Localization of the sulfate/anion exchanger in the rat liver

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Quondamatteo, Fabio, Wolfgang Krick, Yohannes Hagos, Marie-Helen Krüger, Katrin Neuber-Saile, Rainer Herken, Giuliano Ramadori, Gerhard Burckhardt, and Birgitta C. Burckhardt. Localization of the sulfate/anion exchanger in the rat liver. J Physiol Gastrointest Liver Physiol 290: G1075–G1081, 2006.—Aldehyde sulfate (PAPS), the activated form of sulfate and a substrate for sulfotransferases (7). Because sulfate is a divalent hydrophilic anion, it cannot pass freely through the phospholipid bilayer of plasma membranes and is therefore dependent on transport systems to allow its movement into or out of cells.

Na+-dependent as well as Na+-independent sulfate uptake was measured in the isolated perfused rat liver (3) and isolated hepatocytes (34). Sulfate uptake in hepatocytes was further characterized in rat liver sinusoidal (8) and canalicular (22) membrane vesicles. A transmembrane pH difference stimulated sulfate uptake in sinusoidal membrane vesicles, suggesting sulfate/hydroxyl ion exchange (8). cis-Inhibition and trans-stimulation of sulfate uptake were observed with sulfate, thiosulfate, oxalate, and succinate but not with chloride, bicarbonate, monocarboxylates, or glutamate and aspartate (8). In contrast, in rat canalicular membrane vesicles, a bicarbonate (50 mM bicarbonate inside and 5 mM bicarbonate outside) but not a pH gradient (pH 8.0 inside and pH 6.0 outside) stimulated sulfate uptake (22). cis-Inhibition of bicarbonate-stimulated sulfate uptake was observed with sulfate, thiosulfate, and oxalate but not with chloride, nitrate, phosphate, monocarboxylates, or glutathione (22). The sinusoidal transport system exhibited a low affinity for sulfate [Michaelis-Menten constant (Km) ~16 mM] (8), whereas the canalicular system showed a high affinity for sulfate (Km ~0.3 mM) (22).

In recent years, a Na+-dependent as well as a Na+-independent sulfate transporter have been cloned from various tissues and designated as NaSi-1 (19) and sat-1 (2), respectively. Whereas NaSi-1, the Na+-dependent sulfate transporter, was not detected in the liver (19), during the course of our study, a rat Na+-dependent sulfate transporter (rNaS2) was cloned (4). rNaS2 is expressed in the liver (4); however, BLASTing of its mRNA or amino acid sequences showed no sequence homology with sat-1. Sat-1 has been cloned from rats, mice (2, 18, 31). Functional studies in Xenopus laevis oocytes (2, 11, 20, 32, 35) and Sp9 insect cells (11) have suggested that sat-1 translocates sulfate and oxalate. Sulfate uptake was inhibited by selenite, thiosulfate, and the stilbene derivative DIDS. Sat-1 transcripts have been found in the liver and kidneys (2, 32), but localization of sat-1 has only been studied in the kidney (11).

In the liver, the localization of sat-1 is not known. The liver contributes to the biotransformation of drugs via sulfation; therefore, it is important to identify sulfate transport systems either for import of sulfate or export of sulfated compounds. NaSi-1 has not been detected in the liver (19), and rNaS2 has shown limited substrate specificity (4). In addition, the organic anion-transporting polypeptides present in the sinusoidal membrane of hepatocytes do not handle anorganic sulfate (10) but translocate estrone sulfate (ES) (17). Canalicular excretion of organo sulfates is facilitated by multidrug resistance-associated protein 2 (MRP2) (12).

Here, we report on the localization of sat-1 in sinusoids as well as in cell-cell borders of hepatocytes and show that sat-1...
can work as a sulfate/bicarbonate exchanger. Testing of compounds reported to affect sulfate transport in the intact kidney (33) and sinusoidal and canalicular membrane vesicles (8, 22) revealed a low affinity for bulky sulfated compounds.

**MATERIALS AND METHODS**

**In situ hybridization.** This was performed in formalin-fixed paraffin-embedded tissue sections of male rat livers according to our standard protocol (16). Specific localization of sat-1 RNA was obtained by incubation with the digoxigenin-labeled antisense probe, whereas negative controls were performed by incubating sections with a digoxigenin-labeled RNA sense probe substituted for the antisense probe simultaneously with the antisense incubation and by omitting the RNA probes in each experiment. Digoxigenin-labeled RNA probes were yielded by in vitro transcription using the RNA polymerase T7 (sense) and T3 (antisense) starting from a sequence specifically coding for rat sat-1 mRNA (bases 2012–2358, Accession No. L23413). This sequence was amplified from rat liver mRNA using specific primers for rat sat-1 flanked by the T7 and T3 promoter sequences for the forward (5'-TCTTGAGCTACTAATCCAGGCAGGGTTCGGCTCTATCGTTGCACA-3') and reverse primers (5'-GCAATTTAACCCCTCAATTAAAGGCGCTTTTCCTGGCTTCTC-3'), respectively. These amplified a product of 395 bp bridging the 348 bp of the sat-1 sequence. The 392-bp product amplified by these primers was sequenced and shown to correspond to sat-1.

**Sat-1 expression in rat liver cells in vitro.** Hepatocytes, hepatic stellate cells, and endothelial cells from the rat liver were prepared and seeded as previously described. Freshly isolated cells were investigated for sat-1 expression in rat liver parenchyma was clearly detected in hepatocytes and in several sinusoidal and canalicular membrane vesicles (8, 22). In situ hybridization showed that after incubation of the sections with the antisense probes, a specific signal in the liver parenchyma was clearly detected in hepatocytes and in several endothelial cells (Fig. 1, A and B). Incubation with the sense probes (Fig. 1C) yielded no signals.

RT-PCR studies in hepatocytes and endothelial cells as well as in stellate cells were performed in vitro. Sat-1 was expressed in hepatocytes (Fig. 2A), where the level of expression did not change during the time in culture. In freshly isolated endothelial cells, sat-1 transcripts were also detected (Fig. 2B). In stellate cells, clear expression for sat-1 was seen on days 6 and 7 of culture but not on days 2 or 4 (Fig. 2C).
but also sulfated steroids, the uptake of [3H]ES and [3H]DHEA sulfate uptake significantly, only acetazolamide was able to inhibit diuretics (Fig. 4A and 4B). Among the cis-inhibition experiments for their ability to inhibit [35S]sulfate uptake, it was 105.5 ± 5.7 pmol·h⁻¹·oocyte⁻¹ without injection of ES and 116.3 ± 13.8 pmol·h⁻¹·oocyte⁻¹ with injection of ES. Sulfate uptake in water-injected controls was 1.7 ± 0.3 and 8.0 ± 2.3 pmol·h⁻¹·oocyte⁻¹. DHEA sulfate uptake in sat-1-expressing oocytes was 112.1 ± 15.3 and 599.1 ± 178.6 sulfate was investigated. In these experiments, oocytes from the same frog were divided randomly into three groups. One group was incubated for 1 h with ORi solution containing 0.5 mM [35S]sulfate, whereas the two other groups were incubated for 1 h in ORi solution containing 0.1 or 0.5 μM [3H]ES. In three batches of oocytes, sulfate uptake was 189.9 ± 43.9 pmol·h⁻¹·oocyte⁻¹, indicating successfully expressed sat-1. ES uptake into sat-1-expressing oocytes was 0.8 ± 0.1 and 9.1 ± 0.4 pmol·h⁻¹·oocyte⁻¹ at 0.1 and 0.5 μM ES, respectively (Fig. 5A). ES uptake into water-injected oocytes was 0.8 ± 0.1 and 8.7 ± 0.8 pmol·h⁻¹·oocyte⁻¹, respectively, demonstrating the absence of sat-1-mediated ES uptake. Higher ES concentrations could not be tested because of nonspecific binding of ES to the oocytes. ES undergoes enterohepatic circulation and has to exit from the hepatocytes. To test for ES/sulfate exchange, sat-1-expressing oocytes were injected with ES before they were incubated in 0.5 mM sulfate for the [35S]sulfate uptake to be measured. However, [35S]sulfate uptake by sat-1 was not enhanced by preloading the oocytes with ES (Fig. 5B): it was 105.5 ± 5.7 pmol·h⁻¹·oocyte⁻¹ without injection of ES and 116.3 ± 13.8 pmol·h⁻¹·oocyte⁻¹ with injection of ES. Sulfate uptake in water-injected controls was 1.7 ± 0.3 and 8.0 ± 2.3 pmol·h⁻¹·oocyte⁻¹. DHEA sulfate uptake in sat-1-expressing oocytes was 112.1 ± 15.3 and 599.1 ± 178.6 percent of sulfate uptake in the absence of these drugs. The uptake in the presence of these compounds was calculated as a percentage of sulfate uptake in the absence of these drugs. DMPS is used to reduce the content of mercury in the liver (36). DMPS at concentrations of 0.25 and 1 mM enhanced rather than inhibited [35S]sulfate uptake (Fig. 4B). Among the diuretics (Fig. 4C), only acetazolamide was able to inhibit sulfate uptake significantly (P < 0.05).

To test whether sat-1 is not only able to translocate sulfate but also sulfated steroids, the uptake of [3H]ES and [3H]DHEA uptake in the sinusoidal lining (Fig. 3A). Cell-cell borders between hepatocytes (Fig. 3, A and C) were also clearly stained with the exception of the central region, which corresponds to the bile canaliculi (Fig. 3, A and C). In several corners between hepatocytes, at very high magnification (Fig. 3D), it was possible to distinguish a separation of the staining of the hepatocytic membrane (Fig. 3D) from the staining of the sinusoidal lining (Fig. 3D). In negative controls (Fig. 3B), no such staining was seen.

Incubation of 149 oocytes expressing sat-1 and 115 water-injected control oocytes from 18 different frogs in 0.5 mM [35S]sulfate for 1 h led to an uptake of sulfate of 121.7 ± 32.6 and 4.4 ± 1.1 pmol·h⁻¹·oocyte⁻¹, respectively (Fig. 4A). To evaluate the substrate specificity of sat-1, certain drugs were tested in cis-inhibition experiments for their ability to inhibit [35S]sulfate uptake. For a better comparison of the data, sulfate uptake in the presence of these compounds was calculated as a percentage of sulfate uptake in the absence of these drugs. DMPS is used to reduce the content of mercury in the liver (36). DMPS at concentrations of 0.25 and 1 mM enhanced rather than inhibited [35S]sulfate uptake (Fig. 4B). Among the diuretics (Fig. 4C), only acetazolamide was able to inhibit sulfate uptake significantly (P < 0.05).

To test whether sat-1 is not only able to translocate sulfate but also sulfated steroids, the uptake of [3H]ES and [3H]DHEA.
fmol·h⁻¹·oocyte⁻¹ at 500 nM and 500 μM DHEA sulfate, respectively. In water-injected oocytes, it was 101.8 ± 17.1 and 553.1 ± 26.1 fmol·h⁻¹·oocyte⁻¹, demonstrating the absence of sat-1-mediated DHEA sulfate uptake (Fig. 5C). In addition, DHEA sulfate was not able to inhibit [³⁵S]sulfate uptake up to concentrations of 500 μM (data not shown).

For sat-1-mediated sulfate effects, oocytes were microinjected with [³⁵S]sulfate. The initial [³⁵S]sulfate concentration averaged 0.92 pmol/oocyte in sat-1-expressing as well as water-injected oocytes. In these oocytes, the amount of [³⁵S]sulfate did not change (Fig. 6A), indicating that [³⁵S]sulfate did not leak out of the oocytes (15 oocytes from 3 donors). In sat-1-expressing oocytes, [³⁵S]sulfate decreased from 0.92 to 0.52 ± 0.08 pmol/oocyte within 30 min (15 oocytes from 3 donors; Fig. 6A). [³⁵S]Sulfate efflux was slightly enhanced by chloride (Fig. 6A) but was strongly accelerated in the presence of 30 mM bicarbonate (Fig. 6A). Under this condition, nearly all of the injected [³⁵S]sulfate left the sat-1-expressing oocytes by sat-1 within 30 min, whereas [³⁵S]sulfate stayed constant in water-injected controls. In cis-inhibition experiments, [³⁵S]sulfate uptake was only marginally affected by bicarbonate (Fig. 6B): 164.4 ± 19.8 pmol·h⁻¹·oocyte⁻¹ in the absence of
Fig. 5. Comparison of sulfate uptake in the presence and absence of estrone sulfate (ES). A: 1-h uptake studies of 0.1 and 0.5 μM [3H]ES were compared. Data are means ± SE from 3 independent determinations with >6 oocytes for each experimental condition. B: [35S]sulfate uptake was determined after oocytes were injected with either 50 nl of 20 mM ES (shaded bars) or an equivalent amount of water (solid bars). Data are means ± SE of at least 8 oocytes from 3 donors for every condition. C: 1-h uptake studies of 500 nM and 500 μM [3H]dehydroepiandrosterone (DHEA) sulfate. Data are means ± SE from 3 independent determinations with >9 oocytes for each experimental condition.

Fig. 6. Dependence of sat-1 transport function on bicarbonate. A: sat-1-expressing and water-injected control oocytes were injected with 46 nl [35S]sulfate (0.92 pmol/oocyte), and the efflux of sulfate was followed for 30 min in the presence (solid bars) and absence of chloride (shaded bars) and in a solution containing 30 mM bicarbonate in chloride-containing ORi solution (open bars). Chloride was substituted by methane sulfonate and in the bicarbonate experiments, 30 mM bicarbonate was added at the expense of chloride. Experiments were performed with 5 oocytes from 3 donors. B: sat-1-expressing and water-injected control oocytes were incubated for 1 h in ORi solution containing 0.5 mM total sulfate in the absence and presence of 30 mM bicarbonate. Data are means ± SE of at least 8 oocytes for each condition from 4 donors. C: sat-1-expressing oocytes and water-injected controls were preincubated for 4 h in either the absence or presence of 30 mM bicarbonate. Bicarbonate was added at the expense of chloride. Data are means ± SE of at least 8 oocytes from 2 donors.
bicarbonate and 140.2 ± 36.5 pmol·h⁻¹·oocyte⁻¹ in the presence of bicarbonate. The values for water-injected oocytes were 3.9 ± 0.7 and 2.8 ± 0.6 pmol·h⁻¹·oocyte⁻¹, respectively. To demonstrate the direction of transport, oocytes were incubated for 4 h in ORI solution containing 30 mM bicarbonate at the expense of chloride. In two experiments, [³⁵S]sulfate uptake was enhanced by a factor of 1.59 ± 0.12 in oocytes preincubated in bicarbonate-containing solutions (Fig. 6C).

**DISCUSSION**

Despite the cloning of sat-1 as a hepatic sulfate transporter, neither its physiological role nor its localization within the liver has been clarified to date. Sat-1 was identified as a Na⁺-independent sulfate transporter by expression cloning from the rat liver (2), and its characteristics matched in part those identified for the sulfate transport systems from either the canalicular (22) or the sinusoidal (8) membrane of hepatocytes. In sat-1-expressing oocytes, the $K_m$ for sulfate was found to be 0.14 mM (2), which is close to that reported for rat canalicular membrane vesicles (0.3 mM) (22). Because canalicular and sinusoidal membrane vesicles may each contain several sulfate transporters, no safe conclusion from the $K_m$ to the localization of sat-1 can be drawn.

In the present work, we clearly showed that hepatocytes and endothelial cells express transcripts of sat-1. In contrast, stellate cells showed clear sat-1 expression only under conditions that were far from the physiological state. We also showed that sat-1 is localized along the sinusoidal borders and in the borders between hepatocytes. Unexpectedly, the canalicular region was free of staining. Therefore, we conclude that sat-1 is not located in the bile canaliculi. In contrast, our results identify sat-1 as a sulfate transporter present in the sinusoidal membrane of hepatocytes in the interhepatocytic borders and in endothelial cells.

Sulfate serves to form PAPS, a sulfate donor important in detoxification processes (7). However, in the liver, sulfates are provided with a perisinusoidal matrix containing sulfated proteoglycans (6, 25, 26). Proteoglycans participate in many cellular processes such as migration, adhesion, proliferation, and regulation of growth factors (9). The presence of sat-1 in the sinusoidal domain of hepatocytes and in other cell types located in the proximity of the sinusoids such as endothelial cells, as shown in the present work, strongly suggests that sat-1-dependent sulfate transport may be involved in the synthesis of sulfated proteoglycans of the perisinusoidal matrix. In the present work, we also showed that, in stellate cells, sat-1 expression increases with the length of time of culture. This might be suggestive of a general tendency of stellate cells to upregulate sat-1 expression under conditions that are far from the physiological state. In this context, it is interesting to note that nonphysiological conditions in which stellate cells play a key role occur, for example, in chronic liver injury. In the course of this process, stellate cells can be activated by stimuli derived from damaged hepatocytes, endothelial cells, and Kupffer cells. Stellate cell activation results in liver fibrosis with changes in extracellular matrix composition accompanied by production of great amounts of matrix also containing sulfated proteoglycans (1, 6, 26, 30). Whether an upregulation of sat-1 occurs during activation of stellate cells in liver injury in vivo and, therefore, may account for an increased sulfate uptake in this cell type is not yet known.

Liver sulfation capability is not only aimed at synthesising sulfated proteoglycans. Sulfation enhances the water solubility of steroids and drugs and their distribution within the plasma. Sulfation also facilitates the excretion of lipophilic compounds by the kidneys, thereby contributing to detoxification. In this context, the question arises as to whether sat-1 contributes to translocation of sulfated compounds and, therefore, may participate in their excretion. The results of our experiments show that sat-1 does not translocate ES and DHEA sulfate. Therefore, it is conceivable that sulfate steroids are not excreted from the liver via a sat-1-mediated transport. The excretion of these sulfated steroids is rather mediated by other systems localized in the sinusoidal membrane (5, 33a) or, in the bile, by MRPs (12). With the exception of acetazolamide, millimolar concentrations of diuretics were not able to reduce sulfate uptake mediated by sat-1. The preference of sat-1 for inorganic sulfate and the sinusoidal localization of the transporter suggest a role for sat-1 in the uptake of inorganic sulfate from the circulation rather than a function in the excretion of sulfated compounds from the liver. This feature enables sat-1 to continuously support hepatocytes and endothelial cells providing sufficient sulfate for sulfation.

We were also able to demonstrate sulfate efflux from oocytes that was not significantly changed when chloride was replaced by methane sulfonate but was strongly accelerated in the presence of bicarbonate. This indicates sulfate/bicarbonate rather than sulfate/chloride exchange by sat-1. In cis-inhibition experiments, [³⁵S]sulfate uptake was only marginally reduced in the presence of bicarbonate, suggesting that the affinities for bicarbonate at the extracellular site may be less strong than at the intracellular site of sat-1.

In conclusion, sat-1 is a sulfate/bicarbonate exchanger located in the sinusoidal membrane of hepatocytes as well as at their interhepatocytic borders and in endothelial cells.

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