Immunolocalization of sat-1 sulfate/oxalate/bicarbonate anion exchanger in the rat kidney

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IN THE MAMMALIAN PROXIMAL tubule, an anion exchanger that accepts sulfate, bicarbonate, and oxalate as substrates has been identified on both the apical and basolateral membranes (10, 11, 18). It has been proposed that sulfate/oxalate exchange on the proximal tubule basolateral membrane provides cellular exit and entry steps for sulfate and oxalate, respectively (1). On the apical membrane, sulfate/oxalate exchange may participate in oxalate-dependent chloride reabsorption by providing a mechanism of recycling oxalate from the lumen to the cell so that sufficient substrate remains available for transport on the apical membrane chloride/oxalate exchanger (7). It is unknown whether sulfate/oxalate exchange on the apical and basolateral membranes represents transport on identical or different anion exchange proteins.

Recently, the gene encoding a rat liver sulfate/anion transporter (sat-1) was expression cloned in Xenopus laevis oocytes (2). Oocytes injected with the sat-1 cDNA expressed a 200- to 500-fold increase in sulfate uptake compared with oocytes injected with rat liver total RNA. Expressed sulfate uptake was cis-inhibited by oxalate and the anion exchange inhibitor DIDS but not by succinate or cholate, suggesting that it represents the canalicular sulfate/anion transporter (14). Northern blot analysis showed a strong hybridization reaction of liver sat-1 with rat kidney mRNA, demonstrating that sat-1 or a closely related homolog is also expressed in the kidney. The functional similarity between liver sat-1 and the renal apical and basolateral sulfate/bicarbonate/oxalate exchangers suggests that one or both proximal tubule anion transporters are related to the sat-1 protein.

The purpose of the present study was to isolate and identify the sat-1-related clone from the rat kidney. We report that the liver and kidney sat-1 clones probably represent the same gene products. The sat-1 protein has been expressed in SF9 insect cells and the protein used to generate monoclonal antibodies. Immunolocalization of the sat-1 protein in the rat kidney is described.

METHODS

Cloning of rat renal sat-1. To generate a probe, a 1,356-bp fragment was excised from rat liver sat-1 cDNA by digestion with Xho I, which cuts at sat-1 nucleotide 1327, and MuI, which cuts in the multiple cloning site of the pSPORT-1 vector. 29 bases upstream from the 5' end of the sat-1 insert. The fragment was gel purified and labeled by random-primed synthesis with [32P]dCTP. Approximately 1.5 × 106 primary recombinants isolated from a rat renal cortex cDNA library (13) constructed in the plasmid vector pSPORT-1 were screened using standard colony hybridization techniques (21).Male Sprague-Dawley rats were used to prepare the renal cDNA library and were the source of the liver sat-1 cDNA. Nitrocellulose membranes were hybridized overnight at 42°C in a solution of 50% formamide, 5× Denhardt's solution, 5× SSC buffer (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 0.1% SDS, and 0.1 mg/ml herring sperm DNA. Blots were washed for 2 h with 0.1% SDS in 2× SSC buffer at 55°C. Selected colonies were purified through four rounds of screening. The nucleotide sequence of the renal sat-1 was determined using a T7 sequencing kit (Pharmacia-LKB Biotechnologies) with double-stranded DNA as the template.

For RT-PCR, total rat renal cortex RNA was prepared using the method of Chomczynski and Sacchi (3) and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega) for 1 h at 37°C employing a primer corresponding to sat-1 nucleotides 902–918. Transcribed cDNA was amplified by the PCR employing a forward primer consisting of liver sat-1 nucleotides 335–355 with an added 5' EcoR I
restriction site and a reverse primer consisting of sat-1 nucleotides 851–869. Amplification was performed for 30 cycles at 55°C with negative controls performed in the absence of template. PCR products were sequenced on an Applied Biosystem 373A fluorescent sequencer at the University of Iowa DNA Core Facility.

Construction of the histidine-tagged, sat-1 baculovirus transfer vector. Liver sat-1 cDNA was amplified by PCR using pfu polymerase (Stratagene) in a two-step process. The first PCR (30 cycles at 50°C) utilized a forward primer corresponding to published sat-1 nucleotides 335–355 (starting 32 nucleotides upstream from the presumed start codon) and includes an added EcoRI restriction site. The reverse primer corresponded to nucleotide sequence 1559–1578, downstream from the presumed start codon (30 cycles at 50°C) utilized a forward primer corresponding to sat-1 nucleotides 1483–1502 (upstream from the NheI restriction site). The reverse primer for the second PCR included 17 nucleotides immediately upstream of the presumed stop codon (2460–2476) followed by 18 nucleotides encoding 6 histidine residues, the stop codon, and a Bgl II restriction site (5′ GAAAGATCTCTAGTGTTGGTGTTGGTGTTGGAGGGCAGAGTCAGCAG 3′). Following the two PCRs, the two amplified products were digested with NheI, purified by agarose gel electrophoresis, then ligated with T4 ligase. This ligation product includes the entire open-reading frame of sat-1, a COOH-terminal 6-histidine tag, and added restriction sites to assist in cloning into the transfer vector. The recombinant baculovirus was generated by cotransfection with the BaculoGold AconPV virus (PharMingen) according to the manufacturer's instructions. Recombinant virus was amplified three times to a viral concentration of ~10^9 infectious particles per milliliter (16).

Cell culture and protein purification. SF9 cells (Invitrogen) were grown at 27°C in Grace's insect medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. Cells were seeded on 100-mm tissue culture plates at a density of 7 × 10^6 cells per plate and infected with recombinant baculovirus at a multiplicity of infection (MOI) of 0.5. The cells were rocked in the presence of virus for 1 h at room temperature then incubated at 27°C. Three days postinoculation, the cells were harvested by gentle streaming of the washed cell pellet in 1 ml uptake solution. Uptake of radioisotope was initiated by resuspending the washed cell pellet in uptake solution containing tris/oxalic acid, pH 8.0.

Calcium was eliminated from the uptake medium to avoid precipitation with oxalate. In preliminary studies, the uptake of 35SO_4^- was not different in the presence or absence of 2.8 mM CaCl_2. Uptake of radioisotope was initiated by resuspending the washed cell pellet in 1 ml uptake solution. Uptake solutions consisted of cell wash buffer with 50 mM K_2SO_4, 4 mM KCl, 8 mM HEPES, and 5 μM [35S]oxalate, or 5 μM [35S]oxalate in the presence or absence of 5 mM MgCl_2.

Anion transport in Sf9 cells. For transport assays, SF9 cells were seeded on 35-mm cell culture plates at a density of 1 × 10^6 cells per plate and infected with either recombinant baculovirus or wild-type virus at an MOI of 0.2. Two days postinfection, the cells were harvested by gentle streaming of the plates with media and transferred to individual 2.0-ml microcentrifuge tubes. Cells from a single 35-mm plate were used for each individual uptake determination, and uptakes were performed using cells from three different passages.

Cells were pelleted by centrifugation for 10 s at 2,000 g, then washed with 1 ml cell wash buffer consisting of 137 mM NaCl, 20 mM Tris, and 18 mM oxalic acid, pH 8.0, centrifuged again at 10,220 g, and resuspended in 3 ml Tris/oxalic acid buffer per plate (200 mM NaCl, 20 mM Tris, and 18 mM oxalic acid, pH 8.0), centrifuged at 10,220 g, and resuspended in 1.0 ml Tris/oxalic acid buffer per plate. The cells were sheared by multiple passes through a 22-gauge needle, and the cell membranes were pelleted with an Eppendorf microcentrifuge at 14,000 rpm for 5 min. The pellet was subsequently incubated at room temperature for 20 min in 1.0 ml Tris/oxalic acid buffer plus 0.5% SDS, centrifuged at 100,000 g, and the supernatant was removed for analysis of recombinant sat-1 protein.

To purify the histidine-tagged sat-1 protein, 1 ml of Talon metal affinity matrix (Clontech) was typically used for five plates of cells. The Talon beads were washed three times with 2.5 ml Tris/oxalic acid buffer plus 0.5% SDS prior to use. The solubilized SF9 cell protein was added to the Talon beads and incubated at room temperature on a rotating test tube mixer for 1 h. The protein/Talon mixture was transferred to a column and washed three times with 5 ml of Tris/oxalic acid buffer plus 0.5% SDS, followed by 3 ml of 500 mM NaCl, 10% glycerol, and 20 mM Tris/oxalic acid, pH 8.0. Elution of bound protein was achieved with 2.5 ml elution buffer containing 50 mM imidazole, 500 mM NaCl, 10% glycerol, and 20 mM Tris/oxalic acid, pH 8.0. The first 0.4 ml eluting from the column was discarded, and the protein eluting in the next 1.7 ml was saved. Protein concentration of the eluate was determined by the Pierce bicinchoninic acid method. Western blots were performed with the semidry transfer method. Primary antibody was detected with peroxidase-conjugated, antimouse secondary antibody using the Pierce SuperSignal chemiluminescent kit for development.
characterized as subclasses IgG₂ and IgG₁, respectively, and were purified from culture supernatants with immobilized protein A (Pierce).

Inmunohistochemistry and Western blot analysis. Male Wistar rats were anesthetized with intraperitoneal thiopental (100 mg/kg body wt) and perfused retrogradely at a pressure of 1.4 mbar through the abdominal aorta with a fixative of 3% paraformaldehyde and 0.05% picric acid in a 6:4 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mosmol/kg with sucrose) and 10% hydroxyethyl starch. After 5 min, the fixative was replaced by perfusing an additional 5 min with cacodylate buffer. Slices of fixed kidneys were frozen in liquid propane cooled by liquid nitrogen. Sections 3 μm thick were cut at –22°C in the cryomicrotome, mounted on chromalum/gelatin-coated glass slides, thawed, and placed into citrate buffer (0.01 M, pH 6.0). After 10 min of pulsed boiling in the microwave, the sections were transferred to cold PBS buffer. We found that the addition of the microwave antigen retrieval method (22) enhanced significantly the detection of sat-1 with the monoclonal antibodies.

For immunofluorescent staining, sections were preincubated for 5 min at room temperature in 3% dried milk powder in PBS containing 0.05% Triton X-100. Sections were covered for 2 h with monoclonal antibody diluted 1:100 in preincubation solution. The sections were rinsed three times with PBS then incubated for 45 min at room temperature in a 1:200 dilution of goat anti-mouse IgG conjugated to Cy3 (Jackson Immunoresearch). After rinse, the sections were placed under a coverslip with DAKO-Glycergel plus 2.5% 1,4-diazabicyclo[2.2.2]octane applied as a fading retardant. Sections were examined by epifluorescent microscopy using a narrow-band filter system for Cy3.

For localization by immunoblot analysis, microvillus mem-
brane vesicles from the rat and rabbit proximal tubule apical membrane were isolated using the Mg²⁺-aggregation method followed by sucrose-gradient ultracentrifugation as described previously (7). Basolateral membranes were purified from rat and rabbit renal cortices on a Percoll gradient (5). Protein concentrations were determined by the method of Lowry et al. (12) as modified by Peterson (17) using bovine serum albumin as the standard.

Materials. Carrier-free [35S]Sulfate (0.3 mCi/ml) was purchased from ICN. [14C]Oxalate (4.5 mCi/mmol), 1,4-
diazabicyclo[2.2.2]octane, aprotinin, and antipain were obtained from Sigma. Leupeptin, Pefabloc SC, and N-glycosi-
dase F were purchased from Boehringer Mannheim. Grace’s supplemented insect cell medium, fetal calf serum, and the pSPORT-1 vector were from Life Technologies (GIBCO-BRL). DAKO-Glycergel was supplied by Dakopatts, Glustrup, Denmark. Anti-6-histidine monoclonal antibodies were obtained fromDia nova.

RESULTS

Characterization of a cDNA clone encoding renal sat-1. On the basis of the observation that liver sat-1 hybridizes with kidney mRNA on Northern blots (2), the first set of experiments examined whether the rat kidney expresses a sulfate transporter identical or closely related to sat-1. A 32P-labeled fragment derived from the 5’ end of liver sat-1 (nucleotides 0–1327) was used to screen a rat renal cortex cDNA library. Five colonies were hybridization positive on the initial screen. Colony purification, restriction mapping, and sequence analysis of the 3’ and 5’ ends demonstrated that a single clone had been isolated with an insert size of ~2.0 kb. Preliminary sequencing demonstrated that the renal clone was missing the 5’ end of the predicted open-reading frame. To determine the complete coding sequence of the renal transcript, rat renal cortex total RNA was reverse transcribed, then amplified by PCR using a forward, gene-specific primer upstream from the presumed start codon of liver sat-1 and a reverse primer based on sequence 270 bases downstream from the 5’ end of the renal clone. The 3’ end of the resulting RT-PCR product had the expected overlap of 270 bases at the 5’ end of the renal clone, confirming that the RT-PCR product derived from rat renal cortex total RNA represents the 5’ coding region of the renal sat-1 cDNA. The renal clone and the 5’ RT-PCR product were completely sequenced in both directions and compared with liver sat-1.

The combined sequence of the renal clone and RT-
PCR product is identical to the liver sat-1 sequence corresponding to published nucleotides 335–2576 with the exception of two isolated base substitutions. An A-for-G substitution at nucleotide 1849 does not alter the predicted amino acid sequence for sat-1; however, a substitution of A for C found at sat-1 nucleotide 2388 would result in the substitution of a glutamic acid for alanine at position 674 of the renal clone. In addition, the 3’-untranslated region of the renal clone is 1,135 nucleotides shorter than that of liver sat-1. On Northern blot analysis both rat liver and kidney mRNA, probed with a 32P-labeled fragment of liver sat-1, have hybridization signals ~3.7 kb in size (2). The signifi-
cance of the truncated 3’-untranslated region of the renal clone is unknown. Taken together, these results demonstrate that the renal clone and the liver sat-1 clone probably arise from the same gene.

Expression of histidine-tagged sat-1 in Sf9 cells. The sat-1 protein is an epithelial transporter that would not be expected to be found in high abundance under native conditions. Therefore, to simplify purification of the sulfate/anion exchanger, we introduced a 6-histidine tag to the COOH terminus of liver sat-1 to allow for selective purification using metal affinity chromatography (6). Based on the predicted amino acid sequence of sat-1, the addition of a histidine tag to the COOH terminus would not be expected to alter the integrity of the protein; however, prior to this study, a functional requirement for the native COOH terminus has not been tested. The insert was subsequently subcloned into the pVL1393 baculovirus transfer vector and used for expression of the histidine-tagged sulfate/anion exchanger in Sf9 insect cells.

Insect cells infected with recombinant virus strongly express a 68-kDa protein (identified with the arrow in Fig. 1) that is not present in either uninfected Sf9 cells (Fig. 1, control) or in cells infected with the wild-type baculovirus. The molecular mass of the expressed protein is similar in size to that predicted for sat-1 (2). The intense band of ~30 kDa seen in cells infected with wild-type virus represents the highly expressed viral polyhedrin protein. Expression of polyhedrin is blocked in Sf9 cells infected with recombinant virus as a result of the insertion of the sat-1 gene downstream from the
polyhedrin promoter. The last lane in Fig. 1 shows a Western blot of a protein homogenate from Sf9 cells infected with recombinant, histidine-tagged, sat-1 virus. A single 68-kDa band is identified by a monoclonal antibody directed against the 6-histidine tag. The anti-6-histidine antibody does not react with uninfected Sf9 cells or with cells infected with the wild-type virus (data not shown). These results demonstrate that the histidine-tagged, sat-1 protein can be expressed at a high level in Sf9 cells.

Expression of sulfate and oxalate transport in Sf9 cells. To determine whether the expressed histidine-tagged protein is capable of performing anion exchange, we measured the uptakes of $^{35}\text{SO}_4^2-$ and $[^{14}\text{C}]\text{oxalate}$ in Sf9 cells infected with either recombinant or wild-type baculovirus. Sulfate and oxalate are substrates for the sulfate/anion exchanger, and their transport would be expected to be increased in cells expressing a functional sat-1 protein. These experiments were performed in the absence of sodium, to eliminate the influence of endogenous sodium-dependent transport on sulfate or oxalate uptake. As shown in Fig. 2, there is a marked stimulation of sulfate and oxalate uptake in Sf9 cells 48 h postinfection with recombinant virus compared with wild-type virus, consistent with the expression and membrane insertion of functional sat-1. Furthermore, the presence of 5 mM oxalate in the uptake medium abolished the uptake of $^{35}\text{SO}_4^2-$ (Fig. 2A), whereas 5 mM sulfate inhibits $[^{14}\text{C}]\text{oxalate}$ uptake by 73% (Fig. 2B). Cis-inhibition of $^{35}\text{SO}_4^2-$ uptake by oxalate, as well as $[^{14}\text{C}]\text{oxalate}$ uptake by sulfate, provides additional evidence that the transport of these anions is mediated by the sat-1 transporter. In contrast, cis-inhibition of $^{35}\text{SO}_4^2-$ and $[^{14}\text{C}]\text{oxalate}$ uptake is not observed in cells infected with wild-type virus, suggesting that the small component of endogenous sulfate and oxalate uptake in Sf9 cells is mediated by transport proteins unrelated to sat-1.

Purification of the sat-1 protein. To purify the histidine-tagged sat-1 protein from Sf9 cells, it was necessary to first determine the optimal conditions for solubilization of sat-1. Homogenates of infected Sf9 cells were washed 30 min in the presence of various detergents and centrifuged at 100,000 g, and the supernatant and pellet were examined by Western blot analysis using an anti-6-histidine antibody as the probe. Following incubation in Triton X-100 (2%), octyl-glucoside (2%), or 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) (0.8%), the sat-1
protein was detected only in the pellet, demonstrating that sat-1 expressed in SF9 cells is mostly insoluble in these detergents. In contrast, sat-1 was detected in the supernatant and not the pellet when solubilized in 0.5% SDS; therefore, SDS was used in all subsequent studies. The addition of protease inhibitors (1.0 µg/ml aprotinin, 50 µg/ml antipain, 0.5 µg/ml leupeptin, 0.5 mg/ml Pefabloc SC, and 1.0 mM disodium EDTA) during harvesting and solubilization with SDS did not alter the molecular mass of sat-1 (not shown), demonstrating that the 68-kDa sat-1 protein is not a proteolytic fragment of a larger polypeptide.

To purify sat-1, solubilized protein from SF9 cells infected with recombinant baculovirus was applied to a metal affinity matrix (6). Following extensive washing, the bound protein was eluted with 50 mM imidazole at pH 8.0. Shown in lane 1 of Fig. 3 is the starting protein homogenate from SF9 cells infected with recombinant baculovirus. In contrast to lane 1 of Fig. 3, a single protein band with a molecular mass of 68 kDa (lane 3) is observed in the fraction eluted with imidazole. The faint lower molecular weight bands represent artifacts of the silver-stained gel, as similar bands are observed in the absence of added protein (lane 2). Anti-6-histidine antibodies recognize the 68-kDa protein on Western blot analysis (lane 4), confirming that the purified protein represents recombinant sat-1.

Immunolocalization of sat-1 in the kidney by Western blot analysis. To localize the sat-1 protein in the kidney, two monoclonal antibodies, IVH4 and IID4, were generated against the purified, histidine-tagged, sat-1 protein. To determine the sensitivity and specificity of the antibodies, Western blots were performed against protein homogenates from SF9 cells infected with either recombinant or wild-type virus. The immunoblot in Fig. 4 shows that the anti-sat monoclonal antibody IVH4 recognizes a single protein with a molecular mass of 68 kDa in homogenates from SF9 cells infected with recombinant baculovirus (lane 1) but not in homogenates from cells infected with the wild-type virus (lane 2). An immunoblot of protein from SF9 cells infected with recombinant virus and probed with anti-histidine-tag antibodies is shown for comparison (Fig. 4, lane 3) and demonstrates that the IVH4 antibody recognizes the sat-1 protein when expressed in SF9 cells. Similar results were obtained with antibody IID4. In addition, both monoclonal antibodies identify the purified sat-1 protein by dot-blot analysis at dilutions of 1:10,000 (data not shown). Taken together, these results demonstrate that the anti-sat monoclonal antibodies are directed against the histidine-tagged sat-1 protein.

The anti-sat monoclonal antibodies were next used to immunolocalize sat-1 expression in the kidney. Functional studies in membrane vesicles demonstrate sulfate/oxalate exchange on both the apical and basolateral membranes of the proximal tubule (10, 11). To determine whether sat-1 could account for sulfate/oxalate exchange on either the apical or basolateral membranes, isolated membrane fractions were probed with anti-sat antibodies. Figure 5 shows that a single protein is recognized on Western blots of rabbit and rat basolateral membranes (lanes 1 and 3, respectively). Interestingly, for equal amounts of protein, the observed signal is much greater with rabbit basolateral membranes than with rat basolateral membranes, despite the fact that rat sat-1 protein was used to
Immunolocalization of sat-1 in the kidney. Western blots were performed on 40 µg brush border or basolateral membrane protein isolated from the rat and rabbit renal cortex and separated on a 6–12% linear gradient SDS-polyacrylamide gel. Lane 1, rabbit basolateral membrane; lane 2, rabbit brush-border membrane; lane 3, rat basolateral membrane. IID4 anti-sat monoclonal antibody was used as the primary antibody at a 1:100 dilution.

generate the monoclonal antibodies. Relative enrichments of the basolateral membrane fractions were similar for both species. These results suggest that the rabbit has a higher level of expression of sat-1 in the kidney than the rat. In contrast to the results with basolateral membranes, no protein is recognized by anti-sat antibodies on Western blots of either rabbit apical brush-border membranes (Fig. 5, lane 2) or rat brush-border membranes (not shown), suggesting that in the proximal tubule the sat-1 protein is directed only to the basolateral membrane.

The 80-kDa size of the band observed on the immunoblots in Fig. 5 is greater than that expected from the deduced amino acid sequence of sat-1 or that seen on immunoblots of protein expressed in SF9 cells (Fig. 4). To determine whether the higher apparent molecular mass of sat-1 in native membranes is the result of N-glycosylation, we examined the electrophoretic mobility of basolateral membrane sat-1 protein and recombinant sat-1 protein before and after treatment with N-glycosidase F. As can be seen in Fig. 6, treatment with N-glycosidase F did not alter the mobility or appearance of the 68-kDa protein from SF9 cells. In contrast, the electrophoretic mobility of sat-1 from rat or rabbit basolateral membranes is increased following deglycosylation, migrating to a molecular mass of 68 kDa. These results suggest that sat-1 is glycosylated under native conditions but has incomplete or absent N-linked glycosylation when expressed in SF9 cells.

Immunohistochemical localization of sat-1 in the rat kidney. Using anti-sat antibody IVH4 as the probe, immunohistochemical identification of sat-1 in the rat kidney is shown in Fig. 7. At lower power (Fig. 7A), it can be seen that immunostaining is strongest in the proximal convoluted tubules of the cortical labyrinth. Staining is less intense in the proximal straight tubules of the medullary rays, with decreasing intensity toward the outer stripe of the outer medulla. At higher magnification (Fig. 7B), staining is observed only in the proximal tubule, with the most intense staining in the S1 and S2 segments and diminished staining in the S3 segment. No staining is observed in the glomerulus itself or in the distal tubule. As shown in Fig. 7C, the sat-1 protein is clearly confined to the infoldings of the proximal tubule basolateral membrane. The intermittent gaps observed in the basolateral infolding of the S1 and S2 segments probably represent cell nuclei. Similar interruptions have been observed with antibodies directed against other proteins found on the basolateral membrane of the proximal tubule (9, 20). The different pattern of staining in the S3 segment is the result of less infolding of the basolateral membrane in this nephron segment. An identical pattern of staining was observed with anti-sat monoclonal antibody IID4.

**DISCUSSION**

In the present study, we have isolated from the rat kidney a partial cDNA encoding sat-1. The sat-1 transport protein was originally cloned from the rat liver and accepts sulfate, oxalate, and bicarbonate as substrates (2). In the kidney, a sulfate/bicarbonate/oxalate exchanger has been identified on both the basolateral and apical membranes of the proximal tubule (10, 11). The basolateral exchanger serves as an important mechanism for sulfate exit from the cell, with oxalate probably entering the proximal tubule in exchange for sulfate. It has been proposed that intracellular oxalate provides the driving force for the proximal reabsorption of chloride via luminal chloride/oxalate exchange (7). In turn, oxalate is recycled back into the cell, possibly via the apical membrane sulfate/oxalate exchanger, so that intracellular substrate is available to maintain chloride reabsorption (23). Supporting this hypothesis is the observation by Wang et al. (25) that the addition of 1 µM oxalate to luminal and peritubular perfusates stimulates net chloride reabsorption in micropерfused rat kidneys by 38%. This effect was eliminated upon removal of sulfate from the perfusates (25).

Given the functional similarities between sat-1 and the proximal tubule apical and basolateral membrane...
sulfate/anion exchangers, we undertook the present study to determine whether one or both of the sulfate/anion exchangers in the proximal tubule are related to the sat-1 protein. To address this question, we generated anti-sat-1 monoclonal antibodies and have confirmed by both Western blot analysis and immunohistochemistry that the sat-1 sulfate/bicarbonate/oxalate exchanger is present on the basolateral membrane of the rat proximal tubule. The immunofluorescent signal on the basolateral membrane is more intense in the S1 and S2 segments of the proximal tubule compared with S3. However, the early segments of the proximal tubule have a greater basolateral membrane surface area; thus, the number of sat-1 molecules per membrane area may not be different between the early and late segments. Our inability to identify sat-1 on the apical membrane using two different techniques, provides strong evidence that apical sulfate/oxalate exchange is not via the sat-1 protein. However, the RT-PCR method used to detect the 5' end of sat-1 in the kidney may not detect alternatively spliced transcripts and does not exclude the possibility that additional members of the sulfate/anion exchange gene family are present in the kidney. The conclusion that the apical and basolateral exchangers are different proteins is consistent with our previous observation that these two sulfate/oxalate exchangers have different sensitivities to inactivation by the detergent octylglucoside (8).

In addition to the proximal tubule, oxalate-dependent chloride reabsorption has been described in the distal nephron (24). The mechanism is thought to involve the apical exchange of intracellular oxalate for luminal chloride in parallel with Na/H exchange. As in the proximal tubule, for this to occur, an oxalate entry step across the basolateral membrane and a method of oxalate recycling across the apical membrane are required. The sat-1 protein, or a similar anion exchange protein, would be a likely candidate to provide either of these oxalate transport steps. Our immunohistochemical data indicates that sat-1 is present only in the

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**Fig. 7. Immunohistochemical localization of sat-1 in the rat kidney.**

A: overview of perfusion-fixed rat kidney sections treated with anti-sat antibody IVH4. Immunostaining is most intense in proximal convoluted tubules of the cortical labyrinth (CL). In medullary rays (MR), there is decreasing intensity in the proximal straight tubules toward the outer stripe (OS) of the outer medulla. Only faint staining is detected in the late segments of the proximal tubules in the OS. Dashed line delineates the MR from the CL and the boundary between the OS and the inner stripe (IS) of the outer medulla. Bar = 100 µm. B: immunolocalization of sat-1 in the proximal tubule; sat-1 is detected in the S1, S2, and S3 segments of the proximal tubule. Note the positive staining as the proximal tubule begins at the urinary pole of the glomerulus (G). Staining is absent from the glomerulus itself and in the distal nephron (D). Bar = 25 µm. C: at higher resolution, sat-1 is clearly confined to the infoldings of the basolateral membrane of the proximal tubule cells. Bar = 10 µm.
proximal tubule; therefore, the mechanism of oxalate transport in the distal tubule remains to be determined. It is also possible that different methods of immunohistochemical fixation or epitope unmasking would identify sat-1 in other nephron segments.

To purify the sulfate/bicarbonate/oxalate exchange protein, we expressed sat-1 in SF9 cells and added a COOH-terminal, 6-histidine tag to simplify purification. Purification of solubilized sat-1 was achieved in a single step using a metal affinity matrix to bind the histidine-tagged protein. It had been hoped that the purified sat-1 protein could be solubilized and reconstituted, to study its function in an isolated membrane system; however, sat-1 required strong denaturants such as SDS for solubilization. Limited solubility was observed in nondenaturing detergents such as CHAPS, octylglucoside, and Triton X-100. The sat-1 protein is an integral membrane protein with 12 transmembrane domains predicted by hydrophobicity analysis (2). Conceivably, the hydrophobic nature of the protein favors the formation of protein aggregates when sat-1 is expressed at high levels in SF9 cells, making it relatively insoluble in the nonionic detergents tested. In fact, we have observed that anti-sat monoclonal antibody IVH4, when coupled to protein A Sepharose, will immunoprecipitate sat-1 from rabbit renal basolateral membranes solubilized in the nonionic detergent octylglucoside (data not shown). These results are consistent with the limited solubility of sat-1 being related to its expression in SF9 cells but not in the native state.

Treatment of basolateral membranes with N-glycosidase F increased the mobility of sat-1 on SDS-PAGE from an apparent molecular mass of ~80 kDa to a sharp band of 68 kDa. This is similar to the size predicted by its amino acid sequence and suggests that sat-1 is N-glycosylated under native conditions. In contrast, sat-1 is probably not N-glycosylated in SF9 cells, despite the presence of three N-linked glycosylation sites (2). The posttranslational glycosylation of expressed proteins by SF9 cells is highly variable. In general, SF9 cells are capable of recognizing and initiating glycosylation of expressed mammalian proteins but are unable to process immature oligosaccharides to a complex form (16). In some reports, the formation of complex oligosaccharides has been observed during the late phase of expression in SF9 cells, whereas in other reports, proteins that are expressed in the unglycosylated form in SF9 cells are found to be fully glycosylated when expressed in mammalian cell lines (13, 19).

To test the function of sat-1 following expression in SF9 cells, we measured the rate of $^{35}$SO$_4$$_2$$^-$ and [H]$^1$CO oxalate uptake in cells infected with either recombinant or wild-type baculovirus. Cells infected with recombinant baculovirus exhibited a marked increase in sulfate and oxalate uptake compared with cells infected with wild-type virus and demonstrated the expected pattern of cis-inhibition. These studies confirm that the histidine tag does not interfere with the transport function of sat-1. In addition, since our studies have shown that sat-1 is probably not N-glycosylated when expressed in SF9 cells, it would appear that complete N-linked glycosylation is not required for either the insertion of sat-1 into the plasma membrane or for sat-1 to maintain transport function. Additional experiments are required to determine whether the lack of N-linked glycosylation results in an alteration of transport function that may not be detected by the methods employed in these studies.

In summary, we have cloned sat-1 from the rat renal cortex and have identified this as a product of the same gene expressing sat-1 in the liver. The sat-1 protein has been expressed in SF9 cells and retains its transport function, despite incomplete N-linked glycosylation. Immunohistochemistry and Western blot analysis have localized sat-1 to the basolateral membrane of the proximal tubule, demonstrating that the apical and basolateral sulfate/oxalate exchangers represent two different transport proteins.

The rat liver sat-1 cDNA was a generous gift from Dr. B. Hagenbuch and Dr. P. Meier. The generation of the monoclonal antibodies was accomplished with the technical assistance of Krista Wheeler and the University of Iowa Hydridoma facility.

This work was supported by a Senior International Fellowship from the Fogarty Center at the National Institutes of Health, by grants from the Department of Veterans Affairs Office of Research and Development, the American Heart Association, and Ciba-Geigy-Jubilaeums-Stiftung (Basel), and by Swiss National Science Foundation Grants 32-30785.91 and 31-46523.96 (to H. Murer).

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Received 7 November 1997; accepted in final form 6 March 1998.

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