Stable Inducible Expression of a Functional Rat Liver Organic Anion Transport Protein in HeLa Cells*

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Recently we expression cloned a rat liver organic anion transport protein in Xenopus laevis oocytes (J. Acquemini, E., Hagenbuch, B., Stieger, B., Wolkoff, A. W., and Meier, P. J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 133-137). In the present study, we have stably transfected the cDNA encoding this protein into HeLa cells by using a vector containing a zinc-inducible promoter. The parent cells have virtually no baseline transport of [35S]sulfobromophthalein, whereas the induced transfected cells express a novel 74-kDa protein and avidly transport this ligand. Transport by these cells is saturable (Km = 3.3 μM, Vmax = 257 pmol/min/mg protein), bidirectional, and highly temperature-dependent. In the presence of albumin, uptake of [35S]sulfobromophthalein requires the presence of extracellular Cl⁻, whereas in the absence of albumin, this Cl⁻ dependence is not seen. These studies indicate that cellular uptake of sulfobromophthalein does not result from direct interaction with the plasma membrane lipid bilayer but rather requires the presence of a specific plasma membrane transporter.

Hepatocyte uptake of the organic anions bilirubin and sulfobromophthalein (BSP) has kinetics suggestive of a carrier-mediated process (1, 2). These organic anions circulate tightly bound to albumin from which they are rapidly extracted by hepatocytes (2, 3). Previous studies performed in short term cultured rat hepatocytes demonstrated a high affinity low capacity organic anion transporter (4, 5). This transporter extracted BSP from albumin, was electroneutral and temperature-dependent, and was inhibited after depletion of cellular ATP. The ability of this transporter to extract BSP from albumin was modulated by extracellular Cl⁻. Although a number of putative hepatocyte surface membrane organic anion transporters have been described (6-8), we recently cloned a cDNA encoding the organic anion transporting polypeptide (oatp) of rat liver into HeLa cells using a vector containing a zinc-inducible promoter. The parent cells have virtually no baseline transport of [35S]BSP, whereas the induced transfected cells express a novel 74-kDa protein and avidly transport this ligand. This has permitted dissection of the transport characteristics of oatp in a mammalian system without the possible confounding effects of other cell surface organic anion transporters.

As previously reported (10), oatp cDNA was initially cloned in the plasmid pSPORT1 (BRL). Recombinant DNA was digested with NcoI and HindIII (New England Biolabs Inc.), and the oatp cDNA insert was subcloned into the plasmid PrsetB (Invitrogen). This resulting plasmid was digested with KpnI and HindIII. The oatp cDNA insert was isolated and was cloned into the zinc-inducible vector pMEP-4 (Invitrogen). pMEP-4 is a mammalian expression vector that uses the inducible metallothionein IIa promoter and also carries the hygromycin resistance gene as a selectable marker (II). The pMEP4-oatp recombinant was amplified in Escherichia coli M13061 (gift of Dr. Thomas Shrader). The plasmid pMEP4-oatp was purified by the plasmid maxi kit (QIAGEN).

Cell Culture and Transfection

HeLa cells (ATCC) were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc) supplemented with 5% fetal bovine serum (Hyclone Labs Inc.) and 100 units/ml penicillin (Life Technologies, Inc.). Cells were cultured in a 3% CO₂ incubator at 37 °C. Cells were subcultured at a ratio of 1:4–1:5.

Materials and Methods

Construction of Plasmids

As previously reported (9), oatp cDNA was initially cloned in the plasmid pSPORT1 (BRL). Recombinant DNA was digested with NcoI and HindIII (New England Biolabs Inc.), and the oatp cDNA insert was subcloned into the plasmid PrsetB (Invitrogen). 48 h after transfection, cells were cultured in selective medium containing hygromycin (150 units/ml, Calbiochem). After 1–2 weeks, resistant colonies were selected and expanded in selective medium.

Immunoblot Detection of oatp

Cell lysates were resolved on 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and subjected to immunoblot as described previously (14). A rabbit polyclonal antibody made to a synthetic peptide corresponding to the oatp sequence near the carboxyl terminus was used. Antibody was detected using [125I]protein A.

General Methods—Uptake of [35S]BSP by HeLa Cells

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The abbreviations used are: BSP, sulfobromophthalein; oatp, organic anion transporting polypeptide; SFM, serum-free medium; BSA, bovine serum albumin.

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performed in normal SFM and SFM in which NaCl was substituted isosmotically by sucrose (4, 5). Initial uptake of [35S]BSP was determined over 1 min at 37 °C. Data were analyzed by nonlinear least squares regression (SigmaPlot version 5.0, Jandel Scientific, Corte Madera, CA).

Influence of Albumin on [35S]BSP Uptake—Uptake of [35S]BSP (0.8 μM) was determined in SFM alone and in SFM containing BSA (0–3%). Cl− dependence of [35S]BSP uptake was also determined as a function of albumin concentration. This was quantified as the difference in uptake by cells in NaCl-containing medium versus cells in sucrose-substituted medium (4, 5, 9, 10, 17).

Cellular Energy Requirements of [35S]BSP Uptake—Uptake of [35S]BSP was determined as described above at temperatures between 4 and 37°C. In other studies, ATP levels were depleted by preincubation of cells for 15 min in SFM containing 0.1% sodium azide and 50 mM 2-deoxyglucose as we have previously described (4). Uptake of [35S]BSP was then determined. Cellular ATP content was determined in replicate plates (4).

Efflux of [35S]BSP from Preloaded Cells—Cells were preloaded with [35S]BSP (0.8 μM) at 37 °C for 15 min. They were washed four times at 4 °C with 1.5 ml of SFM to remove surface bound and extracellular ligand. Following addition of 1 ml of 5% BSA in SFM, cells were incubated for 5–30 min at 4 or 37 °C (4). At the end of this time, cells were washed twice with 1.5 ml of SFM and harvested, and radioactivity was determined.

**RESULTS**

Plasmid Construction and Cell Transfection—The plasmid pMEP4 contains an Epstein-Barr virus origin of replication allowing replication in eukaryotic cells and a metallothionein-inducible promoter for the expression of heterologous genes (11). pMEP4-oatp was constructed, verified by restriction mapping, and transfected into HeLa cells. Transfected cells were selected on the basis of hygromycin resistance. As seen in Fig. 1, nontransfected cells (H) and cells transfected with vector alone (V) had virtually no uptake of [35S]BSP. Fifteen transfected HeLa cells were donally expanded and were assayed for [35S]BSP transport activity. In the absence of ZnSO4 induction, there was little transport by any of these clones. Following induction, however, all clones transported [35S]BSP (Fig. 1). All further studies were performed with clone 2 or clone 10 cells.

**Time Course of Induction of [35S]BSP Transport**—As seen in Fig. 2, transport of [35S]BSP increased with time of zinc induction over the first day. When additional ZnSO4 was added at 20 h, transport activity continued to increase through 48 h.

**Immunoblot Detection of oatp in Induced pMEP4-oatp-Transfected HeLa Cells**—As seen in Fig. 3, there was no immunoreactivity to proteins in nontransfected HeLa cells or in HeLa cells transfected with vector not containing the oatp sequence. pMEP4-oatp-transfected HeLa cells showed immunoreactivity to an approximately 74-kDa peptide corresponding to the oatp sequence near the carboxyl terminus. Immunoblot Detection of oatp—As seen in Fig. 3, there was no immunoreactivity to proteins in nontransfected HeLa cells or in HeLa cells transfected with vector not containing the oatp sequence. pMEP4-oatp-transfected HeLa cells showed immunoreactivity to an approximately 74-kDa peptide only when induced with zinc.

**Time Course and Temperature Dependence of [35S]BSP Transport**—As seen in Fig. 4, at 37 °C, [35S]BSP uptake by induced pMEP4-oatp-transfected HeLa cells was rapid and approached linearity for 2–3 min. At 15 min, as much as 40–50% of incubated ligand was cell-associated. In contrast, there was little association of [35S]BSP with cells incubated at 4 °C (Fig. 4). At intermediate temperatures, there was a direct relationship of [35S]BSP uptake with incubation temperature (Fig. 5). Preincubation of cells in a mixture of sodium azide and 2-deoxyglucose reduced their ATP concentration from 52.1 ± 1.2 (mean ± S.D.) nmol/mg protein to 29.1 ± 9.0 nmol/mg protein (n = 3). Under this condition, [35S]BSP uptake was reduced by approximately 50% (Fig. 6).

Saturability and Cl− Dependence of [35S]BSP Uptake—In the absence of BSA in the medium, uptake of [35S]BSP was saturable (Fig. 7), with (two studies) K̅m = 3.2 and 3.3 μM and V̅max = 239 and 275 pmol/mg protein/min. Previous studies in cultured rat hepatocytes suggested that high affinity uptake of BSP was Cl−-dependent (5, 17). When NaCl in medium was...
replaced isosmotically by sucrose; however, no Cl⁻ dependence was seen (Fig. 7). In two studies, Kₘ for BSP in the absence of Cl⁻ was 4.4 and 2.3 μM with V_max of 251 and 222 pmol/mg protein/min. Thus, under these conditions, there is no Cl⁻ dependence of BSP transport. In vivo, however, BSP is bound to albumin in the circulation. Because this binding is high affinity, the free fraction of BSP is very low. BSP transport by induced transfected cells was inversely related to the concentration of BSA in the medium (Fig. 8A). This is consistent with interaction of free BSP with the transporter. Although BSP uptake in the absence of BSA was not reduced by elimination of NaCl from the medium, transport of BSP in the presence of BSA was clearly Cl⁻-dependent (Fig. 8B).

**35S**BSP Transport Is Bidirectional—Induced transfected cells were incubated with 0.8 μM **35S**BSP for 15 min at 37°C. Following this loading with BSP, they were washed and incubated in 5% BSA at 4 or 37°C. As seen in Fig. 9, efflux of BSP from cells was rapid at 37°C. There was little release of ligand from cells at 4°C.

**DISCUSSION**

Although hepatocyte transport of organic anions such as bilirubin and BSP has been known for many years to have carrier-mediated kinetics, characterization of this important pathway has been difficult. In part this may be due to multiple transporters of high and low affinities that may be present on the hepatocyte surface (17, 18). In previous studies (4, 5), we have functionally characterized high affinity transport of **35S**BSP by short term cultured rat hepatocytes. We have found that these cells have the ability to extract BSP from a molar excess of albumin. This uptake process is highly temperature-dependent and is markedly reduced following depletion of cellular ATP levels. Whether ATP interacts directly or indirectly with this transporter is not known. Uptake of BSP was not altered by removal of Na⁺ from medium and replacement with other cations such as K⁺, Li⁺, or choline. In contrast, replacement of Cl⁻ in medium by gluconate or HCO₃⁻ significantly reduced BSP uptake. The mechanism for this Cl⁻ dependence of transport is not known. Affinity of BSP for albumin is Cl⁻-independent (5). Although there was a 7-fold increase in affinity of hepatocytes for BSP in the presence as compared with the absence of Cl⁻, it is not clear that this is sufficient to explain the large effect on BSP transport.

Using Cl⁻-dependent extraction of BSP from albumin as an assay of expression, a cDNA encoding an oatp has recently been cloned in Xenopus oocytes (9, 10, 19). In this system, uptake of BSP was Cl⁻-dependent in the presence of albumin but Cl⁻-independent in its absence (10). Because of the relatively low transport signal and the inherent difficulties of the Xeno-
The possibility that an endogenous transporter could complicate interpretation of experiments. As seen in Fig. 1, of 15 cell clones that were randomly selected, all were transporters of BSP upon zinc induction. Transport activity declined between 20 and 48 h of induction, unless additional zinc was added to the medium (Fig. 2). The reason for this finding is not clear at this time but likely represents depletion of the metal from the medium.

As there was virtually no basal BSP transport in control or uninduced transfecants, initial studies were performed in the absence of extracellular albumin. These studies revealed rapid, temperature-dependent uptake of BSP. This transport was not Cl⁻ dependent. In the presence of albumin, the rate of BSP uptake fell considerably (Fig. 8A). However, close to 80% of BSP transport in the presence of albumin was Cl⁻ dependent (Fig. 8B). These data thus strongly suggest that Cl⁻ does not directly modulate transport activity of oatp. Some studies (5, 20) have indicated that although Cl⁻ binds to albumin (21), it does not influence binding of organic anions to albumin. The mechanism resulting in Cl⁻ dependent kinetics that has been described in cultured hepatocytes (4, 5, 17) and perfused rat liver (4) thus requires further clarification.

Evidence presented in this study indicates that induced transfected HeLa cells transport BSP bidirectionally. Efflux of ligand rapid and is temperature-dependent. It is possible, however, that these cells also have an endogenous organic anion efflux mechanism that is detectable only when BSP has entered the cell. This situation has been described for efflux of taurocholate from Xenopus oocytes (22). The fact that BSP effluxes rapidly may complicate interpretation of studies performed in the presence of extracellular albumin. This protein binds BSP avidly and markedly reduces its free concentration (5). The fact that in the absence of albumin BSP accumulation over time reaches an apparent steady state (Fig. 4) suggests that equilibration of free BSP between the medium and the cell is established; that is, influx of ligand equals its efflux at this time. The presence of albumin outside the cell will shift the equilibrium in that direction, reducing cellular accumulation of the ligand. It should be noted that HeLa cells contain several cystolic glutathione transferases that may bind intracellular ligand. It is likely that in vivo, bile canalicular excretion provides the driving force for the net transport of BSP by keeping the system in a state of disequilibrium.

It has been suggested that uptake of the organic anion bilirubin can be explained simply by its direct interaction with the plasma membrane lipid bilayer (24). The present studies clearly show that in the case of BSP this is not so. Parental HeLa cells have virtually no uptake of this ligand. It is not until transfected cells are induced to express oatp that they develop the ability to transport BSP (Fig. 1). Thus, oatp facilitates the movement of BSP across the lipid bilayer. The mechanism by which it does this is as yet unknown. Previous observations in the isolated perfused rat liver suggested that the uptake mechanisms for bilirubin and more soluble organic anions are partially independent (25). Although investigations in transfected Xenopus laevis oocytes revealed inhibition of BSP uptake by bilirubin (19), whether oatp is an efficient transporter of bilirubin (19), whether oatp is an efficient transporter of bilirubin has not as yet been studied directly.

These studies thus establish in a mammalian system that the presence of oatp is sufficient to permit BSP transport. The characteristics of transport resulting from oatp transfection are identical to those that have been described previously in cultured rat hepatocytes. These studies do not imply, however, that oatp is the only basolateral plasma membrane organic anion transporter in the rat hepatocyte. Several putative transporters have been suggested by a number of other investigators.
Elucidation of their possible functional relationship with each other and with oatp remains to be established.

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