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Expression cloning of the cDNA for a polypeptide associated with rat hepatic sinusoidal reduced glutathione transport: Characteristics and comparison with the canalicular transporter

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ABSTRACT **Using the *Xenopus* oocyte expression system, we previously identified an ≈4-kb fraction of mRNA from rat liver that expresses sulfobromophthalein reduced glutathione *S*-conjugate (BSP-GSH)-insensitive and an ≈2.5-kb fraction expressing BSP-GSH-sensitive reduced glutathione (GSH) transport. From the former, a 4.05-kb cDNA was cloned and characterized as the putative rat canalicular GSH transporter. Starting with a cDNA library constructed from the ≈2.5-kb fraction, we have now isolated a single clone that leads to expression of a BSP-GSH- and cystathionine-inhibitable GSH transporter activity with K_m ≈ 3 mM characteristic of the sinusoidal GSH transporter. The cDNA for the rat sinusoidal GSH transporter-associated polypeptide (RsGshT) is 2733 bases with an open reading frame of 1059 nucleotides encoding a polypeptide of 353 amino acids (39,968 Da) with two putative membrane-spanning domains. No identifiable homologies were found in searching various data bases. An ≈40-kDa protein is generated in *in vitro* translation of cRNA for RsGshT. Northern blot analysis revealed a single ≈2.8-kb transcript in rat and human liver with negligible hybridization signal in other organs. The abundance of mRNA for RsGshT did not increase with phenobarbital treatment. Cis-inhibition by BSP-GSH and trans-inhibition by cystathionine and lack of induction by phenobarbital are characteristic of sinusoidal GSH secretion and thus indicate that RsGshT either encodes the sinusoidal GSH transporter itself or a regulatory subunit of the transporter that determines its liver-specific activity.**

Reduced glutathione (GSH) is widely recognized as a key factor in cell defense; it serves as a substrate for GSH *S*-transferases and GSH peroxidases as well as a radical scavenger and regulator of protein thiol-disulfide status (1, 2). Another proposed critical function of GSH is to store and transfer cysteine (1, 2). In the rat, nearly all plasma GSH is derived from the liver (1, 2). Plasma GSH has a very short half-life of disposal (≈1 min) and is rapidly broken down in extrahepatic sites, thereby liberating cysteine (3, 4). Recent modeling of the plasma turnover of GSH, cysteine, and cystine in the mature rat indicates that >80% of the plasma cysteine and cystine is derived from hydrolysis of GSH released by the liver (4). Cysteine itself has a short half-life of disposal (≈2 min) (4), and the maintenance of steady-state plasma cysteine may be the critical purpose of sinusoidal GSH efflux. Cysteine is a preferential precursor for GSH and protein synthesis in cell types that neither take up cystine nor trans-sulfurate methionine; among these are lymphocytes, erythrocytes, and brain endothelial cells.

Hepatocytes are polar epithelial cells. GSH is released at both poles of the hepatocyte, sinusoidal and canalicular, by apparent carrier-mediated transport. Both sinusoidal and canalicular systems are facilitative, bidirectional, and exhibit low affinity and high capacity (5–7). However, differences in certain physiological characteristics of the two low-affinity transport systems permit their functional distinction. Sinusoidal GSH transport is cis-inhibited by sulfobromophthalein GSH *S*-conjugate (BSP-GSH) (7, 8), trans-inhibited by methionine (9–11) or cystathionine (12), and unchanged after phenobarbital treatment (6, 13). The low-affinity canalicular GSH transport is not affected by these inhibitors (12, 14) but is induced by phenobarbital (6, 13). Neither transporter is sodium-dependent or transports amino acids (1, 2, 7, 9–11), indicating that these transporters are likely to be different from the recently cloned amino acid transporters. High-affinity GSH transport activity was recently identified in canalicular membrane, which is inhibited by BSP-GSH and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) but is of low capacity and probably makes little contribution to biliary GSH secretion (15).

We previously identified two size fractions (≈4.0 and ≈2.5 kb) of rat liver poly(A)$^+$ RNA that expressed GSH transport in *Xenopus laevis* oocytes (16). From the ≈4-kb fraction, which exhibited canalicular transporter features (insensitive to BSP-GSH), we recently cloned a 4.05-kb cDNA, which leads to expression of BSP-GSH-insensitive GSH transport in oocytes (17, 18). This clone is referred to as RcGshT for rat canalicular GSH transporter-associated polypeptide. Abundance of its transcript was increased about 10-fold after a dose of phenobarbital, and the transcript was found in liver, kidney, lung, intestine, and brain. We now report the cloning of the cDNA for the GSH transporter or regulator[§] in the ≈2.5-kb size fraction, which exhibits sinusoidal characteristics (sensitivity to inhibition by BSP-GSH and cystathionine); this clone is referred to as RsGshT for rat sinusoidal GSH transporter-associated polypeptide. We compare its structure, function, and distribution to the canalicular clone.

METHODS

RNA Isolation, Oocyte Preparation, and Transport. Isolation of poly(A)$^+$ RNA from rat liver (male Sprague–Dawley rats, 200–250 g), size fractionation, and microinjection of mRNA and cRNA into stage 5 and 6 defolliculated *X. laevis* oocytes were performed as described (16, 17). GSH uptake and efflux were determined as described using [^{35}S]GSH and unlabeled GSH (16, 17) 3 days after injection of mRNA,

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Abbreviations: GSH, reduced glutathione; BSP-GSH, sulfobromophthalein GSH *S*-conjugate; diBSP, dibromosulfophthalein; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; RsGshT, rat sinusoidal GSH transporter-associated polypeptide; RcGshT, rat canalicular GSH transporter.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U16358).

cRNA, or water. Incubations consisted of 600 μl and eight oocytes at 25°C. Uptake utilized 2 μCi (1 Ci = 37 GBq) of [^{35}S]GSH, and for efflux 30 nCi were injected per oocyte. For uptake, 10 mM extracellular GSH with or without 5 mM BSP-GSH or dibromosulfophthalein (diBSP) was used; for efflux, oocytes were injected with 33 nl per oocyte containing either 16 nmol of GSH plus or minus 4 nmol of BSP-GSH or water alone. In some cases, efflux was determined in the presence of 5 mM extracellular cystathionine. In some experiments, the effect of 4°C or DIDS (0.1 mM) on uptake was assessed. In uptake experiments, preliminary studies showed that accumulation of GSH was linear for 1 h. Oocytes were washed seven times in ice-cold medium until no additional radioactivity was detected in the wash. Verification of the molecular form of the radiolabel transported was performed by HPLC (16, 17). As in our previous studies (16, 17), no evidence of breakdown products of [^{35}S]GSH was seen in HPLC analysis of cells or medium in either uptake or efflux experiments. [^{35}S]GSH was obtained from DuPont/NEN (145 Ci/mmol).

cDNA Library Construction and Clone Isolation. The ≈2.5-kb mRNA size fraction that conferred peak stimulation of GSH uptake/efflux (BSP-GSH sensitive) in *Xenopus* oocytes was used to construct a directional cDNA library in the expression vector pcDNA1 (Invitrogen); the library was screened for expression of cRNA in oocytes as described (17), until a single colony expressing GSH transport was isolated.

Northern Blot Analysis of Rat and Human Tissues. Total RNA was prepared from rat tissues by the acid guanidinium/phenol method. The A_{260} was used to calculate RNA concentration. Twenty micrograms of each total RNA was electrophoresed on a 1% formaldehyde/agarose gel and blotted onto nitrocellulose filters (Schleicher & Schuell). The full-length RsGshT cloned cDNA was labeled by using the Prime-It II random primer labeling kit (Stratagene). Hybridization was performed for 2.5 h at 65°C in a rapid hybridization mixture (Amersham). Washing was done in 2× SSC/0.1% (wt/wt) SDS (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) at room temperature for 20 min and in 0.1× SSC/0.1% SDS at 65°C for 15 min for high stringency. Equivalence of loading was verified with either ethidium bromide staining of rRNA in the gel or the blot was reprobed for a "housekeeping" RNA, glyceraldehyde-3-phosphate dehydrogenase, using full-length 1.4-kb human cDNA (Clontech). Autoradiography was performed by exposing blots for 3 days to Amersham Hyperfilm.

***In Vitro* Translation of RsGshT cRNA.** *In vitro* translation of cloned RsGshT cRNA was performed by using a rabbit reticulocyte system (Promega) and [^{35}S]methionine (DuPont/NEN; 1200 Ci/mmol) according to the instructions of the manufacturer. Translated protein was run in SDS/PAGE and autoradiographed as above.

Effect of Phenobarbital on RsGshT mRNA. Fed male Sprague–Dawley rats (250 g) were injected with a single dose of phenobarbital (80 mg/kg in saline, i.p.) or an equal volume of saline 6 h before isolation of total liver RNA. Twenty micrograms of each total RNA was run on an agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled RsGshT, as described above. The same membrane was rehybridized with ^{32}P-labeled rat glyceraldehyde-3-phosphate dehydrogenase cDNA (Clontech).

Sequence Analysis of the cDNA. The insert of cloned cDNA was digested with different restriction enzymes, and the resulting fragments of cDNA were subcloned into the plasmid vector pGEM-f11(−) (Promega). The nucleotide sequence was determined as described (17). Comparison of RsGshT amino acid and nucleotide sequences was performed with the Genetics Computer Group software to search the GenBank, European Molecular Biology Laboratory, Swiss-Prot, and Prosite data bases.

RESULTS

Expression of the RsGshT Clone. The single colony isolated contained a cDNA insert whose cRNA expressed a high enrichment of GSH uptake activity [≈500-fold in comparing 30 ng of poly(A)$^+$ RNA and 0.03 ng of RsGshT (data not shown)]. The expressed transporter exhibited the capacity to take up or efflux GSH depending on the direction of the concentration gradient. Table 1 compares the sensitivity to cis-inhibition by BSP-GSH or trans-inhibition by cystathionine of the expressed RsGshT in direct comparison to water, poly(A)$^+$ RNA, and RcGshT, all individually injected into oocytes prepared from the same two frogs. As expected for the sinusoidal GSH transporter, RsGshT cRNA expressed GSH uptake and efflux that was cis-inhibited by BSP-GSH and efflux that was trans-inhibited by cystathionine (Table 1). In two experiments, methionine exerted an effect similar to cystathionine on RsGshT-expressed GSH efflux, and DIDS did not inhibit uptake or efflux by RsGshT (data not shown). These findings exclude the possibility that we have identified the high-affinity canalicular GSH transporter (15), because it is reported not to be inhibited by methionine (19) but is inhibited by DIDS (15). In contrast to RsGshT, expressed GSH transport by RcGshT in the same two oocyte preparations (Table 1) was not inhibited by BSP-GSH or cystathionine. These findings confirm the inhibitor specificities that were identified for transporters expressed by the original two size fractions of mRNA (16). The expression of GSH efflux indicates that the activity cannot be attributed to binding rather than to transport. Furthermore, uptake by oocytes expressing both transporters was markedly inhibited at 4°C and by 5 mM diBSP, whereas the small uptake in water-injected oocytes was not affected by these conditions (Table 2).

We examined the concentration dependence of GSH transport. Uptake was a saturable function of GSH concentration with $K_m \approx 3$ mM, which was approximately half of that observed with canalicular transporter, both examined in oocytes from the same preparation (Fig. 1). Water-injected controls exhibited a low apparent uptake but, as above, at all GSH concentrations in Fig. 1, this was not decreased at 4°C or

Table 1. Expression of GSH transport in oocytes: Comparison of RsGshT and RcGshT

	Uptake, nmol per h per oocyte				Efflux, nmol per h per oocyte					
	Experiment 1		Experiment 2		Experiment 1			Experiment 2		
Injection	− BSP-GSH	+ BSP-GSH	− BSP-GSH	+ BSP-GSH	− BSP-GSH	+ BSP-GSH	Cyst	− BSP-GSH	+ BSP-GSH	Cyst
Water	1.7	1.3	1.6	1.1	0.4	0.3	0.4	0.5	0.4	0.3
Poly(A)$^+$ RNA	17	11	17	9.9	1.8	1.1	1.1	2.0	1.0	1.1
RsGshT	30	7.5	22	6.6	3.8	0.9	1.2	4.1	0.9	1.7
RcGshT	29	28	27	27	4.6	5.0	4.6	5.2	5.6	5.6

Oocytes were injected with 30 ng of poly(A)$^+$ RNA, 0.3 ng of cRNA for RsGshT or RcGshT, or water and studied after 3 days. GSH uptake employed 10 mM GSH, and for efflux occytes were loaded with 16 nmol per oocyte. BSP-GSH was employed in a cis fashion: 5 mM outside for uptake and 4 nmol injected per oocyte for efflux (co-loaded). Cystathionine (Cyst) was studied in a trans fashion (i.e., effect of 5 mM outside on efflux). See *Methods* for details. Experiments 1 and 2 refer to oocyte preparations from different frogs studied on two separate occasions.

Table 2. Effect of incubation temperature, diBSP, and BSP-GSH on GSH uptake by oocytes

Injection	Uptake, nmol per oocyte per h			
			Inhibition at 25°C	
	25°C	4°C	diBSP	BSP-GSH
RsGshT	35.5 ± 0.5	0.73 ± 0.19	22.0 ± 0.1	22.5 ± 2.9
RcGshT	40.3 ± 3.7	0.60 ± 0.04	14.1 ± 0.1	38.4 ± 4.2
Water	0.27 ± 0.10	0.23 ± 0.03	0.31 ± 0.03	0.26 ± 0.05

For uptake studies, GSH (10 mM plus 2 μCi of [^{35}S]GSH) was incubated for 1 h in 600 μl containing eight oocytes, and uptake was determined 3 days after injection of 3 ng of RsGshT or RcGshT or water. These are different preparations from the data in Figs. 1–3 and represent the mean ± SD for oocytes from three experiments injected and studied on separate days. The concentration of diBSP and BSP-GSH was 5 mM.

inhibited by diBSP (data not shown). This endogenous association of GSH with oocytes most probably represents binding, but some low level temperature-insensitive uptake mechanism cannot be excluded. Thus, we can conclude that the cRNA for the RsGshT clone leads to expression of a transporter with the property of a carrier—namely, saturation kinetics and a moderately low affinity for GSH—comparable to that observed in rat liver. Both RsGshT and RcGshT operate bidirectionally, which means that net transport of GSH is determined by the concentration gradient. Since the physiological condition is high intracellular and low extracellular GSH, both transporters will function to efflux GSH. The low affinity of RsGshT is further evidence that it is a different transporter than the high-affinity canalicular transporter (15).

Characteristics of RsGshT. The nucleotide and deduced amino acid sequences of RsGshT are shown in Fig. 2. The cDNA insert consists of 2733 nucleotides with an open reading frame of 1059 nucleotides, predicting a polypeptide of 353 amino acids with a molecular mass of 39,968 Da. The start codon was identified according to the model of Kozak (20). Kyte–Doolittle analysis (21) was consistent with an integral membrane protein with two typical membrane-spanning domains (amino acid 232–250 and 270–289); several other possible membrane-spanning regions of sufficient length were more weakly hydrophobic (amino acids 17–32, 95–111, and 123–141). RsGshT has no cleavable signal sequence, indicating that the N terminus is likely to be cytoplasmic. Three possible N-linked glycosylation sites are present (amino acids 63, 113, and 297) as well as multiple potential phosphorylation sites (protein kinase C: amino acids 52, 233, 341, and 345) and three potential sites of N-myristoylation (amino acids 101, 166, and 243). No protein kinase A site was identified. No significant amino acid or nucleotide sequence homology could be identified in searching current data bases of nucleotide and amino acid sequences. In addition, no sequence homologies between RsGshT and RcGshT were apparent. Thus, RsGshT represents the cDNA for a gene completely distinct from that represented by RcGshT cDNA; both are new and unrelated genes.

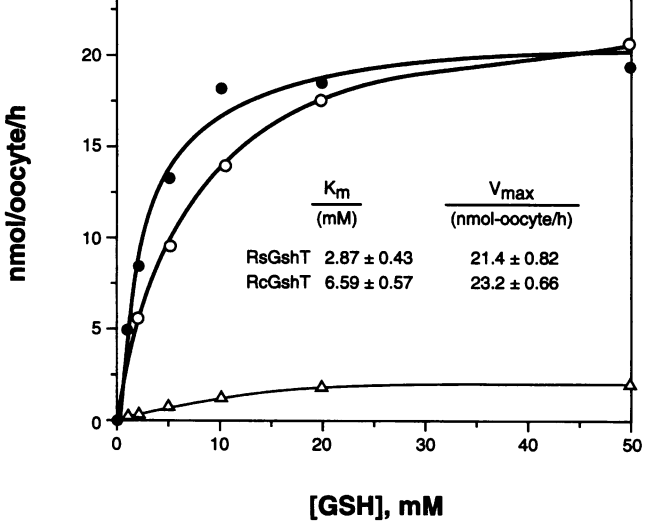

FIG. 1. Concentration-dependent uptake of GSH by oocytes. Oocytes were injected with 0.3 ng of cRNA prepared from RsGshT cDNA (●), RcGshT (○), or water (△), and uptake was determined after 3 days. Results for water-injected oocytes were subtracted from results for cRNA-injected oocytes to yield the corrected values shown for the two clones. Kinetic parameters derived from nonlinear least-squares fit to the Michaelis–Menten equation are given.

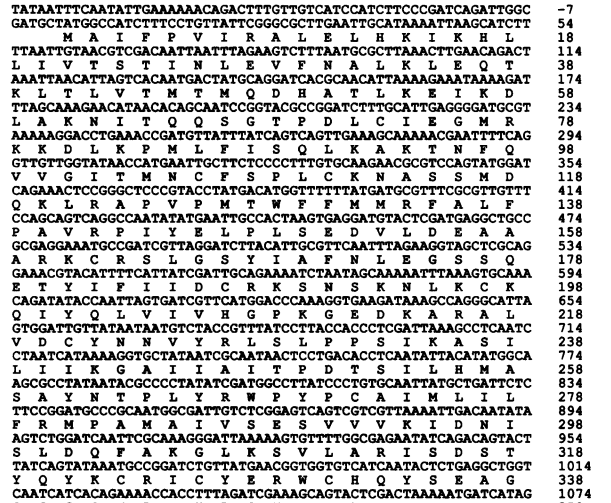

FIG. 2. Nucleotide and deduced amino acid sequences of RsGshT. The first nucleotide and amino acid residue of the start site are designated as position 1, and the termination codon follows the last amino acid residue. The polyadenylylation signal sequence is underlined.

In vitro translation using a rabbit reticulocyte system revealed a single polypeptide of ≈40 kDa on SDS/PAGE (data not shown), in close agreement with the predicted molecular mass calculated from the open reading frame. A single hybridizable transcript of ≈2.8 kb was identified on Northern blot analysis of rat liver (Fig. 3). Only trace signals were seen in other organs. This selective expression of the RsGshT transcript in liver is consistent with the known role of rat liver in supplying nearly all GSH to plasma (1, 2). This localization contrasts with RcGshT, which was identified in most rat organs (17).

Comparison of the response to phenobarbital confirmed an increase in RcGshT mRNA but revealed no concurrent change

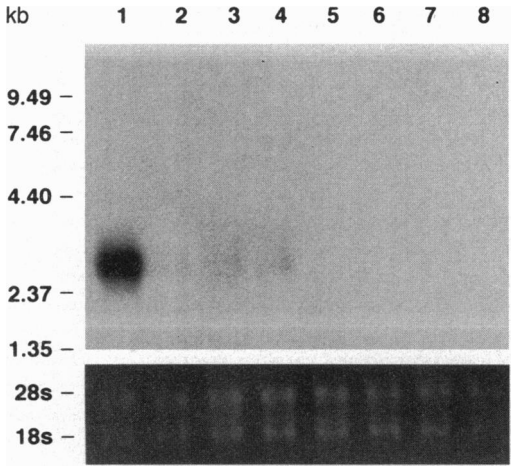

FIG. 3. (*Upper*) Northern blot analysis of rat tissue distribution of RsGshT mRNA. Twenty micrograms of RNA isolated from liver (lane 1), kidney (lane 2), lung (lane 3), skeletal muscle (lane 4), pancreas (lane 5), brain (lane 6), heart (lane 7), and intestine (lane 8) were subjected to electrophoresis on a 6.7% formaldehyde/1% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled full-length insert of the RsGshT cDNA clone. Blots were subsequently washed under high stringency. The scale at the left indicates the positions of standard markers. (*Lower*) Ethidium bromide fluorescent stain of the gel prior to blotting showing rRNA as an index of loading.

in the abundance of RsGshT transcripts in the same livers (data not shown).

Using a commercial human organ blot probed with both RsGshT and RcGshT with high stringency, a single hybridization signal of appropriate size for each was observed (2.8 kb, and 4.0 kb, respectively). As in the rat, RsGshT was selectively expressed in liver, whereas RcGshT was widely distributed (data not shown). Furthermore, these results suggest that the human transcripts are highly homologous with the rat and that, in humans as in rat, the liver supplies nearly all GSH to plasma.

DISCUSSION

We report the cloning of a cDNA that leads to expression of sinusoidal GSH transporter-like activity. In view of the small number of transmembrane-spanning domains of RsGshT (two) and RcGshT (four) (although each might have additional ones), it is possible that these are integral membrane regulatory subunits of the GSH transporters rather than the actual transporters as suggested for the D2 or rBAT amino acid transporter (22, 23). It is of interest that recent evidence suggests that three or four regions of rBAT with weaker hydrophobicity are true membrane-spanning domains (24), pointing out the uncertainty of hydropathy criteria. Furthermore, sodium (25, 26) and potassium ion channels (27, 28) with two membrane-spanning domains and a bile acid transporter (29) with only a single transmembrane domain have been identified. Thus, it may be that homooligomeric forms of RsGshT and RcGshT directly govern GSH transport as suggested for the ion channels (25–28). The identification of RsGshT as either the sinusoidal transporter or its regulatory subunit is based on the characteristic cis-inhibition by BSP-GSH and trans-inhibition by methionine and cystathionine of the expressed transport activity, which are known unique physiological properties of the sinusoidal GSH transporter. The RsGshT sequence is unique and bears no homology to the previously cloned RcGshT, ruling out a single gene with alternative processing or posttranslational modifications to account for the two transporters. In contrast to the wide distribution of RcGshT, RsGshT appears to be nearly liver-specific. Thus, regardless of whether RsGshT is the transporter or its regulator, it appears to determine the liver-specific expression of sinusoidal GSH efflux.

Having isolated the cDNA for a polypeptide associated with sinusoidal GSH transport, it will be of great interest to determine its regulation and the influence of diseases on its expression and organ-specific GSH transport. Low plasma GSH has been described in liver disease (30) and in human immunodeficiency virus infection (31, 32). Since plasma GSH is derived mainly from the liver and is governed by sinusoidal GSH transport, down-regulation of this polypeptide may contribute to the profound systemic effects, including brain and immune dysfunction, seen in liver disease and chronic viral infections such as human immunodeficiency virus. Conversely, up-regulation of this transporter may explain the increased sinusoidal GSH efflux observed in stress and endotoxemia (33) and play a role in bolstering antioxidant defenses in plasma as well as in extrahepatic cells. In view of the importance of sinusoidal GSH efflux, the cloning of the sinusoidal GSH transporter-associated polypeptide represents an important step in permitting future studies of the role of this transporter in the pathophysiology of disturbances of sulfur amino acid metabolism and antioxidant defenses found in many diseases.

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