Protein-Mediated Facilitated Uptake Processes for Fatty Acids, Bilirubin, and Other Amphipathic Compounds (43987)

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Passage of various substances across cell membranes must be highly selective since it allows the cell to maintain a constant internal environment. Essential molecules such as glucose, amino acids, and lipids must be able to readily enter the cell, whereas waste compounds must leave the cell. This selective permeability of the plasma membrane is due to different transport proteins within the lipid bilayer (1). Those transporters can be roughly divided into pumps, carriers, and channels, each of which is characterized by their different ways of using energy and transporting solutes.

In this article, we will focus on the transport of fatty acids, bilirubin and other amphipathic compounds such as bile acids and phospholipids which were long thought to enter cells by simple diffusion. We will start with describing fatty acid uptake as an example of receptor-mediated transport and will discuss the criteria by which active transport is distinguished from passive diffusion. In the following sections, discussion of the uptake of the other organic anions will be limited to a description of the isolated transport proteins.

Fatty Acids

The mechanism of the translocation process of fatty acids through the plasma membrane is controversial. On the one hand, it is suggested that uptake represents passive diffusion and is only determined by the rate of metabolism (2–4). On the other hand, it is argued that translocation involves the interaction of specific receptors within the plasma membrane. The vast majority of experimental findings suggest that fatty acid uptake is protein facilitated.

Before considering the actual process of fatty acid uptake, one should take into consideration how fatty acids are exposed to cells. As the major energy source of the organism, fatty acids circulate in the blood, where they are tightly bound to albumin which keeps them soluble. Concentration of fatty acids in blood plasma varies between 90 and 1200 μM, with an average value of around 300–500 μM under equilibrium conditions. Only 0.02% of the fatty acids are not bound to albumin and thus termed "free" fatty acids. The molar ratio of albumin:fatty acids normally varies between 0.15 and 2 with basal values of 0.5–0.8. At physiologic pH, fatty acids exhibit a net negative charge (5).

Complexed to albumin, fatty acids reach the sites of their consumption (e.g., heart, liver, and skeletal muscle). Within a single blood passage at least 30% of the fatty acids are extracted by those organs. In order to be taken up, fatty acids have to dissociate from albumin, since albumin does not enter cells in comparable amounts. As a consequence the uptake process involves:

1. dissociation from albumin
2. entering the plasma membrane
3. translocation across the membrane
4. release at the inner surface

Because of the high concentration of albumin in blood and its high affinity for fatty acids ($K_d = 10^{-8} M$), it is unlikely that the efficient cellular uptake of fatty acids is only the result of a free exchange of unbound fatty acids between the extra- and intracellular space.

To determine whether fatty acid uptake is receptor mediated, one has to define criteria for the existence of a transport protein. Such a receptor generally is defined by:

1. high-affinity binding sites at the membrane surface
2. saturable transport kinetics
3. specific inhibitors, possibly competitive
4. elimination of transport function upon protease digestion.

Based on studies of uptake kinetics of fatty acids in liver cells, it was first suggested that a specific recep-

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tor for albumin mediates the dissociation of albumin–fatty acid complexes followed by uptake of the ligand (6, 7). In order to determine whether a specific albumin receptor mediates the dissociation of the albumin–fatty acid complex, binding studies with [^{125}I]-labeled albumin and isolated rat liver plasma membranes were performed. In those experiments, no high-affinity binding site for albumin could be detected in the plasma membranes (8). However, it could be demonstrated that high-affinity binding sites for a representative fatty acid, ^{3}H-oleate, exist within the plasma membrane and that binding was saturable with 10^{15} binding sites per milligram of membrane protein and an affinity constant of $2 \times 10^{-8} M$ (9, 10). Furthermore, it was demonstrated that the "albumin effect" was not specific, as it was shown that uptake kinetics of fatty acids are similar to other ligand binding proteins such as β-lactoglobulin (11) or ligandin (12). Moreover, studies in elasmobranchs, species that lack albumin, demonstrated the same uptake kinetics as in mammals (12).

Taken together, these findings demonstrate that no interaction of albumin with the plasma membrane is required for fatty acid uptake, but that interaction of the fatty acid molecule with the plasma membrane seems to be necessary.

One strong argument against the diffusion theory of fatty acid uptake came from experiments done on muscle cells. The skeletal muscles are one of the major consumers of long-chain fatty acids. They are composed of different fiber types: fast-twitch glycolytic (FG), fast-twitch oxidative glycolytic (FOG), and slow-twitch oxidative (SO) fibers (13). The concentration of fatty acids in muscles composed mostly of FG, FOG, and SO fibers was reported to be 77.8 ± 9.4, 60.6 ± 13.6, and 119.8 ± 24.6 nmol/g, respectively (14). Calculating serum/muscle gradients for different acids in different muscle types showed that the gradients depend both on the fatty acid and on the muscle type, but they are not dependent on a muscle's oxidative capacity. Moreover, van der Vusse *et al.* determined serum/myocardium concentration gradients for different fatty acids and showed them to be dependent both on the type of fatty acid and on the species examined. In humans and dog it was highest for oleic and linoleic acid, whereas in rat it was highest for palmitic and oleic acid. The gradient for arachidonic acid was several-fold lower than for oleic acid, for one (15). If the plasma membrane only played a passive role in the process of fatty acid transport, one would not expect such great interacid differences in this gradient. This is not consistent with the diffusion theory.

In muscle it could also be shown that contractile activity evoked by stimulation of the sciatic nerve markedly increased incorporation of ^{14}C-palmitic acid into different lipid fractions, including that of free fatty acids in each muscle type. This rapid flux of palmitic acid during contractions occurred in the presence of unchanged plasma fatty acid concentration, thus an unchanged albumin/fatty acid molar ratio (16). It has also been shown that fatty acid uptake by muscle cells exhibits saturation kinetics (17) and that training markedly increases the V_{max} of fatty acid transport (18). Those data strongly argue for the existence of a facilitated transport system in skeletal muscle cells.

Most of the studies of fatty acid transport have been performed with hepatocytes. The requirement for fatty acid uptake, however, varies among different tissues and sometimes has to increase rapidly, such as in skeletal muscle cells at the onset of contractions. Whether regulation of fatty acid transport across the plasma membrane depends on tissue function or not is therefore an open question.

Kinetics of Fatty Acid Transport

Another argument against passive diffusion of fatty acids comes from kinetic studies of fatty acid uptake. Cellular uptake rates of fatty acids as a function of their external concentration can distinguish simple diffusion, which exhibits a linear relationship, from facilitated transport, which has to be saturable if membrane permeation is rate limiting. Studies using short-term cultured hepatocytes revealed that uptake of oleate is maximal and linear over the first 30-sec incubation period. This initial uptake rate representing cellular influx was shown to be independent of intracellular metabolism, including oxidation and esterification (19–21). In order to modulate the unbound concentration of the fatty acid used, ^{3}H-oleate was bound to albumin in various molar ratios without exceeding its solubility. At 37°C, hepatocellular influx increased as a function of the unbound oleate concentration in a saturable way with a K_m of 90 nM and a V_{max} of 835 pmol/min × mg cell protein (20). Similar results could be obtained with cardiomyocytes (22), jejunal enterocytes (23), adipocytes (24), and skeletal muscle cells (17). Furthermore, uptake of oleate was markedly reduced in the presence of other long-chain fatty acids and markedly inhibited after preincubation of hepatocytes with trypsin (21). In the presence of phloretin, an inhibitor of transport processes, influx of oleate was decreased by more than 75% (19). A similar effect was seen with DIDS, a compound known to inhibit transport of inorganic and monocarboxylate anions in many cell types, when incubated with adipocytes (24). Abumrad showed that epinephrine and insulin influenced the bidirectional transport of fatty acids across the membrane (25–27), an effect that could not be explained if fatty acids entered the cell *via* simple diffusion.

To overcome the problems involved in indirectly measuring fatty acid uptake simply by determining the amount of radioactivity taken up by different cells, we used a confocal laser scanning microscope to visualize fatty acid uptake. This was made possible by using a fluorescent fatty acid derivative, 12-NBD-stearate (12-*N*-methyl-7-nitobenzo-2-oxa-1,3-diazol-amoni stearate), which still behaves like naturally occurring fatty acids (28). In recent studies, this derivative could be shown to enter hepatocytes by the same uptake mechanism as that described for unmodified fatty acids (28).

Uptake studies with freshly isolated rat hepatocytes revealed that uptake of 12-NBD-stearate was maximal and linear over the first 60 sec of incubation time, representing unidirectional cellular influx (29). Initial uptake rates were shown to increase in parallel with the total concentration of 12-NBD-stearate at a constant albumin concentration of 25 μM. Plotting of total 12-NBD-stearate concentration versus uptake velocity revealed a linear relationship that is in accordance with observations obtained with radiolabeled oleate (29). In the presence of albumin, uptake of 12-NBD-stearate is rapid, with pronounced staining of the plasma membrane. The accelerated membrane-mediated uptake mechanism was not albumin specific, since it could also be observed with other fatty acid–binding proteins such as β-lactoglobulin and ligandin. Previous studies by Storch *et al.* with 3T3F442 adipocytes and fluorescent fatty acid derivatives using a conventional fluorescence microscope showed that uptake of fatty acids without albumin occurred (30). Today it is clear that Storch *et al.* were unable to distinguish between accumulation of fluorescence at the outer surface of the plasma membrane and actual uptake into cells. With the confocal laser scanning microscopy technique, which we used to reexamine those experiments, such differentiation is possible, because out-of-focus signals are eliminated by scanning a defined optical section (less than 0.5 μm in thickness). It was shown that without albumin rat hepatocytes take up fatty acids very slowly and that fluorescence lipid droplets arise and fuse with the outer surface of the membrane. These effects can only be abolished by the addition of albumin or other fatty acid–binding proteins such as β-lactoglobulin or ligandin. The efficient uptake in presence of fatty acid–binding proteins is due to optimal presentation of fatty acid molecules to the cell surface. The rapid intracellular accumulation of 12-NBD-stearate in presence of albumin was preceded by a prominent staining of the plasma membrane. In order to exclude unspecific partitioning into the lipid bilayer, experiments were repeated with human erythrocytes, where no membrane staining was observable. This observation also argues against simple diffusion, since otherwise erythrocytes should have taken up fatty acids in a comparable manner.

Driving Forces for Fatty Acid Uptake

If fatty acids were taken up simply by diffusion, the rate of transport would be directly proportional to the concentration gradient across the membrane. In this case no other driving forces would be expected. However, data from various laboratories have shown that fatty acid uptake into hepatocytes is energy dependent and Na^+ linked, but it seems as if Na^+ itself is not taken up as a companion molecule (31–33). Recently, it was shown that fatty acid interaction with its carrier protein requires the presence of Na^+ and H^+, indicating that it is the protonized form of fatty acids which interacts with the membrane (34). That would facilitate transport in so far as protonized fatty acids rapidly enter and flip-flop across phospholipid bilayers, although they cannot leave the inner surface to enter the cytoplasm. In contrast, fatty acid anions rarely partition into membranes, which represents the initial step in uptake (35).

Studies conducted in plasma membrane vesicles and perfused rat liver could demonstrate that fatty acid uptake is electrogenic and is accelerated by a more negative intracellular potential (31, 32). Studies using short-term cultured hepatocytes evaluated the effect on fatty acid uptake of a proton gradient across the plasma membrane (36). Hepatocytes that were acidified by an ammonium chloride prepulse technique showed a significant increase in K_m from 220 to 378 μM at a constant V_{max} value, indicating a decrease in fatty acid transport activity. This effect could be twisted after alkalinization of the cytoplasm by addition of ammonium chloride into the medium. Influx of NH_3 into the cell results in an alkalinization of the cell interior, whereas the remaining protons will acidify the plasma membrane outer surface. Under those conditions, K_m was reduced to 112 nM at a constant V_{max}, indicating an increase of membrane fatty acid transport activity. To evaluate whether the observed effect was due to differences in membrane potential, experiments were repeated with 1 mM $BaCl_2$, which inhibits pH-sensitive K^+ channels, thus keeping the membrane potential under those experimental conditions relatively constant (36). The results obtained were similar to those without $BaCl_2$. Another hint that fatty acid uptake is accelerated by accumulation of protons at the outer surface of the plasma membrane came from an experiment in which the Na^+-H^+-antiporter was blocked with 1 mM amiloride. This antiporter is activated if cells are acidified by the ammonium chloride prepulse technique and will pump excessive protons out of the cell until neutralization is achieved.

After blocking the Na^+-H^+-antiporter, fatty acid influx was further diminished to 46% of control levels (36). Accumulation of protons at the outer surface of the plasma membrane seems to stimulate fatty acid influx. Moreover, it could be shown that dissociation of fatty acids from albumin is significantly faster at a lower pH.

The observed phenomena indicate that uptake of fatty acids cannot be due to simple diffusion, since with simple diffusion uptake of fatty acids would be driven solely by a solute's concentration gradient across the membrane and would not be influenced by proton gradients.

Identification and Characterization of Putative Fatty Acid Transport Proteins

In the procaryote *Escherichia coli*, a long-chain fatty acid transporter (FadL) within the outer membrane has been cloned and sequenced by Paul Black's group. At least two genes, *fadL* and *fadD*, are required for translocation (37–43). The product of the *fadL* gene (FadL) is located in the outer uptake, where it binds long-chain fatty acids and facilitates their transfer across this membrane layer. The *fadD* gene encodes fatty acyl-CoA synthetase which activates exogenous fatty acids to CoA thioesters *via* a process that is postulated to be concomitant with transport across the inner membrane. There is some evidence for an oleic acid–binding protein within the inner membrane postulated to be a H^+/fatty acid cotransporter, but it has not yet been identified. Several laboratories have tried to identify and characterize the eukaryotic fatty acid transport protein from various cell types and most of them came up with putative transport proteins, although they are all different from each other (44–49).

The latest approach by Schaffer and Lodish took advantage of combined expression cloning and flow cytometric analysis (50). They used a 3T3L1-adipocyte cDNA library to transfect COS7 cells. 3T3L1-adipocytes differentiate from 3T3L1-fibroblasts after stimulation with insulin, dexamethasone, and methylisobutyl-xanthine. This is accompanied by a 10-fold increase in fatty acid uptake (51, 52). Assuming that the observed increase is due to enhanced expression of a fatty acid transport protein, one would expect an elevated level of a specific mRNA in 3T3L1-adipocytes. Schaffer and Lodish measured the uptake of the fluorescent long-chain fatty acid derivative BODIPY 3823 posttransfection and collected the most 0.03% fluorescent cells with a fluorescent-activated cell sorter. They identified six plasmids with two classes of inserts after extraction of plasmid DNA from these cells and retransfection into COS7 cells. One of these plasmids share 94% identity with rat liver fatty acyl-CoA synthetase (FACS), the same enzyme that is needed for translocation of fatty acids in *E. coli*. The other contained a novel cDNA, encoding a 646–amino acid fatty acid transport protein (FATP), with a predicted molecular weight of 71 kDa and an apparent molecular weight of 63 kDa. It had six potential membrane-spanning domains and three potential glycosylation sites. Among mouse tissues examined, expression of FATP was highest in skeletal muscle, heart, and fat, and lower levels were observed in brain, kidney, and liver, whereas no expression was found in intestine. Stable 3T3 fibroblast cell lines expressing FATP exhibited a 3- to 4-fold increase in oleic acid uptake. FATP was shown to be an integral membrane protein and appears to be similar to other cloned transporters with multiple membrane-spanning regions.

The detection of FATP is the most important and direct proof that uptake of long-chain fatty acids is membrane protein mediated. In contrast to the other methods used so far, that of Schaffer and Lodish is the first that resulted in the isolation of a protein with a functional assay, thus directly demonstrating its physiological role.

Transport of Bile Acids

Transport of bile acids into and out of different epithelial cells, namely hepatocytes and ileal enterocytes, has to occur with a directed passage through those cells (53–55). Bile acids are detergent-like compounds that serve to facilitate intestinal absorption of dietary lipids and lipid soluble vitamins. They are synthesized from cholesterol in liver and secreted into bile. In the small intestine, bile acids are absorbed and returned to liver *via* the portal circulation and again are reexcreted into bile (enterohepatic circulation). In addition to enterocytes and hepatocytes, bile acids are also taken up by renal tubule cells; all of these cell types posses transport systems that facilitate uptake.

Bile acid transport involves at least three different steps: uptake, translocation across the cell, and secretion. Recently, it has been shown that uptake of bile acids by the proximal ileum of hamster is facilitated by a 348–amino acid protein (38 kDa) termed ileal bile acid transporter (IBAT) (56). This result was obtained after expression cloning of hamster ileal cDNA in COS cells. A cDNA clone was isolated that coded for a protein with seven potential trans-membrane domains and three possible N-linked glycosylation sites. After transfection of COS cells, this cDNA stimulated transport of the conjugated bile acid taurocholate in a strict Na^+-dependent, saturable fashion with an apparent K_m of 33 μM. Inhibition of taurocholate uptake by the Na^+/bile acid cotransporter could be obtained by various bile acids but not by taurine or other organic anions. The apparent molecular weight of 38 kDa is clearly different from the previously identified proteins, which are 90 and 99 kDa in size, respectively

(57, 58). Those proteins were described by photoaffinity labeling using photolabile derivatives of taurocholate, and their relation to IBAT remains to be elucidated. In contrast, IBAT was shown to have 35% identical and 63% similar amino acid sequence to the rat liver Na^+/bile acid transporter identified by Peter Meiers group (59). They used the expression cloning strategy of rat liver mRNA in *Xenopus laevis* oocytes and isolated a cDNA clone coding for a 33- to 35-kDa protein with five glycosylation sites and seven transmembrane domains. This was in apparent contrast to the previously isolated 49- and 54-kDa proteins, which have been detected *via* photoaffinity labeling in hepatocyte sinusoidal plasma membranes (60–62). Those studies were done by Daniel Levys group, who used the bile acid derivative (7,7-azo-3,12-dihydroxy-5β-cholan-24-oyl)-2-amino-ethane sulfonic acid (7-ADTC) as photolabile substrate. Taurocholate uptake was inhibited by 7-ADTC, and the kinetic uptake of the derivative was shown to be similar to that of taurocholate. Using glycocholate-Sepharose 4B for affinity chromatography, they could isolate proteins in the range of 49–54 kDa from sinusoidal plasma membranes. This protein fraction led to a stimulation of bile acid uptake in reconstitution experiments. Monoclonal antibodies raised against this fraction were cloned in order to isolate those that inhibit bile acid uptake. As a result, one monoclonal antibody that recognized a 49-kDa protein was able to block Na^+-dependent uptake of conjugated bile acids. Sequence analysis done on the first 33 amino acids of the N terminus revealed homology to microsomal epoxide hydrolase, which also has a molecular weight of 49 kDa (63). Inhibitors of epoxide hydrolase activity did not influence taurocholate uptake, whereas known taurocholate uptake inhibitors did not show any effect on epoxide hydrolase activity. It was therefore assumed that epoxide hydrolase is a bifunctional protein. Transfection with an appropriate cDNA clone resulted in elevated transport of taurocholate.

Although bile acid uptake into hepatocytes was shown to be receptor mediated, which of the isolated putative liver Na^+/bile acid cotransporters is responsible for the translocation process still has to be proven.

Transport of Phospholipids into Bile

In bile, bile acids are associated with a specific population of lipids, mainly phosphatidylcholine and cholesterol, to form mixed micelles (64). Phospholipids were long thought to transverse the canalicular membrane by simple diffusion (65), but now there is striking evidence that they are transported by a special transport protein (66). This protein, the murine *mdr*2 *P*-glycoprotein belongs to a family of highly homologous *mdr* genes composed of three members in rodents, *mdr*1, *mdr*2, and *mdr*3, and by two members in humans, MDR1 and MDR2 (67–69). The *mdr* gene family itself belongs to the superfamily of ABC transporters to which CFTR (cystic fibrosis transmembrane conductance regulator) also belongs (70, 71). They share a common structural and functional unit formed by two homologous halves, each encoding a nucleotide-binding domain and six predicted transmembrane domains. Mdr *P*-glycoproteins can be divided into two functionally distinct groups: the first with Mdr1 and Mdr2 including human MDR1 can directly confer drug resistance to drug-sensitive cells, whereas the second group with Mdr2 and the human MDR2 is not able to export cytotoxic compounds. Immunological studies have shown that Mdr2 expression is largely limited to liver and is restricted to the canalicular membrane of the epithelial cells lining the lumen of the bile canaliculi and biliary ductules (72, 73).

In order to investigate the function of Mdr2, Smits group has generated Mdr2 knock-out mice (66). These develop severe liver damage that appears to be caused by the complete inability of the liver to secrete phospholipids into bile. Mice heterozygous for the mutant allel do not shown any liver pathology but have half the level of phospholipids in bile. Since concentration of bile acids in mice is very high (approximately 55 m*M*) and if phospholipids are missing, to form mixed micelles bile acids will elute phospholipids and cholesterol from membranes (74). This would explain the destructive cholangitis and the other observed damages of the liver. It was suggested that Mdr2 participates in the translocation of phosphatidylcholine within or across the canalicular membrane and perhaps functions as a flippase. These observations are in agreement with biochemical studies that implied the existence of such a phosphatidylcholine translocator in canalicular plasma membrane vesicles (75).

Philippe Gros group used another approach to investigate the physiological role of Mdr2 (76). They studied Mdr2 expression in secretory vesicles (SVs) from a yeast mutant and used a fluorescent phosphatidylcholine derivative to determine the lipid distribution within the lipid bilayer. It could be shown that Mdr2 expression caused a time- and temperature-dependent enhancement of phosphatidylcholine translocation to the inner leaflet of the membrane. Translocation was ATP and Mg^{2+} dependent and abrogated by the ATPase inhibitor vanadate and the *P*-glycoprotein mediator verapamil. It was concluded that Mdr2 *P*-glycoprotein can function as a lipid translocator or flippase within the lipid bilayer. The physiological reason for a phospholipid flippase in canalicular membranes could be that secreted bile acids do not need to elute phospholipids from the membrane in order to form mixed micelles. The epithelium of the canaliculi would be protected from the detergent action of bile

acids. It can be hypothesized that Mdr2 *P*-glycoprotein creates the asymmetrical distribution of phosphatidylcholine in the outer leaflet as observed previously (77).

These experiments clearly establish that phospholipids are transported into bile *via* an energy-consuming protein-mediated process and not by mere diffusion.

Transport of Bilirubin

Bilirubin belongs to a class of substances usually termed cholephilic organ anions, because their concentration is 10- to 1000-fold higher in bile than in blood (78, 79). Other cholephilic organic anions are phthalein dyes as BSP (bromosulphthalein) and dibromosulphthalein, aromatic dyes as phenol red and ICG (indocyanine green) and bilirubin mono- and diglucuronide. Common to this heterogenous group of substances are (i) their negative charge at physiologic pH, (ii) their binding to albumin in blood (with different affinities), and (iii) their efficient removal from the portal circulation by hepatocytes. Various experimental findings suggest that hepatocellular uptake of those substances involves a facilitated membrane transport mechanism (80, 81).

Eighty percent of bilirubin, the main degradation product of heme catabolism, is derived from hemoglobin of senescent erythrocytes. Its clearance from blood occurs against an apparent concentration gradient and thus implies the presence of a facilitated transport system. Kinetic studies performed with isolated hepatocytes as well as in the intact animal or perfused rat liver showed that uptake had the characteristics of a carrier-mediated process (82, 83). Binding of BSP to rat liver plasma membranes was shown to be saturable with a high-affinity binding site, and pretreatment of the membranes with trypsin resulted in an inhibition of binding (84). Since these results strongly argue for receptor-mediated uptake, several groups using different methods, tried to isolate the putative bilirubin transport protein. As a result, three different proteins were identified, all of which exhibit affinity for bilirubin.

Paul Berk's group isolated a BSP/bilirubin–binding protein with an apparent molecular weight of 54 kDa from Triton ×-100–solubilized rat liver plasma membrane fraction (84). The protein was eluted from a bilirubin or a BSP-agarose affinity chromatography column. Under native conditions, the protein has a molecular weight of about 100 kDa so that dimer formation may occur. The BSP/bilirubin–binding protein is glycosylated with a pI value around 3.5. Antibodies raised against the protein have been able to inhibit BSP or bilirubin uptake in isolated hepatocytes, whereas uptake of oleate, taurocholate, or cholate has not been influenced (82).

Allan Wolkoff's group has used detergent-solubilized (0.4% deoxycholate) liver plasma membrane as starting material, which was then subjected to affinity chromatography (glutathione-BSP-agarose gel) (85). As a result, they have isolated a protein termed organic anion–binding protein (OABP) with the same size as the BSP/bilirubin–binding protein. It is glycosylated, but has a pI value of 7. Although OABP– and BSP/bilirubin–binding protein are very similar in size and glycosylation, they are not the same, as has been proven by a comparison of their antibodies (86). Moreover, recent studies revealed homology of the former protein to the β subunit of mitochondrial F1-ATPase and of the latter to arginase (87–89).

A third protein with high affinity to bilirubin, termed bilitranslocase, has been isolated by Claudio Tiribellis group (90). They started with an acetone extract of a crude plasma membrane preparation, followed by gel permeation and exchange chromatography based on the proteins ability to bind BSP. In the presence of detergents, the protein showed a molecular weight of 170 kDa as determined by gel permeation, whereas removal of detergent at high ionic strength led to an apparent molecular weight of 105 kDa. SDS-PAGE under reducing conditions has revealed that the protein consists of two subunits, α and β, of 37 and 35.5 kDa, respectively, with an apparent molar ratio of 2:1 (91). After isolation of bilitranslocase under reducing conditions from liver plasma membrane vesicles, the β subunit could no longer be detected (92). Reconstitution experiments in liposomes have shown that the β subunit is not necessary for transport activity. Antibodies raised against bilitranslocase have been able to inhibit uptake of bilirubin.

Bilitranslocase is a basic protein with a pI value >9 and must be stabilized with β-mercaptoethanol in aqueous solutions to maintain its native structure, which implies that SH groups are required for function (92). Immunologically the protein has been detected at the sinusoidal pole of liver parenchymal cells, in basolateral plasma membrane vesicles from kidney cortex, in the intestine, testis, heart, and adrenal cortex, and in the HepG2 hepatoma cell line (93, 94). In a comparative study, Claudio Tiribelli's group has shown that uptake of bilirubin by bilitranslocase is electrogenic whereas such uptake by the BSP/bilirubin–binding protein is electroneutral. These authors concluded that the two proteins operate in parallel, where the relative role of each on the overall transport depends on substrate concentration (95).

Peter Meier's group tried to isolate a transport protein for cholephilic anions *via* expression cloning in *Xenopus laevis* oocytes (96). They isolated a cDNA encoding a rat liver organic anion-transporting polypeptide (oatp). The cloned oatp not only mediated Na^+-independent uptake of BSP but also of cholate and taurocholate. Sequence analysis revealed an open

reading frame of 2010 nucleotides coding for a protein of 670 amino acids with a calculated mass of 74 kDa. There are four possible glycosylation sites and 10 putative trans-membrane domains. *In vitro* translation experiments have shown that the protein is indeed glycosylated and its apparent molecular weight is around 59 kDa.

None of the isolated proteins/cDNA are identical, so which of the putative transporters have a physiological function still has to be elucidated.

Perspectives

Biochemical identification of transport proteins has proven quite controversial. It is now necessary to clone the described carriers in order to determine their primary structure as well as their genomic localization and regulation. With the help of cDNA probes, different transporters can be compared to determine whether they belong to certain classes of transport families that already have been described (97–99). The possibility of site-directed mutagenesis will then lead to an understanding of the reactive sites of the different transport proteins and an overall understanding of transport processes.

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