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Cloning and characterization of the transport modifier RS1 from rabbit which was previously assumed to be specific for Na^+-D-glucose cotransport

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Abstract

Previously we cloned membrane associated polypeptides from pig and man (pRS1, hRS1) which altered rate and glucose dependence of Na^+-D-glucose cotransport expressed by SGLT1 from rabbit and man. This paper describes the cloning of a related cDNA sequence from rabbit intestine (*rbRS1*) which encodes a gene product with about 65% amino acid identity to pRS1 and hRS1. Hybridization of endonuclease-restricted genomic DNA with cDNA fragments of *rbRS1* showed that there is only one gene with similarity to *rbRS1* in rabbit, and genomic PCR amplifications revealed that the *rbRS1* gene is intronless. Comparing the transcription of *rbRS1* and *rbSGLT1* in various tissues and cell types, different mRNA patterns were obtained for both genes. In *Xenopus* oocytes the V_{max} of expressed Na^+-D-glucose cotransport was increased or decreased when *rbRS1* was coexpressed with *rbSGLT1* or *hSGLT1*, respectively. After coexpression with *hSGLT1* the glucose dependence of the expressed transport was changed. By coexpression of *rbRS1* with the human organic cation transporter *hOCT2* the expressed cation uptake was not altered; however, the expressed cation uptake was drastically decreased when *hRS1* was coexpressed with *hOCT2*. The data show that RS1 can modulate the function of transporters with non-homologous primary structures.

Keywords: Transport modifier RS1; Primary structure; Expression; Na^+-D-Glucose cotransporter SGLT1; Organic cation transporter OCT2; Rabbit

1. Introduction

Employing immunoscreening with a monoclonal antibody that altered phlorizin binding to renal brush border membranes (R4A6) we cloned a 623-amino acid polypeptide from pig (pRS1) which modified the expression of Na^+-D-glucose cotransport in *Xenopus* oocytes [1,2]. By coexpression of *pRS1* with the rabbit Na^+-D-glucose cotransporter *rbSGLT1* the V_{max} of the expressed transport was increased and the apparent glucose dependence of the transport was altered. Since immunohistochemical data suggested that the antibody R4A6 binds to the extracellular side of brush-border membranes [3,4] and the primary structure of RS1 does not pre-

Abbreviations: AMG, methyl-α-D-glucopyranoside; DIG, digoxigenin; GAPDH, glyceraldehyde phosphate dehydrogenase; RACE, rapid amplification of cDNA ends; TEA, tetraethylammonium

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dict membrane-spanning domains we raised the hypothesis that pRS1 might interact with rbSGLT1 at the extracellular side of the plasma membrane. Specificity of this interaction with SGLT1 or homologous proteins was suggested because coexpression of *pRS1* with some transporters from other protein families did not influence the respective transport activities, whereas the transport expressed by the SGLT1 homologous Na^+-*myo*-inositol cotransporter SMIT was also altered [1,5]. In pig, mRNA with similarity to pRS1 was not only detected in small intestine and renal outer medulla where SGLT1 had been localized, but also in renal outer cortex where expression of SGLT1 had not been described [1,2,6–8]. Therefore it was unclear whether a number of RS1-type proteins interact with different SGLT1-homologous Na^+ cotransporters, whether one RS1 protein interacts with several SGLT1-type transporters, or whether the effects of pRS1 are less specific as originally assumed. For a detailed investigation of the role of RS1 it was necessary to study interactions of RS1 with SGLT1 and other transporters from the same species and to determine whether different RS1-type proteins exist in these species.

Considering the significant species differences concerning functional properties and regulation of SGLT1 [9] we decided to investigate the effects of RS1 in human and rabbit. Recently we cloned an intronless gene from man with similarity to *pRS1* (*hRS1*) which altered the expression of Na^+-D-glucose cotransport by human SGLT1 (*hSGLT1*) [10], albeit with a different dose response as the previously observed effects of *pRS1* on rabbit *SGLT1* (*rbSGLT1*) [1]. Southern blots with restricted genomic DNA from man suggested the existence of only one human *RS1*-type gene. In the present paper we report the cloning of a *RS1*-type gene from rabbit small intestine (*rbRS1*) and show that *rbRS1* is the only *RS1*-type gene in rabbit. In addition we present data which contradict our previous hypothesis that RS1 is a stoichiometric modulator or subunit of SGLT1.

2. Materials and methods

2.1. Materials

[^{14}C]Methyl-α-D-glucopyranoside (10.3 Gbq/mmole), [α-^{32}P]dATP (111 Tbq/mmol), and [α-^{35}S]dATP (37 Tbq/mmole) were obtained from Amersham Buchler (Braunschweig, Germany) and [^{14}C]tetraethylammonium (1.9 Tbq/mmole from Biotrend (Cologne, Germany). Ficoll, polyvinylpyrrolidone 40, M-MLV reverse transcriptase (Superscript), *Taq* DNA polymerase and terminal deoxynucleotidyl transferase were purchased from Gibco (Eggenstein, Germany). Restriction enzymes, DNA polymerase I, dNTPs, rNTPs, ddUTP-DIG, dUTP-DIG, antidigoxigenin antibody from goat coupled with alkaline phosphatase, Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were provided by Boehringer (Mannheim, Germany). All other chemicals were obtained as described earlier [1].

2.2. Construction and screening of the cDNA library

From mucosal scrapings of rabbit jejunum mRNA was isolated by affinity purification [11] and a unidirectional, oligo(dT)-primed cDNA library in λ ZAP phages was prepared by employing a commercial kit (Stratagene, Heidelberg, Germany). Hybridizations were performed under high stringency conditions (0.1×SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 0.1% (w/v) SDS, 65°C) employing a random primed or nick translated radioactively labelled 1.8 kb cDNA fragment of *pRS1* [1]. Positive plaques were isolated and Bluescript phagemids were prepared in *Escherichia coli* XL1 cells using the helper phage R408 [12]. The insert sizes were determined and the restriction patterns of the cDNA fragments analysed.

2.3. Isolation of nephron segments

A rabbit kidney was first perfused with dissection solution (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 0.34 NaH_2PO_4, 1 mM $CaCl_2$, 4 mM $NaHCO_3$, 5 mM D-glucose, 1 mg/ml BSA) and then with dissection solution containing 1 mg/ml of collagenase. Thin tissue sections were cut along the corticomedullary axis and incubated for 25 min (45 min for microdissection in the inner medulla) at 35°C in aerated dissection solution containing 1 mg/ml of collagenase. Then the tissue was rinsed in ice-cold dissection solution and kept at 4°C. Microdissection of glomeruli, convoluted proximal tubules

from outer cortex, straight proximal tubules from outer medulla, thin limbs of Henle's loop, thick ascending limbs of Henle's loop, cortical collecting ducts and inner medullary collecting ducts was performed as described [13]. Identical types of tubules (10–40 mm) were pooled, washed free from contaminating cells or debris and transferred into denaturating solution (25 mM sodium citrate, pH 7.0, 4 M guanidinium thiocyanate, 0.5% (w/v) sarcosyl, 0.1 M β-mercaptoethanol, and 15 μg of yeast tRNA). From the pools total RNA was extracted using a microadaptation of the guanidinium thiocyanate-phenol-chloroform method [13,14].

2.4. Polymerase chain reactions

For the amplification of cDNA fragments single-stranded cDNA was reversely transcribed from mRNA after oligo(dT) priming. For genomic amplifications DNA was isolated from rabbit skeletal muscle [11]. PCR was performed in the presence of 67 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5–2.3 mM $MgCl_2$, 0.2 mM of each dNTP and 0.2 μM of each primer. After *Taq* polymerase was added 30–35 PCR cycles (1 min, 94°C; 1 min between 48 and 52°C; 1 min 72°C) were performed. To clone the 5′-end of *rbRS1* the 5′-RACE methodology was employed [15]. After priming with $G5^-$ (for primers see Table 1) single-stranded cDNA was synthesized and tailed with poly(dA). For synthesis of the second cDNA strand the oligo(dT)-containing adapter primer H1 was employed. After purification of the double-stranded cDNA we performed the first round of PCR using the primers $G4^-$ and H2 and verified the specificity of the amplification product by hybridization with $G3^-$. After gel elution of the amplification products [16] another round of amplification was performed using the same primers for amplification and hybridization. The final amplification product was subcloned in pBluescript SKII and sequenced.

2.5. Subcloning and DNA sequencing

PCR amplification products were gel-eluted, filled in with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase, blunt end-subcloned into pBluescript SKII plasmid and propagated in electrotransfected *E. coli* DH 10B cells. *rbRS1* was constructed from a cDNA fragment isolated from the rabbit cDNA library and a PCR product (GenBank accession No. X82876, nucleotides 588–2150 and 1–662, respectively). The PCR product was amplified with the primers $G1^+$ and $G7^-$, subcloned and sequenced. Both cDNAs in pBluescript SKII plasmids were restricted with *Xba*I and *Sma*I, ligated and subcloned into pBluescript SKII. DNA sequencing was performed by the dideoxynucleotide chain termination method employing [α-^{35}S]dATP. T7 DNA polymerase and *Taq* polymerase were used for sequencing of double-stranded cDNA in pBluescript SKII [17] and for cycle sequencing of PCR products, respectively. Both cDNA strands of *rbRS1* were sequenced using standard vector-derived sequencing primers and sequence-related oligonucleotides.

2.6. In vitro synthesis of cRNA

For injections into *Xenopus* oocytes 5′7me-Gppp5′G capped cRNA was prepared, purified, evaluated and stored as described earlier [1,18]. To prepare sense cRNA from *rbSGLT1* [19], *hSGLT1* [20], *hOCT2* [21], *hRS1* and *rbRS1*, the purified plasmids were linearized with *Not*I (*rbSGLT1*, *hOCT2*), *Eco*RI (*hSGLT1*), *Xba*I (*hRS1*) and *Kpn*I (*rbRS1*) and cRNA was synthesized by T3 polymerase (*rbSGLT1*, *hSGLT1*) and T7 polymerase (*hOCT2*, *hRS1*, *rbRS1*). For in situ hybridization digoxigenin (DIG)-labelled sense and antisense cRNA was prepared from an *Apa*I-*Xho*I restriction fragment of *rbRS1* (see nucleotides 543–966) which was subcloned in pBluescript SKII plasmid employing T3 and T7 polymerase, respectively, and NTPs plus digoxigenin-(11)dUTP.

2.7. Hybridizations

For Northern blotting mRNA from different tissues (5 μg per lane) was fractionated on a formaldehyde-containing agarose gel [22], alkaline-denatured, transferred to Hybond-N membrane (Amersham) and fixed with ultraviolet light. The membranes were hybridized with 400 ng of random-primed ^{32}P-labelled cDNA fragments of *rbRS1* (GenBank accession No. X82876 nucleotides 543–966). The hybridizations were performed for 18 h at 42°C in 50%

formamide, 5×SSPE (0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA), 5×Denhardt's solution, 0.5% (w/v) SDS and 100 μg ml^{-1} herring sperm DNA. The blots were washed to final stringency of 0.1×SSPE, 0.1% (w/v) SDS at 60°C. For Southern blots prehybridization was performed at 65°C with 5×Denhardt's solution, 6×SSC, 0.5% (w/v) SDS and 100 μg ml^{-1} herring sperm DNA. The hybridizations (65°C) were done with ^{32}P- or DIG-dUTP-labelled cDNA fragments of *rbRS1* (nucleotides 543–966, 1–2061) dissolved in 5×Denhardt's solution, 1% (w/v) bovine serum albumin (BSA), 1% (v/v) Ficoll and 1% (v/v) polyvinylpyrrolidone 40 and washed up to a stringency of 0.1% (v/v) SSC, 0.1% (w/v) SDS (65°C). In situ hybridizations with DIG-labelled sense and antisense cRNA fragment of *rbRS1* (nucleotides 543–966) were done on 7 μm sections of rabbit small intestine and kidney. For fixation the organs were perfused and the sections incubated with 4% (w/v) paraformaldehyde in PBS. Then the sections were washed with PBS, successively incubated with 0.1 M HCl (10 min), PBS (5 min), 0.1 M triethanolamine-HCl, pH 8, containing 0.25% (v/v) acetic anhydride (10 min), dehydrated in ethanol and air dried. For prehybridization and hybridization the sections were incubated without (90 min, 37°C) and with (12 h, 37°C) 20 μg ml^{-1} DIG-labelled cRNA probes dissolved in 5×Denhardt's solution containing 12.5 mM triethanolamine-HCl, pH 7.2, 25 mM EDTA, 25 mM NaCl, 50% (v/v) deionized formamide, 200 μg ml^{-1} heat-denatured herring sperm DNA and 20 mg ml^{-1} tRNA from yeast. The sections were washed 15 min (37°C) with 5×SSC, 50% (v/v) formamide, 15 min (22°C) with 0.5×SSC, and 5 min with 0.1×SSC. They were incubated with alkaline phosphatase conjugated anti-digoxigenin antibody from goat (diluted 1/500), washed with 150 mM NaCl, 100 mM Tris-HCl, pH 7.5 and rinsed with 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5. The colour was developed by incubation with Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.8. Oocyte assays

Oocytes were removed from female *Xenopus laevis* clawed toads, selected and injected with cRNAs or water as described earlier [1]. The oocytes were injected with 50 nl of water (control) and with water containing transporter cRNAs alone (*rbSGLT1*, *hSGLT1* or *hOCT2*) or together with different amounts of *rbRS1*-cRNA or *hRS1*-cRNA. For translation the oocytes were incubated for 48 h (18°C) in ORi (5 mM HEPES-Tris, pH 7.4, 110 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$). Initial uptake rates of glucose were measured by incubating the oocytes 60 min at 22°C with ORi containing [^{14}C]methyl-α-D-glucopyranoside ([^{14}C]AMG) without and with phlorizin [1] or 100 μM [^{14}C]tetraethylammonium without and with 75 μM cyanine 863. Transport was stopped and the oocytes were washed and analysed for radioactivity as described before [1,18]. The uptake rates inhibited by phlorizin or cyanine 863 of 8–10 paired oocytes were calculated. Means ± S.E.M. are presented.

3. Results

3.1. Cloning of a pRS1-homologous cDNA from rabbit

A ZAP cDNA library from rabbit small intestine was screened with a cDNA fragment of *pRS1* ([1], nucleotides 302–2128). *pRS1* was previously isolated from pig kidney and shown to modify the activity of Na^+-D-glucose cotransport which is expressed by *SGLT1* derived from rabbit [19]. Two 5′-truncated cDNA clones were obtained which appeared to be fragments of the same cDNA (GenBank accession No. X82876, nucleotides 311–2150 and 873–2150). The missing 5′-nucleotide sequence was cloned by PCR employing the RACE methodology and linked to the longer cDNA fragment as described in Section 2. The complete clone termed *rbRS1* is 2150 nucleotides long and contains an open reading frame between nucleotides 25 and 1794. One consensus polyadenylation AATAAA signal (nucleotides 2096–2101) is located 27 nucleotides upstream from the poly(A) tail. The predicted protein has a relative molecular mass of 62 011 Da and contains 590 amino acids. It is 27 amino acids shorter than hRS1 [10] and 33 amino acids shorter than pRS1 [1] (Fig. 1). The amino acids of rbRS1 are 64% and 65% identical to pRS1 and hRS1, respectively. 304 amino acids are conserved in rbRS1, pRS1 and hRS1 including the

Table 1
Oligonucleotide primers which were used for PCR experiments

G1$^+$	5′-*GGT AAA GAA TCT ACC TCA*-3′	(*rbRS1*, 1–18)
G3$^-$	5′-*CTG CTT CAG ATG AAT GCC*-3′	(*rbRS1*, 319–301)
G4$^-$	5′-*CAG ATG CCT CCA GGC*-3′	(*rbRS1*, 349–329)
G5$^-$	5′-AGA GAG AGA ACT AGT CTC GAG *AAT CGG AGG CCC TGG GTG CG*-3′	(*rbRS1*, 379–358)
G6$^+$	5′-*GCA TGA GGC GGG CCC TC*-3′	(*rbRS1*, 534–550)
G7$^-$	5′-*GCG TCC TGC TGC AGA CG*-3′	(*rbRS1*, 662–646)
G8$^-$	5′-*CGT ATG CTC TGT CTG TC*-3′	(*rbRS1*, 949–933)
G10$^+$	5′-*GAC CGA AAG TGA GCG TC*-3′	(*rbRS1*, 1167–1183)
G11$^-$	5′—*GCT CAC TTT CGG TCC ATC*-3′	(*rbRS1*, 1180–1162)
G12$^+$	5′-*CAG ACT TGT GAG CAG AC*-3′	(*rbRS1*, 1477–1493)
G16$^-$	5′-*CTT CCA CAG TTA CGT AGG*-3′	(*rbRS1*, 1806–1789)
H1	5′-AAG GAT CCG TCG ACT GCA GAA TTC AAG CTT $(T)_{17}$-3′	
H2	5′-AAG GAT CCG TCG ACT GC-3′	
S1$^+$	5′-*GC GCC AGC ACC CTC TTC AC*-3′	(*SGLT1* from rabbit [19], 1222–1240)
S2$^-$	5′-*CAG CCC ACA AAA CAG GTC*-3′	(*SGLT1* from rabbit [19], 1837–1854)

+ and − indicate whether the sequences belong to the plus or minus strand. Nucleotides derived from *rbSGLT1* [19] or from *rbRS1* (GenBank accession No. X82876) are printed in italics. Restriction sites are underlined.

42 C-terminal amino acids, five consensus sequences for phosphorylation by casein kinase II and four consensus sequences for phosphorylation by protein kinase C (Fig. 1). The most conserved amino acid residues were: glutamate (42), leucine (39), serine (37), proline (34), alanine (27), glutamine (26), threonine (21), and valine (20). Five potential *N*-glycosylation sites in rbRS1 are not conserved in pRS1 and hRS1.

3.2. Genomic organization of rbRS1 and search for rbRS1-homologous genes in rabbit

In order to identify introns in the *rbRS1* gene, genomic DNA was isolated from skeletal muscle and overlapping fragments from the coding region of *rbRS1* were amplified. The fragments were analysed for their size and identified by hybridization with *rbRS1* (Fig. 2). With all primer pairs single specific amplification products were obtained. The sizes of the amplification products were always identical to those predicted from the cDNA sequence of *rbRS1*. The data indicate that the *rbRS1* gene contains no intron. The same result was recently obtained for the human gene *hRS1* [10]. To elucidate whether the rabbit genome contains one or more RS1-type genes, genomic DNA was restricted with different endonucleases and Southern blots were performed with different cDNA fragments of *rbRS1*. In Fig. 3a–c the genomic DNA was digested with *Sac*I, *Rsa*I or *Taq*I and hybridized with the coding region of *rbRS1*. The genomic DNA was also digested with pairs of restriction enzymes that cut *rbRS1* cDNA only once: *Sac*I-*Taq*I (Fig. 3f), *Apa*I-*Rsa*I (Fig. 3g) or *Apa*I-*Taq*I (Fig. 3h), and hybridized with the respective restriction fragments of *rbRS1*-cDNA (see Fig. 3, bottom panel). The conditions employed for hybridization of the rabbit DNA (see Section 2) allowed the detection of *RS1* from other species (*pRS1* and *hRS1*). The hybridization signals obtained with the restricted genomic DNA show that there is only one gene in rabbit which codes for proteins with high similarity to *rbRS1*, and that the *rbRS1* gene is intronless.

3.3. Tissue distribution and cellular localization of rbRS1

In Fig. 4 the tissue distribution of *rbRS1*-mRNA was determined by Northern blots. After high stringency washing strong hybridization signals at 3.9 and 9.5 kb were obtained in renal outer medulla, renal inner medulla, duodenum, ileum and jejunum (Fig. 4, top panel). Intermediate signals were revealed in renal outer cortex, renal papilla, brain and liver, and weak signals were found in lung and spleen. In heart muscle, skeletal muscle, colon and stomach no significant signals were detected. The Northern blots

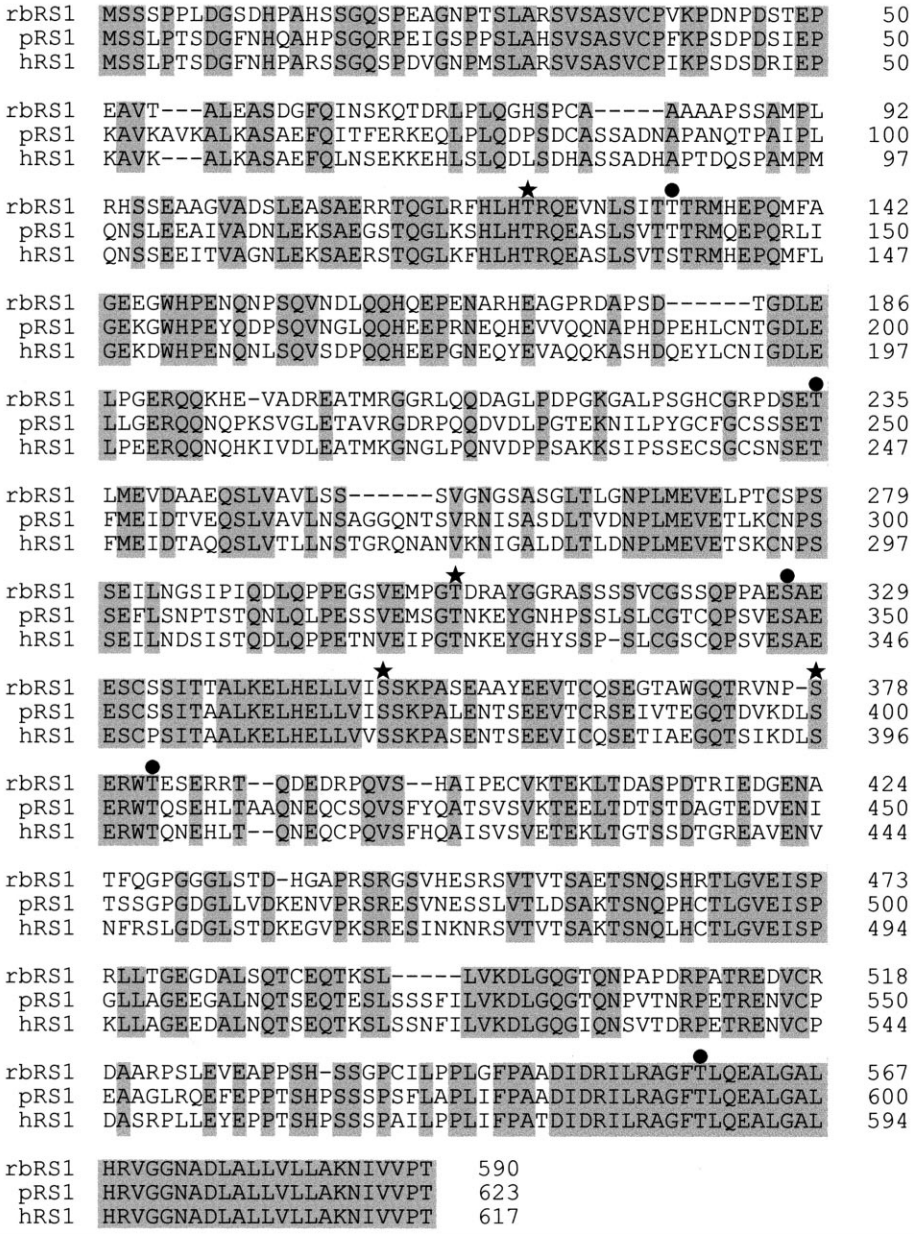

Fig. 1. Alignment of amino acid sequences of rbRS1, pRS1 and hRS1. Identical amino acids are shadowed. Conserved consensus sequences for protein kinase C dependent and casein kinase II dependent phosphorylation sites are indicated by asterisks and points, respectively.

showed a different distribution of *rbRS1* than has been described for *SGLT1* in rabbit [6] where no significant amounts of *SGLT1*-mRNA were detected in renal outer cortex, renal papilla, brain, spleen and liver. These tissues were investigated by RT-PCR for the transcription of small amounts of SGLT1. It was found that specific fragments of *rbSGLT1* could be amplified from renal outer cortex, renal papilla, and from brain, spleen and liver (data not shown) indicating that small amounts of *SGLT1*-mRNA are transcribed in these tissues.

By in situ hybridization with *rbRS1* in small intestine and kidney we investigated whether *rbRS1* is transcribed in the same cells as SGLT1. Fig. 5e shows hybridization of *rbRS1* with enterocytes in villi and crypts of rabbit jejunum. A similar distribu-

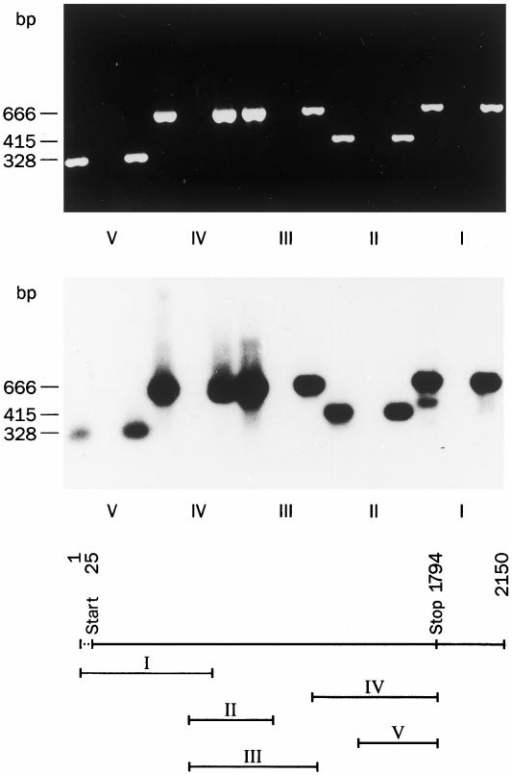

Fig. 2. PCR amplification of overlapping *rbRS1* fragments from genomic DNA. Employing DNA from skeletal muscle overlapping cDNA fragments of *rbRS1* were amplified by PCR. As negative and positive controls no DNA or *rbRS1*-cDNA was added during the PCR, respectively. The amplification products (I–V) were separated by agarose gel electrophoresis. The amplification products from the genomic cDNA were applied to the left, the negative controls to the middle and the positive controls to the right. The gels were stained with ethidium bromide (top panel) or hybridized with digoxigenin-labelled *rbRS1* (middle panel). In the bottom panel a scheme of the amplified fragments is shown. The following primers (see Table 1) were used for amplification of the different cDNA fragments: $G1^+$, $G7^-$ (I); $G6^+$, $G8^-$ (II); $G6^+$, $G11^-$(III); $G10^+$, $G16^-$ (IV); $G12^+$, $G16^-$ (V).

tion of *SGLT1*-mRNA has been demonstrated in rat small intestine [7]. At variance *SGLT1*-mRNA is nearly exclusively localized in the villi in rabbit small intestine [23,24]. The localization of *rbRS1*-mRNA in rabbit kidney is shown in Fig. 5a–d. A significant hybridization was observed in proximal convoluted and straight tubules of the inner cortex and in medullary collecting ducts. No distinct reaction was found in distal tubules. In glomeruli, convoluted proximal tubules of the outer cortex and in cortical collecting ducts a weak hybridization signal was observed which could not be clearly distinguished from the background reaction (data not shown). To investigate whether *rbRS1* is actually transcribed in these locations and whether SGLT1 may be transcribed at the same places we dissected different nephron seg-

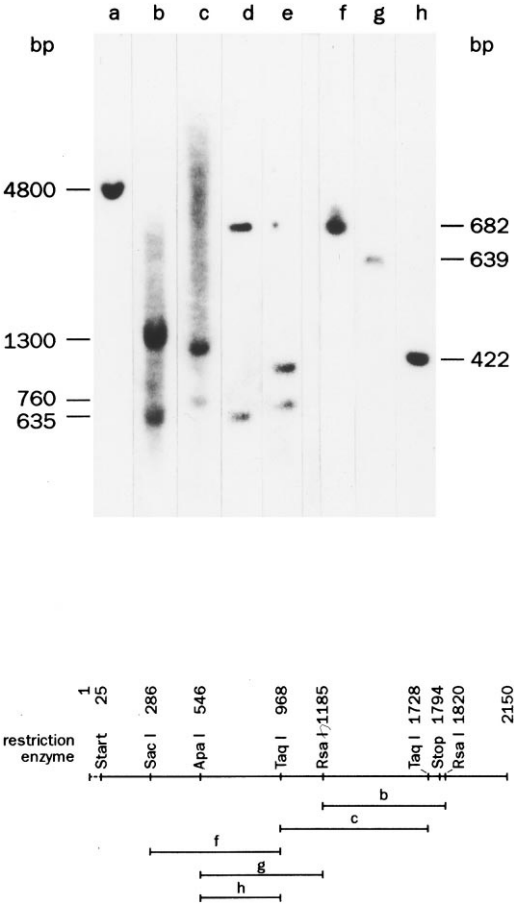

Fig. 3. Hybridization of restricted genomic DNA with specific cDNA probes. 15 μg per lane of genomic DNA (lanes a,b,c,f,g,h) and as positive controls 5 ng per lane of *rbRS1*-cRNA (lanes d,e) were restricted with *Eco*RI (lane a), *Rsa*I (lanes b,d), *Taq*I (lanes c,e), *Sac*I plus *Taq*I (lane f), *Apa*I plus *Rsa*I (lane g) or *Apa*I plus *Taq*I (lane h). The samples were separated on agarose gels which were stained with ethidium bromide to control the restriction reaction for completeness (not shown), or hybridized with the digoxigenin-labelled cDNA fragments of *rbRS1* (top panel). Hybridization was performed with the open reading frame of *rbRS1* (lanes a–e) or with the respective restriction fragments f, g and h which are indicated in the bottom panel (lanes f,g,h). Marker DNAs for lanes a–e are indicated on the left, those for lanes f–h on the right. The scheme in the bottom panel indicates the positions of the relevant restriction sites in *rbRS1* and shows the expected restriction fragments of *rbRS1* (b, *Rsa*I 635bp; c, *Taq*I 760 bp; f, *Sac*I-*Taq*I 682 bp; g, *Apa*I-*Rsa*I 639 bp; h, *Apa*I-*Taq*I 422 bp).

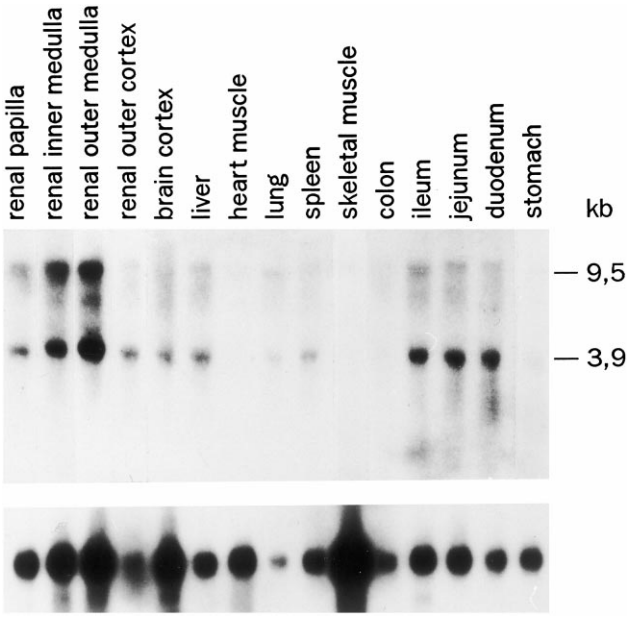

Fig. 4. Tissue distribution of *rbRS1*-mRNA. From different rabbit tissues mRNA was isolated, 5 μg mRNA per lane were separated on a denaturing agarose gel and transferred to Hybond-N membrane. The membrane was hybridized under high stringency conditions (washing at 0.1×SSPE, 0.1% (w/v) SDS, 60°C) with a ^{32}P-labelled 424 bp cDNA fragment of *rbRS1* (nucleotides 543–966). The hybridization is shown in the top panel. The mRNA loading of the different lanes was controlled by rehybridization with a human glyceraldehyde-3-phosphate dehydrogenase cDNA probe (bottom panel).

ments and analysed the transcription of *rbRS1* and *rbSGLT1* by RT-PCR employing the primers G12$^+$, G16$^-$ and S1$^+$ and S2$^-$, respectively. Fig. 6 suggests different amounts of *rbRS1* mRNA and *rbSGLT1* mRNA in most nephron segments. From proximal convoluted tubules, cortical collecting ducts and inner medullary collecting ducts large amounts of *rbRS1*-cDNA and small amounts of *rbSGLT1*-cDNA were amplified by RT-PCR. At variance larger amounts of *rbSGLT1* than *rbRS1* were amplified from proximal straight tubules of outer medulla. Small amounts of *rbRS1* and *rbSGLT1* were ampli-

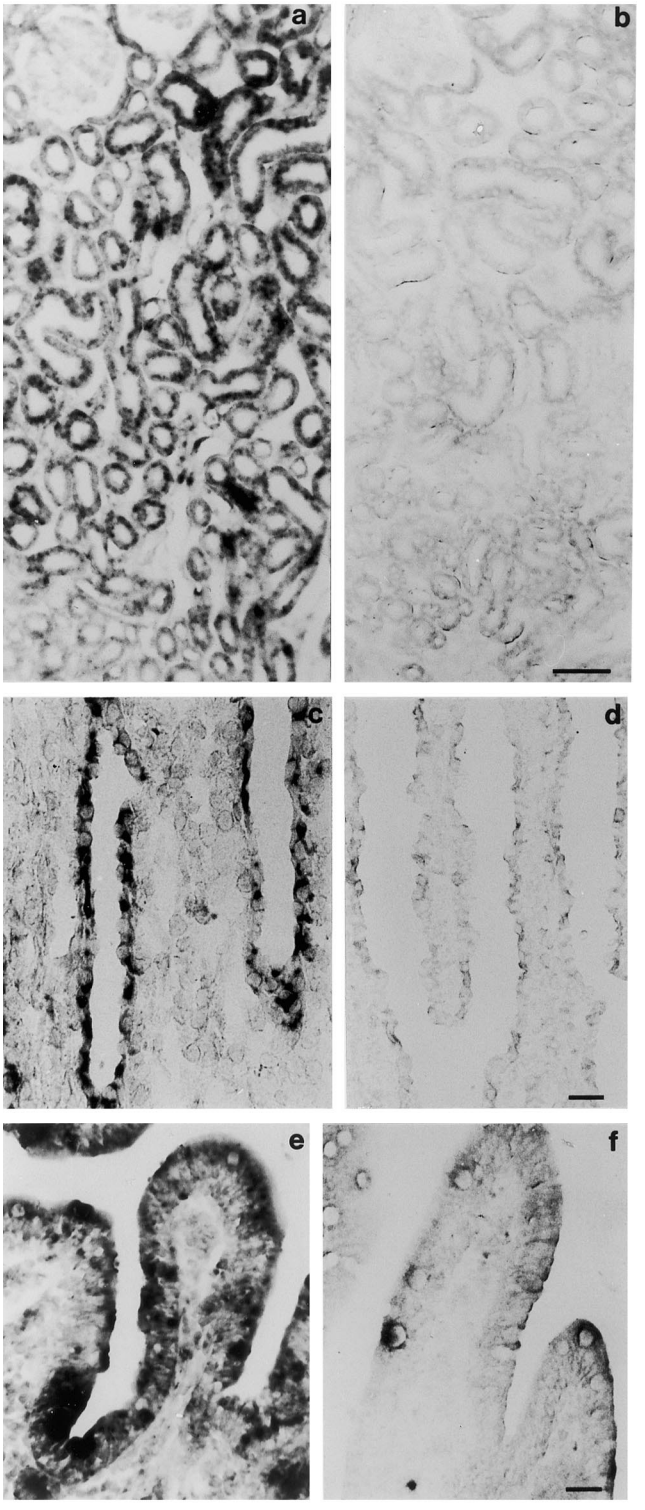

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Fig. 5. Localization of *rbRS1*-mRNA in rabbit kidney and small intestine. 7 μm cryostat sections of rabbit kidney (a–d) and rabbit jejunum (e,f) fixed with paraformaldehyde were prepared and in situ hybridization was performed with a 424 bp long antisense (a,c,e) or sense (b,d,f) cRNA fragment of *rbRS1* which was labelled with digoxigenin (nucleotides 543–966 of *rbRS1*). Longitudinal sections through renal inner cortex and outer medulla (a,b), renal papilla (c,d) and cross-sections through the jejunum are shown (e,f). Bars 50 μm in b, 100 μm in d, 10 μm in f.

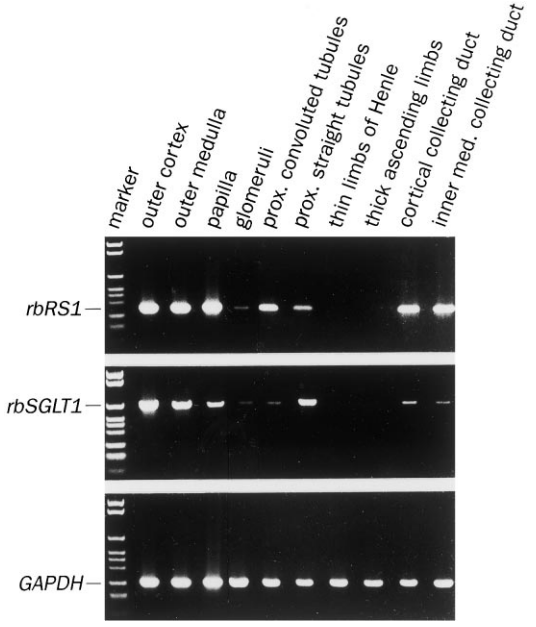

Fig. 6. Detection of *rbRS1*-mRNA and *rbSGLT1*-mRNA in microdissected nephron segments. RNAs were isolated from some regions of rabbit and from pools of different dissected nephron segments and the mRNAs were reversely transcribed. Polymerase chain reactions were performed employing the *rbRS1* specific primers $G12^+$ and $G16^-$ (panel *rbRS1*), the *rbSGLT1* specific primers $S1^+$ and $S2^-$ (panel *rbSGLT1*) and employing primers which were specific for glyceraldehyde phosphate dehydrogenase (panel *GAPDH*). The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Differing amounts of the expected cDNA fragments of *rbRS1* (330 bp) and *rbSGLT1* (630 bp) were amplified from renal glomeruli, proximal convoluted tubules of outer renal cortex, proximal straight tubules of outer renal medulla, cortical collecting ducts and inner medullary collecting ducts. No amplification of *rbRS1* and *rbSGLT1* fragments was obtained from thin limbs and thick ascending limbs of Henle's loops. The expected 310 bp cDNA fragment of GAPDH could be amplified from all RNA preparations. One of four experiments with the same result is shown. In one of each experiment the specificity of the amplified cDNA fragment of *rbRS1* and *rbSGLT1* was verified by hybridization and amplifications from genomic DNAs were excluded by control experiments without reverse transcription (data not shown).

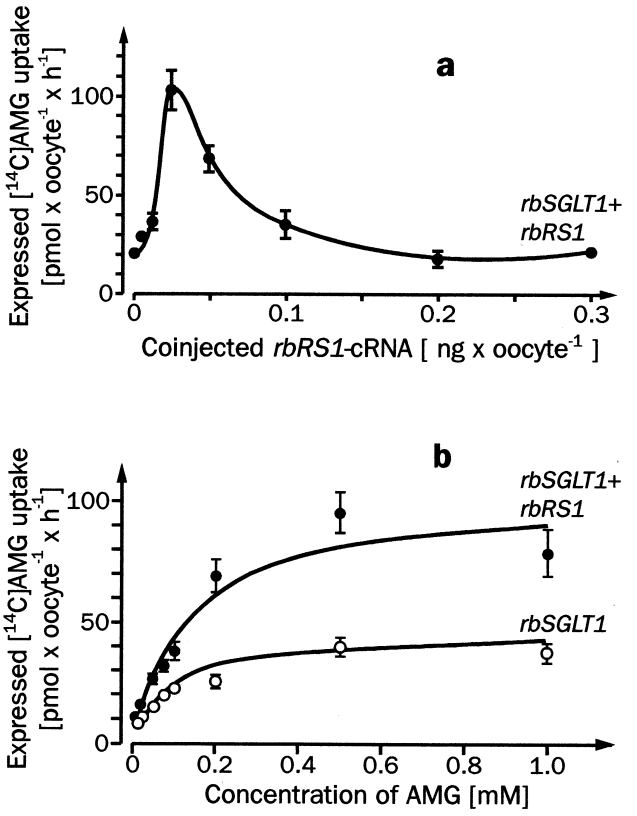

Fig. 7. Coexpression of *RS1* from rabbit with *SGLT1* from rabbit. *Xenopus* oocytes were injected with 0.1 ng *rbSGLT1*-cRNA per oocytes alone or together with cRNA of *rbRS1*. (a) Different amounts of *rbRS1*-cRNA were coinjected. (b) 0.1 ng of *rbSGLT1*-cRNA is injected alone (open symbols) or together with 0.025 ng *rbRS1*-cRNA (closed symbols). As controls for endogenous uptake oocytes were injected with water. The oocytes were incubated for 2 days. The uptake of (a) 50 μM [^{14}C]AMG or of (b) different AMG concentrations was measured in the absence and presence of 100 μM phlorizin. The expressed phlorizin inhibitable uptake rates were corrected for the endogenous uptake which was always less than 5%. Means ± S.E.M. are presented. The Michaelis-Menten equation was fitted to the data in b. The following K_m and V_{max} values ± S.D. were obtained: K_m(SGLT1) = 0.08 ± 0.02 mM, K_m (SGLT1 plus rbRS1) = 0.14 ± 0.04 mM, V_{max}(SGLT1) = 43 pmol oocyte^{-1} h^{-1}, V_{max}(SGLT1 plus rbRS1 = 102 ± 11 pmol oocyte^{-1} h^{-1}.

fied from glomeruli, and no amplification of both gene fragments could be obtained from thin and thick ascending limbs of Henle's loops. The data indicate different relations of the mRNA concentrations of *rbRS1* and *rbSGLT1* in different tissues and cells.

3.4. Functional characterization by coexpression experiments in oocytes of X. laevis

The presumed functional role of RS1 was previously investigated by coexpression experiments of *pRS1* with *rbSGLT1* and of *hRS1* with *hSGLT1* [1,10]. We showed that the V_{max} of Na^+-D-glucose cotransport expressed by injection of *rbSGLT1*-

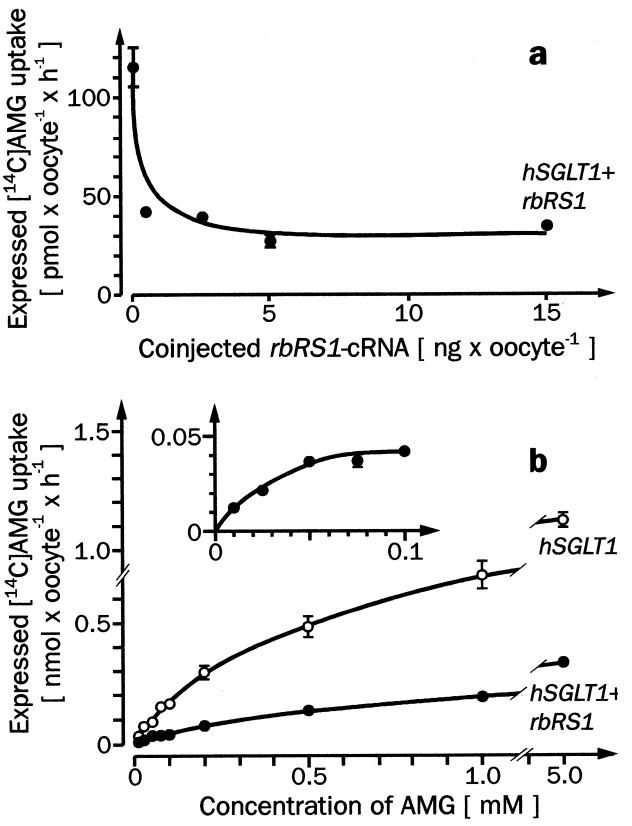

Fig. 8. Coexpression of *RS1* from rabbit with *SGLT1* from man. *Xenopus* oocytes were injected with 2.5 ng *hSGLT1* cRNA alone or together with *rbRS1* cRNA. After 2 days of incubation the uptake of [^{14}C]AMG was measured in the absence and presence of 200 μM phlorizin and corrected for the endogenous uptake which was measured in water injected oocytes. (a) Phlorizin inhibitable uptake of 50 μM [^{14}C]AMG which was expressed after coinjection of different amounts of *rbRS1* cRNA. (b) Substrate dependence of the phlorizin inhibited [^{14}C]AMG uptake which was expressed after injection of 2.5 ng *hSGLT1* cRNA (open symbols) or of 2.5 ng *hSGLT1* cRNA plus 5 ng *rbRS1* (closed symbols). The Michaelis-Menten equation could be fitted to the data obtained after injection of *hSGLT1* cRNA alone, and to the first five data points measured at low substrate concentrations after coinjection of *hSGLT1* cRNA plus *rbRS1* (inset in b). $K_{0.5}$ values (mM) of 0.65 ± 0.05 (*hSGLT1*) and 0.03 ± 0.01 (*hSGLT1* plus *rbRS1*) were obtained and V_{max} values (nmoles oocyte^{-1} h^{-1}) of 1.25 ± 0.03 (*hSGLT1*) and 0.06 ± 0.01 (*hSGLT1* plus *rbRS1*). When an equation describing two coexisting transport components was fitted to the whole set of data obtained after coinjection of *hSGLT1* and *rbRS1* fixing the above parameters for the high affinity component (program SigmaPlot, Jandel Scientific, Erkrath, Germany), for the low affinity component a $K_{0.5}$ value of 0.94 ± 0.05 mM and a V_{max} value of 0.35 ± 0.01 nmoles oocyte^{-1} h^{-1} was calculated.

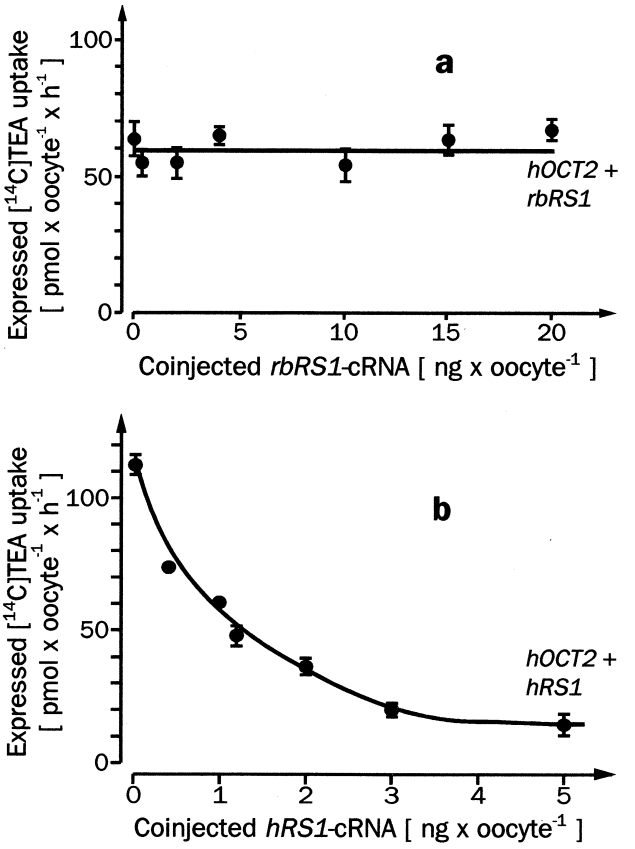

Fig. 9. Coexpression of the human polyspecific cation transporter *hOCT2* with *rbRS1* or *hRS1*. *Xenopus* oocytes were injected with 5 ng of *hOCT2* alone or together with different amounts of *rbRS1*-cRNA or *hRS1*-cRNA. After 2 days incubation the initial uptake of 100 μM [^{14}C]TEA was measured in the absence and presence of 75 μM cyanine 863 and the inhibited uptake rates were calculated. (a) Uptake rates after coexpression of *rbRS1*; (b) uptake rates after coexpression with *hRS1*.

cRNA into *Xenopus* oocytes was increased if an appropriate amount of *RS1*-cRNA from pig (*pRS1*) was coinjected and that the apparent glucose dependence of the expressed transport was altered. At variance, the V_{max} of Na^+-D-glucose cotransport expressed by human *SGLT1* (*hSGLT1*) was decreased if cRNA of human *RS1* (*hRS1*) was coinjected. In this case the apparent glucose dependence was also altered [10]. In the present study coexpression experiments were performed with *rbRS1* and *rbSGLT1*, with *rbRS1* and *hSGLT1*, with *rbRS1* and the human organic cation transporter *hOCT2* [21], or with *hRS1* and *hOCT2*. When 0.1 ng of *rbSGLT1*-cRNA per oocyte was injected and the oocytes incubated for

Table 2
Coexpression experiments with RS1

Expressed proteins	Effects on the expressed transport		
	Uptake rate	V_{max}	Substrate dependence
rbSGLT1 plus pRS1	stimulation	stimulation	change
rbSGLT1 plus rbRS1	stimulation	stimulation	no change detected
hSGLT1 plus hRS1	inhibition	inhibition	change
hSGLT1 plus rbRS1	inhibition	inhibition	change
hOCT2 plus rbRS1	no change	not determined	not determined
hOCT2 plus hRS1	inhibition	not determined	not determined

Effects of RS1 from pig (*pRS1*), man (*hRS1*) and rabbit (*rbRS1*) on the expression of AMG transport by SGLT1 from rabbit (*rbSGLT1*) and man (*hSGLT1*) are summarized (this paper and [1,10]). In addition, measurements on the effects of *rbRS1* and *hRS1* (this paper) on the expression of tetraethylammonium transport by hOCT2 are indicated.

2 days, the expressed phlorizin inhibitable uptake of 50 μM [^{14}C]AMG was 22 ± 3 pmol oocyte^{-1} h^{-1}. By coinjection of 0.025 ng *rbRS1*-cRNA with 0.1 ng *rbSGLT1*-cRNA the expressed uptake increased between two- and sixfold in different batches of oocytes. Fig. 7a shows a typical experiment which indicates that the effect of *rbRS1* was only observed with an optimal amount of coinjected cRNA. At variance to our previous coexpression experiments with *pRS1* and *rbSGLT1* no significant change of the glucose dependence after coexpression of *rbRS1* and *rbSGLT1* was resolved. Fig. 7b shows an experiment where the glucose dependence of AMG uptake was measured after injection of 0.1 ng/oocyte of *rbSGLT1*-cRNA alone or together with 0.025 ng *rbRS1*-cRNA. In this batch of oocytes the V_{max} was increased about twofold by *rbRS1*.

To test whether the different effects of *rbRS1* (on *rbSGLT1*) and of *hRS1* (on *hSGLT1*) are due to different properties of SGLT1 or RS1 from different species *rbRS1* was also expressed together with *hSGLT1* (Fig. 8). When increasing amounts of *rbRS1*-cRNA were coinjected with *hSGLT1*-cRNA the expressed AMG uptake was altered as observed with *hRS1* [10]: the expressed uptake decreased with increasing concentrations of coinjected *rbRS1*-cRNA and no apparent requirement for an optimal ratio between the coinjected cRNAs was detected (Fig. 8a). By coinjection of *rbRS1*-cRNA with *hSGLT1*-cRNA the apparent glucose dependence of AMG uptake was changed as has been described after coexpression of *hRS1* and *hSGLT1* or of *pRS1* and *rbSGLT1* [1,10]. Whereas a monophasic glucose dependence with an apparent $K_{0.5}$ value of 0.65 ± 0.05 mM was observed after expression of *hSGLT1* alone a biphasic glucose dependence with apparent $K_{0.5}$ values of 33 ± 8 μM and 0.95 ± 0.05 mM was observed when *rbRS1*-cRNA was coinjected (Fig. 8b).

Because the ubiquitous transcription of *rbRS1* suggested a more general function of RS1 rather than being an SGLT1 associated protein we reevaluated the specificity of RS1. Fig. 9 shows coexpression experiments of *rbRS1* and *hRS1* with *hOCT2*, the recently cloned polyspecific organic cation transporter from man [21]. Cation transport expressed by the injection of *hOCT2*-cRNA into *Xenopus* oocytes was not altered after coinjection of different amounts of *rbRS1*-cRNA (Fig. 9a); however, the expressed cation uptake was significantly inhibited when *hRS1*-cRNA was coinjected (Fig. 9b). The data indicate that RS1 is not specific for the Na^+-D-glucose cotransporter SGLT1.

4. Discussion

The physiological role and functional mechanism of the RS1 protein which was first cloned from pig (pRS1) is not understood. Previously we raised the hypothesis that RS1 is an extracellular membrane-bound modulator or subunit of the Na^+-D-glucose cotransporter SGLT1 and/or of structural related transporters [1,2]. It turned out to be very difficult to verify or discard this hypothesis since the interaction of RS1 with SGLT1 had to be investigated within the same species and it was not clear whether homologous proteins are expressed in each species, neither were anti-RS1 antibodies available which

could be used for coprecipitation experiments. Because significant functional differences have been described between the high affinity Na^+-D-glucose cotransporter from rabbit (*rbSGLT1*) and man (*hSGLT1*) we cloned pRS1 homologous proteins from these species and investigated their effects on the expression of *rbSGLT1* and *hSGLT1*. Cloning of an intronless human gene on chromosome 1p36.1 (*hRS1*) that encodes a protein with 74% amino acid identity to *pRS1* has been reported [10]. In man no *hRS1*-homologous genes were detected, and coexpression of *hRS1* and *hSGLT1* revealed that the V_{max} of the expressed Na^+-D-glucose cotransport was inhibited by *hRS1* whereas the apparent K_m value of the expressed glucose transport was decreased.

The present paper describes the cloning of a rabbit gene which encodes a polypeptide with 64% amino acid identity to pRS1 and 65% identity to hRS1. As also observed in man there is only one *RS1* gene with similarity to *pRS1* in rabbit. The possibility of splicing variants can be excluded since the *RS1* genes of man and rabbit are intronless. The localization of the *rbRS1* message described in this paper does not support our previous hypothesis that RS1 is a SGLT1-specific modifier or a subunit of SGLT1. Comparing the mRNA levels of *rbRS1* and *rbSGLT1* by Northern blotting and in situ hybridization no indication was obtained for a correlation between the expression of RS1 and SGLT1. In some localizations where the expression of *rbSGLT1* had not been described but *rbRS1*-mRNA was found in Northern blots we detected minor amounts of *rbSGLT1*-mRNA by RT-PCR experiments. For example, we observed mRNA and protein of *rbSGLT1* in neurones of brain [25] and found that *rbSGLT1* is also transcribed in renal glomeruli, proximal convoluted tubules of outer renal cortex and in renal collecting ducts (this paper). However, the amounts of the transcribed mRNAs did not appear to be correlated. The previously hypothesized specificity of RS1 for SGLT1 or for structurally related transporters has been contradicted by an experiment which shows that coexpression of *hRS1* inhibited not only the expression of glucose transport by *hSGLT1* but also the expression of organic cation transport by *hOCT2*.

Recently we obtained immunological data that falsified our previous assumption that RS1 is associated with the extracellular membrane side. Employing a specific affinity-purified antibody against pRS1 we investigated the labelling of expressed pRS1 protein in *Xenopus* oocytes with lipid-permeable and lipid-impermeable reagents (M. Valentin, H. Koepsell, unpublished data). These experiments showed that RS1 is localized at the inner side of the plasma membrane rather than extracellularly as was previously concluded from immunohistochemical data with a monoclonal antibody which had been used to isolate pRS1 from an expression library [1]. Apparently this IgM antibody cross-reacts with a non-related extracellular protein.

In Table 2 coexpression experiments of RS1 from different species with different plasma membrane transporters are summarized. The data show that RS1 may interact with structurally non-related plasma membrane transporters and that the observed effects on transport are dependent on the target transporter and RS1 species. Since the interaction of RS1 with the transporters is apparently not restricted to a specific primary structure of the target transporter additional proteins may be involved that may have adaptor function. The presence of conserved consensus sequences for protein kinase C dependent and casein kinase II dependent phosphorylation sites in RS1 suggests that functional properties of RS1 could be modulated by phosphorylation of RS1. Our coexpression experiments in *Xenopus* oocytes demonstrating an interaction of RS1 with plasma membrane transporters suggests some flux of information between RS1 and the transporters. They do not allow to determine the physiological direction of this flux: whether RS1 modulates transporter activities after activation by an intracellular signal, or whether the transporters may act as extracellular sensors which activate RS1. We would like to prefer this latter possibility since we recently observed that RS1 may leave the plasma membrane (unpublished data).

The physiological role of RS1 is not understood. However, its interaction with structurally different plasma membrane transporters makes it an important factor for the regulation of plasma membrane transporters which should be further investigated.

Acknowledgements

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