Expression of Rat Renal Sulfate Transport Systems in Xenopus laevis Oocytes

FUNCTIONAL CHARACTERIZATION AND MOLECULAR IDENTIFICATION*

(Received for publication, July 15, 1993, and in revised form, October 4, 1993)

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Renal proximal tubular sulfate reabsorption is mediated by brush border membrane Na+/sulfate-cotransport and basolateral Na+-independent sulfate transport. Injection of rat kidney cortex mRNA into Xenopus laevis oocytes induced Na+-dependent as well as Na+-independent sulfate transport. The inhibition pattern of Na+-dependent uptake coincided with that known for the brush border membrane; the inhibition pattern of Na+-independent uptake suggested that this activity could be related to the basolateral cell surface. By Northern blot hybridization of size-fractionated mRNA, we provide evidence that the Na+-dependent uptake is induced by an mRNA species related to a recently cloned cDNA encoding rat renal cortex Na+/SO4 cotransport (NaSI-1; Markovich, D., Forgo, J., Stange, G., Biber, J., and Murer, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8073–8077); the Na+-independent sulfate transport activity seems to be related to an mRNA species encoding a rat liver Na+-independent sulfate transporter (Bissig, M., Hagenbuch, B., Steiger, B., Koller, T., and Meier, P. J. (1994) J. Biol. Chem. 269, 3017–3021). Hybrid depletion experiments using antisense oligonucleotides provided further evidence for the association of the expressed transport activities to NaSI-1 and sat-1, respectively.

Inorganic sulfate is freely filtered at the renal glomerular capillaries and to a large extent reabsorbed in the proximal tubule (1); brush border and basolateral membrane transport steps have been characterized by a variety of techniques (2–20) (for review, see Ref. 21). A Na+-sulfate-cotransporter mediates brush border membrane sulfate uptake (10, 14, 16–18); the stilbene derivative DIDS is a poor inhibitor of this transporter (10, 22) (for review, see Ref. 21). At the basolateral cell surface, a stilbene-sensitive, Na+-dependent transporter is mediating sulfate transport (9–11, 13, 15) (for review, see Ref. 21). In some species, including rat, rabbit, and bovine, an anion exchange system seems also to be present in brush border membranes (23–25).

Two cDNAs most likely involved in sulfate transport were recently identified: a cDNA (NaSI-1) related to rat renal cortex Na+/sulfate cotransport (26) and a cDNA (sat-1) related to a hepatocanalicular Na+-independent sulfate transport system (27). Sequence comparison between NaSI-1 and sat-1-deduced proteins results in 44.6% similarity and 18.5% identity (26, 27) (Beslif, GCG-program). Northern blot analysis of sat-1 cDNA against rat kidney cortex mRNA showed very strong levels of hybridization (27).

The purpose of the present study was to use the Xenopus laevis oocyte system to characterize rat kidney cortex mRNA-induced sulfate transport. The data obtained from: 1) substrate specificities of the expressed uptake activities, 2) the close association of transport activities with Northern blot hybridization, and 3) the effects of hybrid-depletion, together document that Na+-dependent and Na+-independent sulfate transport activities are entirely related to the above cDNAs (NaSI-1 and sat-1).

MATERIALS AND METHODS

X. laevis Oocytes and Transport Assay—All techniques and methods concerning the handling of oocytes, as well as the transport assay, have been described earlier (28–31). Oocytes were injected with 50 nl of H2O, containing no or 0.005–1 µg/ml rat renal cortex mRNA, size-fractionated rat renal cortex mRNA (5–10 ng/oocyte), NaSI-1 cRNA (1 ng/oocyte), or sat-1 cRNA (0.1 ng/oocyte). After 1–6 days, uptake of 36SO42− (DuPont NEN) was measured, either in the presence or absence of sodium as described earlier (28–33). Hybrid depletion was performed, as described earlier (34), using the following oligonucleotides. NaSI-1 oligonucleotides: 5′ sense (5′s) at position 92–107 bp: 5′ GGA GAA GAT CAG GTT 3′; 3′ sense (3′s) at position 1042–1057 bp: 5′ ATC GTG ACC 'ITG 3′; 5′ antisense (5′A) at position 1680–1695 bp: 5′ CAC CACAGC 'ITG 3′; 3′ antisense (3′A) at position 1042–1057 bp: 5′ ATC GTG ACC 'ITG 3′; 3′ antisense (3′A) at position 1680–1695 bp: 5′ CAC CAC AGC ACC A 3′; sat-1 oligonucleotides: 5′ antisense (5′A) at position 593–608 bp: 5′ TGC CAA TGA CCAATC C 3′; 3′ sense (3′s) at position 605–624 bp: 5′ GGC ATT ATC GTG CCT GTG CA 3′; 3′ antisense (3′A) at position 2388–2403 bp: 5′ AAC AGC AGC TCC TCT G 3′; 3′ sense (3′s) at position 2384–2401 bp: 5′ ACT GCA GAG GAG CCT GTG 3′.

Statistics—Significance of differences was calculated by analysis of variance (ANOVA). The Fisher's least significance difference test was used as a multiple comparison method and considered significant when p ≤ 0.01 (99% probability). When the Michaelis-Menten and Hill equations were used, calculations were performed using non-linear regression.

Isolation of RNA, mRNA, and Sucrose Fractionation—Kidney cortex tissue was extracted from male Sprague-Dawley rats (180–200 g). Total RNA was extracted from rat kidney cortex according to the protocol described elsewhere (31, 32), and mRNA was isolated and size-fractionated by sucrose density gradient centrifugation as described earlier (29, 31, 34).

Northern Analysis—mRNA (0.5 µg/lane) was denatured, electrophoresed in 1% agarose, formaldehyde gels and transferred to GeneScreen membranes (DuPont NEN). cDNA probes of NaSI-1 (full-length) and sat-1 cDNA (full-length) were labeled by random priming (Pharmacia LKB Biotechnology Inc.) using α-32PdCTP (Amersham...
RESULTS

The injection of rat renal cortex mRNA into X. laevis oocytes gave rise to a saturable stimulation of sulfate transport, both in the presence and absence of Na⁺ (Fig. 1A). This suggests the presence of two separate SO₄ transport systems: a Na⁺-dependent and a Na⁺-independent pathway. The oocytes themselves (water-injected) showed an intrinsic >10-fold Na⁺-stimulated SO₄ transport (Na⁺ medium), as compared to the Na⁺-independent SO₄ transport rate (choline medium).

Rat renal cortex total mRNA was size-fractionated through a continuous sucrose-density gradient (Fig. 1B). Maximal net Na⁺-dependent stimulation of sulfate uptake (subtraction of choline values) was obtained with fractions 13 and 14, whereas maximal Na⁺-independent SO₄ transport was with fractions 10 and 11. Fraction 11 gave rise to the highest total SO₄ uptake, as well as Na⁺-independent SO₄ uptake, but produced a lower net Na⁺-dependent SO₄ uptake than fractions 13 and 14.

For SO₄-interaction, a saturable Na⁺-dependent and Na⁺-independent SO₄ transport was observed (Fig. 2A). Using simple Michaelis-Menten kinetics, the following values were obtained: for net Na⁺-dependent SO₄ uptake, \( V_{\text{max}} = 37.3 \pm 2.2 \) pmol/oocyte/h, \( K_m = 0.6 \pm 0.1 \) mm; for Na⁺-independent SO₄ uptake, \( V_{\text{max}} = 15.8 \pm 2.2 \) pmol/oocyte/h, \( K_m = 3.4 \pm 0.9 \) mm. For Na⁺-interaction of Na⁺-dependent SO₄ uptake, a sigmoidal pattern was observed (Fig. 2B), with the following values determined, according to the Hill equation: \( V_{\text{max}} = 26.1 \pm 2.3 \) pmol/oocyte/h, \( K_m = 33.7 \pm 3.4 \) mm and the Hill-coefficient, \( n = 2.5 \pm 0.5 \). The values calculated for Na⁺- and sulfate interactions of mRNA-induced Na⁺-dependent SO₄ uptake, are in agreement with studies observed in brush border membrane vesicles (14, 16, 17, 18, 19, 22). Similarly, the values for the sulfate-interaction of mRNA-induced Na⁺-independent SO₄ uptake, are comparable, in order of magnitude (\( K_m > 1 \) mm), to studies performed on the basolateral membrane system, either with vesicles (15) or in microperfusion studies (10). Above kinetic parameters of mRNA-induced Na⁺-dependent and Na⁺-independent sulfate uptake are different from intrinsic oocyte transport activities; e.g. a Hill-coefficient for Na⁺ interaction below unity has been observed (30).

Using thiosulfate, a competitive inhibitor of SO₄ transport (10) (for review, see Ref. 21), almost complete inhibition of Na⁺-independent and Na⁺-dependent SO₄ transport was observed with 5 mm thiosulfate (Fig. 3A). A still more specific, DIDS (an anion exchange inhibitor), almost complete inhibition of Na⁺-independent SO₄ transport was observed with 3 mm DIDS, whereas very weak inhibition of Na⁺-dependent SO₄ transport was observed with 5 mm DIDS (Fig. 3B). For specificity of transport, PO₄ was used as a control (at concentrations 0–5 mm as inhibitor) and showed no effect on either Na⁺-dependent or Na⁺-independent SO₄ transport (data not shown). The above data suggested that the induced transport signals were most likely produced by two distinct transport systems. Na⁺-independent sulfate uptake may have a dual membrane localization (i.e. on the apical and basolateral membrane). Strong inhibitors of the rat basolateral SO₄/HCO₃ anion-exchange system are oxalate, probenecid, and phenol red, as well as numerous organic and inorganic compounds (23); whereas probenecid and (chloro)phenol red have been shown to have a weak or negligible effect on the rat apical SO₄/HCO₃ exchanger (10). In agreement with these properties, probenecid (1 mm), phenol red (1 mm) and oxalate (1 mm) showed strong inhibition of mRNA-induced Na⁺-independent SO₄ transport in oocytes (Fig. 4). These compounds showed no inhibition of the mRNA-induced Na⁺-dependent sulfate transport. Furthermore, a weak or negligible effect (1 mm) showed no significant inhibition of either mRNA-induced Na⁺-dependent or Na⁺-independent SO₄ uptake (Fig. 4). These results suggested that the expressed Na⁺-independent sulfate transport activity is most likely attributed to the basolateral membrane.

To determine whether the two observed mRNA-induced transport activities were related to two recently cloned sulfate transporters (see Introduction) (26, 27), we performed the following experiments. First, we compared the inhibition pattern (using various inhibitors) of NaSi-1-cRNA-induced uptake with that of mRNA-induced Na⁺-dependent SO₄ uptake and found them to be different (4). Similarly, sat-1 cDNA-induced uptake showed an identical inhibition pattern to that of mRNA-induced Na⁺-independent SO₄ transport (i.e. inhibition by probenecid, phenol red, and oxalate, but not by succinate or cholate; Fig. 4). Second, using both NaSi-1 and sat-1 cDNAs, we
performed Northern blot hybridization (Fig. 5), against a blot containing rat renal cortex mRNA fractions (from Fig. 1B). NaSi-1 hybridized most strongly with mRNA fractions 13–15 and sat-1 with mRNA fractions 9–12 (Fig. 5). Third, we performed hybrid depletion experiments. Antisense oligonucleotides derived from NaSi-1 and sat-1 cDNA sequences led independently to a prevention (at least to its major extent) of mRNA-induced Na+/SO4 cotransport and Na+-independent SO4 transport, respectively (Fig. 6), whereas sense oligonucleotides had no effect on the expression of either transport system.

**DISCUSSION**

In our experiments, we observed expression of both Na+-dependent and Na+-independent SO4 transport induced by rat kidney cortex mRNA injected into X. laevis oocytes. These two expressed transport activities showed different inhibition patterns: Na+-independent (but not Na+-dependent) SO4 transport was inhibited by the stilbene derivative DIDS (and SITS; data not shown). In addition, mRNA-induced Na+-independent SO4 uptake was inhibited by probenecid and phenol red, which in-
Asterisks thiosulfate 1 Na+-dependent after injection in a sodium-containing (NaCl 100 m) and sodium-free uptake; oocyte), or sat-1 cRNA (0.1 ng/oocyte). Transport was measured as percentage of control for 7-10 oocytes per condition, with the controls 50 nl rat kidney cortex mRNA (20 ng/oocyte), Nasi-1 cRNA (1 ng/m, phenol red 1 m, oxalate 1 m, succinate 1 m, cholate 1 m, thiosulfate 1 m, and control, with no inhibitor present. Data are shown as percentage of control for 7-10 oocytes per condition, with the controls (100%) having the following uptake rates: Na+-independent SO transport (mRNA, Cho): 9.1 pmol SO/oocyte-h; sat-1 cRNA-induced Na+-independent SO transport (sat-1, Cho): 312 pmol of SO/oocyte-h; net Na+-dependent SO transport (mRNA total SO uptake minus choline uptake; mRNA, Na): 9.3 pmol of SO/oocyte-h; Nasi-1 cRNA-induced Na+-dependent SO transport (Nasi-1, Na): 1870 pmol of SO/oocyte-h. Asterisks (*) represent significance versus control, for 99% probability (p < 0.01). The data are representative of three similar experiments.

**Fig. 5. Northern blot hybridization using Nasi-1 and sat-1 cDNA probes against sucrose gradient fractionated rat kidney cortex mRNA.** The same fractionated mRNA assayed for transport (as for Fig. 1B), was electrophoresed through a 1% formaldehyde agarose gel (0.5 μg mRNA/lane), blotted to GeneScreen membrane, and hybridized individually with random primed α-[32P]dCTP-labeled probes of full-length Nasi-1 cDNA and full-length sat-1 cDNA. The sat-1 cDNA hybridizes to a 3.6-kb signal, most strongly, in mRNA fractions 9–12; Nasi-1 cDNA hybridizes to two signals 2.3 and 2.9 kb, most strongly in mRNA fractions 13–15. Blots were washed at high stringency.

**Fig. 6. Hybrid depletion of the mRNA-induced SO transport using single-stranded oligonucleotides.** Oocytes were either injected with 50 nl of water, 50 nl of mRNA (15 ng/oocyte), or 50 nl of mRNA (15 ng/oocyte) annealed with a sense (S) or antisense (A) oligonucleotide (20 μm (~2.5 ng)), located either at the 5'- or 3'-end (within the open reading frame) of Nasi-1 (left panel) or sat-1 (right panel) cDNAs, respectively (see “Materials and Methods”). Transport was measured 4 days after injection, and the data are presented as net Na+-dependent SO transport (left panel; choline values subtracted from total SO uptake values (100 mM NaCl medium)) and Na+-independent SO uptake (right panel; 100 mM choline chloride medium) using K2SO4 (0.5 mM) as substrate, plus 35SO4 at an activity of 20–40 μCi/ml. Data are shown as means ± S.E. for 7–10 oocytes per condition, and are representative of at least two experiments.
duced transport function is perhaps modified by an “accessory” protein, also being present in the native tissue and co-expressed in oocytes after the injection of kidney cortex mRNA. Such “modification” of sat-1-related transport function seems to be kidney-specific and does not occur in the liver, as the apparent $K_m$ values are similar for sat-1 cRNA- and liver mRNA-induced, as well as for canalicular vesicle Na$^+$-independent sulfate uptake (27). The reduced sulfate affinity ($K_m$) of the putative kidney sat-1 protein is in agreement with $K_m$ values obtained from studies performed on renal basolateral SO$_4^{2-}$ uptake, both in vesicles (15) and microperfused isolated tubules (10).

In conclusion, we were able to demonstrate both by functional studies (hybrid depletion; pattern of inhibition) and by structural homologies (Northern blot analysis), that NaSi-1- and a sat-1-like mRNA transcript are most likely responsible for the sulfate transport activities observed upon injection of rat renal cortex mRNA into oocytes. Thereby, a NaSi-1-related mRNA transcript encodes the rat renal brush border Na$^+$SO$_4^{2-}$ cotransporter and a kidney cortex-homologue of the liver sat-1-like mRNA transcript most likely encodes the basolateral Na$^+$-independent SO$_4^{2-}$ anion exchanger.

Acknowledgments—Our gratitude goes to C. Gasser for help in preparing the figures and K. Mani for secretarial work.

REFERENCES