EXPRESSION CLONING OF TWO NOVEL GENES (Ostα, Ostβ) THAT TOGETHER MEDIATE ORGANIC SOLUTE AND STEROID TRANSPORT IN THE LIVER OF THE LITTLE SKATE, *RAJA ERINACEA*

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Epithelial cells continuously extract large amounts of organic solutes, drugs, and other xenobiotics from circulating blood plasma. Some of the transporters responsible for this organic solute clearance have recently been characterized at the molecular level, and include four families of ATP-independent transport proteins: NTCP, OATP, OAT, and OCT. The first member of each of these families was identified by expression cloning in *Xenopus laevis* oocytes, and additional members have subsequently been identified by homology screening. All of these transporters consist of single polypeptides, which when expressed in heterologous systems are able to mediate organic solute transport. However, because of the large number of endogenous and exogenous compounds that must be transported by the liver, kidney, intestine, and other tissues, it is likely that other transporters and transporter families are also present, but have not yet been described. Indeed, recent estimates indicate that approximately 6% of the human genome (i.e., about 2,000 genes) may encode membrane transport proteins; however, less than 500 have thus far been identified. In an attempt to identify novel organic solute transport proteins, we used a comparative approach, and screened a cDNA library from the liver of an evolutionarily ancient vertebrate species, the little skate, for bile salt ([^{3}H]taurocholate) transport activity using the expression cloning strategy.

Total RNA was isolated from skate liver using guanidinum thiocyanate extraction followed by cesium chloride gradient centrifugation, and poly(A^+) RNA was separated by running the total RNA fraction over an oligo(dT)-cellulose column twice. The poly(A^+) RNA was size-fractionated by centrifugation through a linear sucrose gradient (6-26% w/w), and individual poly(A^+) RNA fractions were checked for taurocholate uptake activity by expression in *Xenopus* oocytes. A cDNA library was constructed using the SuperScript Plasmid System kit (GIBCO BRL). The synthesized cDNA was ligated into pSPORT1 vector.

[^{3}H]Taurocholate transport activity in oocytes injected with size-fractionated skate liver mRNA was highest in size fractions 4 (1.2-2.3 kb) and 6 (0.6-1.5 kb), and intermediate in fraction 5. This bimodal distribution indicated that either two different sized mRNA molecules are able to stimulate taurocholate transport, or that two separate genes from these partially overlapping mRNA size fractions must be co-expressed to generate the transport signal. To evaluate these possibilities, mRNA from fractions 4 and 6 was combined, a cDNA library was constructed, and the synthesized cDNA library was screened for [^{3}H]taurocholate transport activity. During the initial screening, it was noted that some of the cRNA-stimulated transport activity was not stable, and was lost when positive pools of clones were divided into progressively smaller groups of clones. We hypothesized that this loss of transport activity may be due to the segregation into different cDNA pools of two or more genes that may be required to form a heteromultimeric transporter. We therefore initiated studies to identify the two or more clones that may be interacting to generate the active transport system, by making smaller subdivisions of positive pools. After multiple rounds of screening, a pool containing only 13 clones was identified that exhibited strong taurocholate transport activity. However, when the cRNA from the individual clones of this pool were injected into oocytes they failed to stimulate transport, supporting the hypothesis that two or more gene products may be required. To evaluate which of the 13 clones were required, a mixture of cRNA was prepared from either all 13 clones, or from 12 clones by sequentially deleting each clone from the mixture. Taurocholate uptake was observed under all conditions, except when clone 4 or clone 12 were deleted, indicating that both of these are required for transport. When the cRNA from

clone 4 or clone 12 were injected individually, there was no transport; however, when they were injected simultaneously in various ratios there was strong taurocholate transport activity. Clone 4 was denoted organic solute transporter-α (Ostα) and clone 12 was named Ostβ.

The total cDNA inserts of Ostα and Ostβ consist of 1228 and 882 nucleotides, and predict polypeptides of 352 and 182 amino acids, respectively, with calculated molecular masses of approximately 39 and 20 kDa. Ostα is predicted to have 7 transmembrane (TM) domains, and a single N-glycosylation site (Asn-22). In contrast, Ostβ is predicted to have 2 TM domains and 2 possible *N*-linked glycosylation sites (Asn-42 and 48): however, analysis of Ostβ with the SignalP V1.1 Program indicated that its first 27 amino acids form a signal peptide. Because this region contains a putative TM domain, the mature Ostβ protein may only have 1 TM domain. A search of the available databases for both nucleotide and amino acid sequences (March 2001) failed to identify significant homology for either Ostα or Ostβ, indicating that they are novel sequences. However, we identified proteins of unknown function in the human, *C. elegans*, *D. melanogaster*, and *A. thaliana* databases that exhibit about 15-20% predicted amino acid identity with Ostα and up to 30% identity with each other. Many of these sequences also predict 7-helix proteins, suggesting that they may have a common membrane topology and perhaps a common evolutionary origin. The Ostβ sequence was also unique, although it does exhibit 22% amino acid identity with the C-terminal 182 amino acids (i.e., the single TM domain and intracellular C-terminus) of protocadherin-γ, a cell suface glycoprotein. Northern blot analysis of Ostα showed a single major band at 1.4 kb in skate liver, but no significant expression was detected in any of the other skate tissues examined. By RT-PCR a weak signal for Ostα was also detected in skate kidney but all other tissues examined were negative. Ostβ was also only detected in skate liver by Northern blot, at a size of approximately 0.9 kb, whereas RT-PCR analysis indicated its presence in several skate tissues, including liver, kidney, intestine, and heart, but not pancreas and brain. The reason for this discrepancy is not clear, but it is possible that Ostβ or a closely-related sequence may be expressed in other tissues at low levels that are not detected by Northern blot using our full-length cDNA probe under high-stringency conditions.

In addition to mediating taurocholate transport, oocytes injected with Ostα and Ostβ cRNA (1 ng each) were also able to take up estrone sulfate, digoxin, and prostaglandin E_2, but not *p*-aminohippurate or *S*-dinitrophenyl glutathione, indicating that this transport system is polyspecific and may participate in cellular uptake of conjugated steroids and eicosanoids. Transport was also sodium-independent and saturable, although the apparent Michaelis constants (K_m) for taurocholate (785±43 μM), estrone sulfate (85±16 μM), and digoxin (148±30 μM) were high when compared to those reported for the OATP transporters, which are generally about one order of magnitude lower for these substrates. Because these K_m values are relatively high it is likely that these compounds are not the preferred substrates for this transporter. Ostα/Ostβ-mediated uptake of [^{3}H]taurocholate and [^{3}H]estrone sulfate was inhibited by a variety of bile acids, steroids and other organic anions. Sulfated steroids, including the major skate bile salt scymnol sulfate, were effective inhibitors of transport.

In summary, the present study describes the isolation and functional characterization of a multispecific organic solute and steroid transporter that requires the co-expression of two gene products, Ostα and Ostβ. Ostα/Ostβ is the first polygenic organic anion transporter to be identified, and may be the first heteromultimeric organic anion transporter. Because the cDNA and predicted amino acid sequences of Ostα and Ostβ are novel, they are likely to be the first members of a new family of transporters. (Supported by ES03828, ES01247, DK34989, DK25636, DK48823, NSF DBI 9820400, and by the Burroughs Wellcome Foundation).