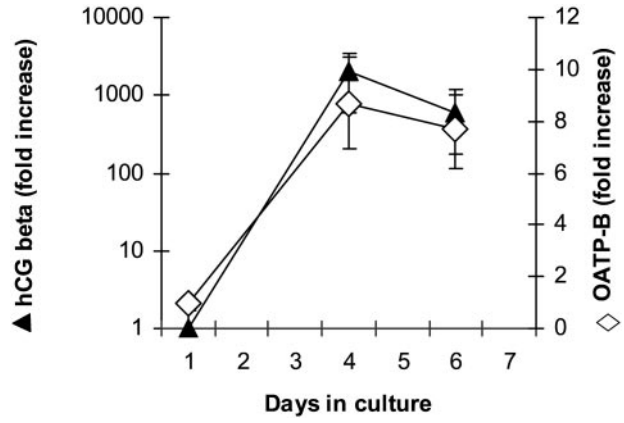

FIG. 6. Real-time quantitative PCR of hCGβ and OATP-B in cytotrophoblasts in culture. Total RNA (20 ng) isolated from trophoblasts after 1, 4, and 6 d in culture was reverse transcribed and amplified by real-time PCR using primers specific for the differentiation marker, hCGβ (▲), and OATP-B (◇). The data were calculated by subtracting the signal threshold cycles (C_T) of the internal standard (ribosomal 18S) from the C_T of OATP-B or hCGβ. Data were normalized for the differences obtained at d 1 and are mean ± SD of triplicate measurements in three placental preparations.

time points (0.25 to 1 min), was 17.7 ± 4.3 pmol/min per milligram protein (mean ± SD from three experiments) (Fig. 7).

Discussion

The syncytiotrophoblast forms both a barrier and conduit between the maternal and fetal circulations. Carriers at the apical surface include the glucose transporter (24), the Na^+-dependent multivitamin transporter (25), amino acid transporters (26), and transporters for catecholamine (27) and carnitine (28) that mediate the essential uptake of nutrients and metabolites from the maternal blood. ATP-dependent pumps such as P-glycoprotein (29) and multidrug-resistant protein 2 (15), which are also strategically located apically at the maternal-fetal interface, may prevent solutes present in the maternal blood from reaching the fetal circulation. The distribution of transporters on the basal surface of the syncytiotrophoblast appears to be less well studied. Given the importance of delivery of sulfated steroid precursors from the fetal blood to the biosynthetic machinery of the syncytiotrophoblast for the normal progression of pregnancy, such an identification and characterization is imperative.

We showed by Western blotting and immunohistochemistry that OATP-B is expressed at the basal surface of the syncytiotrophoblast throughout gestation and is also localized in cytotrophoblasts before differentiation to the syncytiotrophoblast. The precise physiological role of placental OATP-B has yet to be determined, but the abundance and pattern of its expression would suggest a natural substrate that is retrieved from the fetal circulation into the trophoblast cells. A rather restricted substrate profile has been assigned to OATP-B, compared with other members of the multispecific OATP family (8, 30). Estrone-3-sulfate and the model organic anion, bromosulphothalein, are relatively high-affinity substrates (Km = 6 and 1 μmol/liter, respectively). Accordingly, the transport of estrone-3-sulfate was tested in basal syncytiotrophoblast membrane vesicles. Saturable, temperature-dependent estrone-3-sulfate uptake was observed (Fig. 4), albeit with a lower-than-expected affinity, which likely reflects the presence of multiple uptake mechanisms. Uptake of estrone-3-sulfate was also demonstrated in isolated cytotrophoblasts, in which the initial uptake rate of 17.7 ± 4.3 pmol/min per milligram protein for 1 μmol/liter substrate was similar to the value of 19.9 ± 7 pmol/min per milligram protein previously reported for DHEAS studied at the same concentration (20).

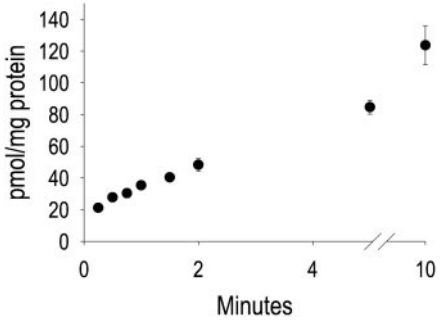

FIG. 7. Uptake of [^{3}H]-estrone-3-sulfate in isolated cytotrophoblasts in suspension. Time course of accumulation of 1 μmol/liter estrone-3-sulfate into cytotrophoblasts at 37 C isolated from term placenta. Reactions were stopped by centrifugation through silicon oil. Radioactivity in the cell pellet was measured by liquid scintillation counting. The initial uptake rate (17.7 ± 4.3 pmol/min per milligram protein, n = 3 placental preparations) was linear until 1 min. Data shown are mean ± SD of one representative experiment, measured in quadruplicate.

A second multispecific organic anion transporter (OAT-4) that transports estrone-3-sulfate has previously been reported in placenta but has not yet been localized (31). The OAT carriers (gene symbol *SLC22*) are encoded by a gene superfamily distinct from that of the OATPs (gene symbol *SLC21*) and are not homologous. Unlike OATP-B, OAT-4 does transport DHEAS with high affinity. Additional comparisons are therefore required to determine the relative contribution of each of these carriers to the uptake of steroid sulfates demonstrated in isolated membrane vesicles and in trophoblasts. The fetal liver can biosynthesize estrone-3-sulfate from androstenedione (32), and circulating levels of the conjugate are in the order of 100 nM (33). Moreover, 17β-hydroxysteroid dehydrogenase (type 2), which oxidizes E2 to estrone, is expressed in fetal capillary endothelial cells of the placenta and is thought to regulate and limit exposure of the fetus to E2 (34). The close proximity of the OATP-B carrier that mediates uptake of estrone-3-sulfate into the syncytiotrophoblast suggests that OATP-B and 17β-hydroxysteroid dehydrogenase may act in tandem to control the local levels of E2 at the placental-fetal interface.

Because the delivery of anionic steroid precursors is a fundamental requirement throughout gestation, additional physiological ligands for OATP-B are very likely. There is a larger circulating reservoir of 16-hydroxy-DHEA sulfate (approximately 10 μM) (4) and pregnenolone sulfate in the fetal compartment than of estrone-3-sulfate. The partial inhibition of estrone-3-sulfate transport by pregnenolone sulfate in both basal syncytiotrophoblast vesicles and in OATP-B cRNA-injected oocytes suggests that pregnenolone sulfate interacts with OATP-B *in vivo* and may be a substrate. Interestingly, the uptake of estrone-3-sulfate into placenta vesicles was inhibited by only 50% with a 5- to 25-fold excess of pregnenolone sulfate, whereas the same concentrations inhibited OATP-B mediated transport in oocytes by 80% (Table 2 and Fig. 5). A plausible explanation is the presence in the placenta of at least one other uptake mechanism, such as OAT-4 (31), which may be only weakly inhibited by pregnenolone sulfate. Additional experiments with radiolabeled pregnenolone sulfate are required to explain these discrepancies.

The expression levels of OATP-B mRNA in cultured trophoblasts show a positive correlation with differentiation that is from isolated cytotrophoblasts to the more differentiated multinucleated syncytiotrophoblast (Fig. 6). The differentiation into syncytiotrophoblast can be monitored functionally by the increase in production of hCGβ, a hormone abundantly synthesized and released from the syncytiotrophoblast, and which in turn stimulates trophoblastic differentiation and placental steroidogenesis (35). The immunohistochemical evidence for a lower expression of OATP-B in the proliferating cytotrophoblasts of the first-trimester placenta than in the syncytiotrophoblast (Figs. 1 and 2) is consistent with the changes in mRNA expression observed in

differentiating trophoblasts in culture. Similar changes in the levels of organic anion transporters have been reported in the regenerating rat liver, in which a selective down-regulation in hepatocytes of the organic anion transporters, Oatp-1 and Oatp-2, was evident at both protein and mRNA levels during the early replicative stages of regeneration (36). Additional studies are required to establish a link between transcriptional factors that control OATP-B expression, cell replication, and cell differentiation.

In conclusion, we report the characterization of OATP-B in the human placenta. It is abundantly expressed at the basal surface of syncytiotrophoblast throughout gestation and transports estrone-3-sulfate. The steroid hormone precursor, pregnenolone sulfate, partially inhibits the transport of estrone-3-sulfate and requires testing as an additional physiological substrate of OATP-B that may be relevant to gestational needs. The identification of the placental mechanisms of translocation of sulfated steroids and precursors among their sites of synthesis, sites of sequential metabolism, and target tissues will offer a more comprehensive view of placental steroidogenesis. Moreover, it may add to our understanding of the link between an altered steroid hormone balance and parturition (37) (38) or preeclampsia (39, 40).

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