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The transport modifier RS1 is localized at the inner side of the plasma membrane and changes membrane capacitance

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Abstract

Previously we cloned membrane associated (M_r 62 000–67 000) polypeptides from pig (pRS1), rabbit (rbRS1) and man (hRS1) which modified transport activities that were expressed in *Xenopus laevis* oocytes by the Na^+-D-glucose cotransporter SGLT1 and/or the organic cation transporter OCT2. These effects were dependent on the species of RS1 and on the target transporters. *hRS1* and *rbRS1* were shown to be intronless single copy genes which are expressed in various tissues and cell types. Earlier immunohistochemical data with a monoclonal IgM antibody suggested an extracellular membrane association of RS1. In the present paper antibodies against recombinant pRS1 were raised and the distribution and membrane localization of RS1 reevaluated. After subcellular fractionation of renal cortex RS1 was found associated with brush border membranes and an about 1:200 relation between RS1 and SGLT1 protein was estimated. Also after overexpression in *X. laevis* oocytes RS1 was associated with the plasma membrane, however, at variance to the kidney it was also observed in the cytosol. Labeling experiments with covalently binding lipid-permeable and lipid-impermeable biotin analogues showed that RS1 is localized at the inner side of the plasma membrane. Western blots with plasma membranes from *Xenopus* oocytes revealed that SGLT1 protein in the plasma membrane was reduced when hRS1 was coexpressed with human SGLT1 which leads to a reduction in V_{max} of expressed glucose transport. Measurements of membrane capacitance and electron microscopic inspection showed that the expression of hRS1 leads to a reduction of the oocyte plasma membrane surface. The data suggest that RS1 is an intracellular regulatory protein that associates with the plasma membrane. Overexpression of RS1 may effect the incorporation and/or retrieval of transporters into the plasma membrane.

Keywords: Transport modifier RS1; Subcellular distribution; Expression; Na^+-D-glucose cotransporter SGLT1; Membrane capacitance; *Xenopus laevis* oocyte

Abbreviations: IPTG, isopropyl-β-D-thioglucopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; pRS1-ab, polyclonal antibody raised against recombinant pRS1; hSGLT1-ab, peptide antibody against human SGLT1; rbSGLT1-ab, peptide antibody against SGLT1 from rabbit; AMG, methyl-α-D-glucopyranoside; l-biotin, *N*-hydroxysuccinimido-biotin; w-biotin, sulfosuccinimidyl-6-(biotinamido)-hexanoate

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1. Introduction

Previously we isolated a hydrophilic M_r 67 000 polypeptide with a hydrophobic C-terminus (pRS1) by screening an expression library from pig kidney with a monoclonal IgM antibody (R4A6) which stimulated high affinity phlorizin binding to renal brush border membranes [1]. Coexpression of pRS1 with the rabbit Na^+-D-glucose cotransporter rbSGLT1 in *Xenopus* oocytes stimulated the expressed glucose uptake and altered the substrate dependence of the transporter. In Western blot experiments with renal and intestinal brush border membranes R4A6 bound predominantly to a polypeptide with an apparent molecular mass of 75 kDa and ultrastructural immunohistochemical data suggested R4A6 binding to the extracellular side of the brush border membranes [1–4]. Because initial coexpression experiments of pRS1 with some other transporters suggested specificity for SGLT1 and an extracellular localization of RS1 was assumed, we raised the hypothesis that RS1 is a subunit of the Na^+-D-glucose cotransporter [5]. Recently this hypothesis became more and more improbable. Firstly, we found that RS1 is encoded by intronless single copy genes in rabbit and man and has a different tissue distribution than SGLT1 [6,7]. Secondly we found that RS1 is not specific for SGLT1 since the activity of both, human SGLT1 and the human cation transporter OCT2 (hOCT2) was reduced when hRS1 was coexpressed in *Xenopus laevis* oocytes [7]. On the other hand some selectivity of RS1 was suggested since the effects of RS1 on the expression of hOCT2 were dependent on the species from which RS1 was derived, and rabbit RS1 exhibited differential effects on the expression of SGLT1 from rabbit and the human [7].

In the present study the subcellular localization of RS1 was reevaluated employing affinity purified antibodies against recombinant RS1 from pig (pRS1). With these antibodies it was found that pRS1 expressed in *Xenopus* oocytes was associated with the intracellular side of the plasma membrane. Capacitance measurements in *Xenopus* oocytes and the ultrastructural inspection showed that the plasma membrane surface was changed after overexpression of RS1.

2. Materials and methods

2.1. Materials

An antiserum against SGLT1 from rabbit (rbSGLT1-ab) was provided by Dr. S. Shirazy-Beechey which had been raised against residues 402–420 of rabbit SGLT1 [8]. [^{14}C]methyl-α-D-glucopyranoside (10.3 GBq/mmol) was obtained from Amersham Buchler (Braunschweig, Germany), PNGase F from Boehringer (Mannheim, Germany) and Ni^{2+} nitriloacetic acid agarose from Quiagen GmbH (Hilden, Germany). Peroxidase-conjugated goat anti-rabbit IgG antiserum, goat anti-mouse IgM antiserum and *N*-hydroxysuccinimido-biotin (l-biotin) were purchased from Sigma (Deisenhofen, Germany), and sulfosuccinimidyl-6-(biotinamido)-hexanoate (w-biotin) and peroxidase-conjugated streptavidin were obtained from Calbiochem (Bad Soden/Taunus, Germany). In Western blots 'high molecular weight markers' of Sigma or prestained proteins (BenchMark) from Life Technologic (Karlsruhe, Germany) were used for calibration. All other reagents were obtained as described earlier [1,9].

2.2. Expression of pRS1 in Escherichia coli and purification

pRS1 [1] was cloned into the histidine-tagged expression plasmid pET22b(+) (Novagen, Madison, WI, USA) using the *Nhe*I and *Xho*I restriction sites of the vector. The nucleotide sequence of *pRS1* in pET (pET-*pRS1*) and correct in frame cloning were verified by DNA sequencing. *E. coli* strain BL21[DE3] [10] was transformed with pET-*pRS1* and grown in the presence of 100 μg/l ampicillin. Induction was activated by adding 0.4 mM isopropyl-β-D-thiogalactoside (IPTG). After 4 h the cells were harvested by centrifugation and frozen at −70°C. For purification the cells were sonicated (3 min at 20°C, 400 Watt), spun down at 10 000×*g*, treated with 2 M urea (1 h at 0°C in 0.1 M Tris–HCl pH 8.0) and spun down again. Inclusion bodies were dissolved by incubating the pellet with 8 M urea and 0.1 M β-mercaptoethanol (1 h at 21°C in 0.1 M Tris–HCl pH 8.0). After 15 min centrifugation at 25 000×*g* the supernatant was incubated overnight

at 4°C with Ni^{2+} nitrilotriacetic acid agarose in the presence of 0.1 M Tris–HCl pH 8.0, 3 M urea and 5 mM β-mercaptoethanol (binding buffer). The gel was filled into a column and washed with 200 ml of binding buffer and with 10 ml of binding buffer containing 0.8 mM imidazole. pRS1 protein was eluted with binding buffer containing 60 mM imidazole. For further purification 0.5 mg of affinity purified pRS1 protein was separated on a 7.5% (w/v) SDS–polyacrylamide gel [11], stained with sodium acetate [12] and the pRS1 band was dissected. Elution was performed by homogenizing the gel strips and suspending them 16 h at 37°C in 50 mM Tris–HCl pH 8.0 containing 8 M urea and 50 mM β-mercaptoethanol [13]. For immunization the eluent was dialyzed against phosphate buffered saline containing detergent absorbent from Boehringer (Mannheim, Germany).

2.3. Antibody production and purification

Rabbits were immunized with purified pRS1 protein. The immunization was performed subscapular (on day 1: oil–water suspension containing 100 μg pRS1 and 100 μg of *N*-acetylmuramyl-ala-D-iso-glutaminyl-*N*-ε-stearyoyl-lysin, on day 21: oil–water suspension containing 100 μg pRS1) and intraveneously (on days 28, 32, 36, 40, 61, 65, 69 and 73: 50 μg pRS1 in PBS). The raised antibodies against pRS1 (pRS1-ab) were affinity purified on ELISA plates which had been coated with the affinity purified pRS1 protein (75 μg/ml) [14]. The antibodies were stored at 4°C at neutral pH in the presence of 2% (w/v) skimmed milk powder and 0.01% (v/v) thimerosal. Antibodies against human SGLT1 (hSGLT1-ab) were raised against the peptide QEGQKETIEIETQVPEKKKGC that represents residues 580–600 of the human SGLT1 [15]. The peptide was coupled to ovalbumin, the antibodies were raised in rabbits and the antiserum was affinity purified on the antigenic peptide that was coupled to epoxy-activated Sepharose [9].

2.4. SDS–polyacrylamide gel electrophoresis and Western blotting

SDS–polyacrylamide gel electrophoresis (4% stacking gel and 7.5% or 9% resolution gel) was performed as described by Laemmli [11]. For standard sample preparation the protein samples were mixed with an equal volume of 46 mM Na_2HPO_4, 54 mM $Na_2H_2PO_4$ pH 6.8, 2% (w/v) SDS, 8 M urea, 0.5 M β-mercaptoethanol, 0.001% (w/v) bromophenol blue (sample buffer) and incubated for 1 h at 37°C. In some experiments the proteins were acetylated by incubating 30 min at 37°C in sample buffer containing 50 mM iodoacetic acid. Silver staining was performed as described [16]. For Western blotting the proteins were electrophoretically transferred to nitrocellulose membrane using a semi-dry procedure [17]. The membranes were blocked by 1 h incubation at 25°C in 50 mM Tris–HCl pH 8.0 containing 137 mM NaCl, 2.7 mM KCl, 0.05% (w/v) Tween-20 and 2% (w/v) skimmed milk powder (blocking buffer). For antibody reactions the blots were incubated for 1 h at 25°C with primary antibodies. The primary antibodies were diluted either with blocking buffer (pRS1-ab antiserum 1:2500, rbSGLT1-ab antiserum 1:1000, affinity purified hSGLT1-ab 1:1000) or with blocking buffer containing 1 M D-glucose, 10% (v/v) glycerin and 1% (v/v) Tween-20 (monoclonal IgM antibody R4A6 [1,3] 1:100). After washing three times with blocking buffer the blots were incubated (1 h at 25°C) with peroxidase-conjugated goat anti-rabbit IgG antiserum or with goat anti-mouse IgM antiserum. Both secondary antibodies were diluted 1:5000. After washing three times with blocking buffer the immunoreactivity was visualized by enhanced chemiluminescence detection. The amount of pRS1 protein in renal brush border membranes was estimated from Western blots by digitizing the stained pRS1 band on the X-ray films, subtracting the background staining (program Sigmascan from Sigma, Deisenhofen, Germany) and compared with the staining obtained from increasing amounts of purified pRS1 protein expressed in *E. coli*. To verify the specificity, the pRS1-ab antisera were blocked by immunoabsorption with affinity purified pRS1 protein which had been separated by polyacrylamide gel electrophoresis, blotted to nitrocellulose and band dissected.

2.5. Expression of RS1 and SGLT1 in Xenopus oocytes and capacitance measurements

To prepare sense cRNAs for expression experi-

ments in *Xenopus* oocytes purified plasmids of the respective clones were linearized with *Not*I (*rbSGLT1*), *Eco*RI (*hSGLT1*), *Xho*I (*pRS1*) or *Sac*I (*hRS1*) and 5′7meGppp5′G capped cRNAs were synthesized by T3 polymerase (*rbSGLT1*, *hSGLT1*, *pRS1*) or T7 polymerase (*hRS1*) [1,6]. For the expression of *pRS1* [1], *hRS1* [6], *rbRS1* [7] and *rbSGLT1* [18] or *hSGLT1* [15], *Xenopus* oocytes were injected with 50 nl water diluted sense cRNAs and incubated for 3 days as described [1]. The expressed phlorizin inhibitable uptake of 50 μM [^{14}C]methyl-α-D-glucopyranoside (AMG) was measured as reported earlier [1,6]. Biotin labeled and unlabeled oocytes were fractionated by differential centrifugation.

The membrane capacitance of *Xenopus* oocytes was measured as described [19]. Single oocytes were placed in a plexiglass chamber and superfused at 2 ml/min with 90 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$ and 5 mM *N*-2-hydroxylethylpiperazine-*N*′-ethanesulfonic acid (HEPES), pH 7.6 and the membrane capacitance (C_m) was measured under voltage-clamp conditions applying a sine wave of voltage changes with a frequency of 73 Hz.

2.6. Subcellular fractionations

Brush border membrane vesicles from porcine renal cortex and duodenum of pig were isolated by Ca^{2+} precipitation and differential centrifugation [20,21]. For a crude fractionation of cellular membranes and cytosolic proteins, different tissues were homogenized in the presence of 280 mM sucrose, 20 mM Tris–HCl pH 7.5, 5 mM EGTA, 5 mM $MgSO_4$ and 1 mM PMSF (sucrose buffer) and centrifuged 10 min (8°C) at 2000×*g*. The pellets were discarded and the supernatants centrifuged for 60 min (8°C) at 40 000×*g*. The remaining pellet (crude membrane fraction) and supernatant (cytosolic fraction) were employed for Western blotting.

The subcellular fractionation of *Xenopus* oocytes was performed as described by Geering and coworkers [22]. Briefly the oocytes were homogenized in the presence of 10 mM HEPES pH 7.9, 83 mM NaCl, 1 mM $MgCl_2$ (homogenization buffer) containing 1 mM PMSF, 0.05 ng/ml aprotinin, 0.05 ng/ml leupeptin and 10 mM benzamidine, and large debris was removed by two times centrifugation for 10 min (4°C) at 1000×*g*. This cleared homogenate was 20 min centrifuged at 10 000×*g* (4°C). The obtained pellet (P1) was washed two times with homogenization buffer to minimize cytosolic contaminations. The 10 000×*g* supernatant (S1) was centrifuged for 90 min at 200 000×*g* (4°C). The supernatant was centrifuged a second time under the same conditions and the remaining supernatant named S2. Both 200 000×*g* pellets were combined and washed by resuspending in homogenization buffer and pelleting at 200 000×*g* (P2). P1, P2 and S2 were analyzed by electron microscopy and Western blotting. The protein was quantified according to Bradford [23] using bovine serum albumin as standard.

2.7. Enzymatic deglycosylation

Renal brush border membranes (1.5 mg protein/ml) were denatured by 10 min incubation at 100°C with 25 mM sodium phosphate pH 7.2, 10 mM EDTA, 0.1% (w/v) SDS and 5 mM β-mercaptoethanol. After cooling to 37°C, 1 mM PMSF, 1% (w/v) Triton X-100 and 0.6 units of PNGase F were added and the sample was incubated for 16 h at 37°C. After addition of another 0.6 units of PNGnase F, the samples were incubated for a second 16 h period. Controls without addition of PNGase F were run in parallel.

2.8. Affinity labeling with biotin

3 days after injection with water or cRNAs, 150 oocytes of *X. laevis* were washed three times with homogenization buffer and incubated for 30 min at 21°C with 0.5 mM of the membrane-permeable biotin derivate *N*-hydroxysuccinimido-biotin dissolved in dimethylsulfoxide (l-biotin) or with 0.2 mM of the membrane-impermeable derivate sulfosuccinimidyl-6-(biotinamido)-hexanoate (w-biotin) [24]. The labeling reaction was chased by washing the oocytes three times in 500 mM glycine–HCl pH 7.4 and two times in homogenization buffer before the oocytes were fractionated as described above. To enrich the biotinylated proteins 40 μg of the P1 and S2 fractions were denatured by boiling for 10 min in the presence of 25 mM sodium phosphate pH 7.2, 10 mM EDTA, 0.1% (w/v) SDS and 5 mM β-mercaptoethanol. After the uncoupled biotin had been removed by 16 h (25°C) dialysis against PBS, the sam-

ples were again boiled for 10 min, diluted with 9 volumes of 50 mM Tris–HCl pH 7.4, 5 mM EDTA, 150 mM NaCl (TEN buffer) and incubated for 2 h at 4°C with 80 μl of streptavidin resin (Boehringer, Mannheim, Germany). The beads were recovered by sedimentation, washed three times with TEN buffer and the final bead pellet was eluted by boiling 10 min with sample buffer for SDS–polyacrylamide gels (see above). After Western blotting the biotinylated proteins were detected with the antibody against pRS1 (pRS1-ab) or with peroxidase-conjugated streptavidin which was diluted 1:5000 with 50 mM Tris–HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl and 0.05% (v/v) Tween-20.

2.9. Electron microscopy

Subcellular fractions of *Xenopus* oocytes were prepared for electronic microscopy as described by Lourim and Krohne [25]. For electron microscopic inspection of plasma membranes in intact oocytes, the oocytes were fixed for 16 h at room temperature in 0.1 M cacodylate pH 7.3, 1 mM $CaCl_2$, 3% (v/v) glutaraldehyde, 2% (v/v) formaldehyde, 1% (v/v) acrolein, 2.5% (v/v) dimethylsulfoxide and washed at 4°C in 0.1 M cacodylate pH 7.3 containing 1 M sucrose. The fixed oocytes and fixed pellets of subcellular fractions were stained with osmium tetroxide and uranyl acetate, embedded in Epon 812, thin sectioned and analyzed by transmission electron microscopy [26].

3. Results

3.1. Expression of pRS1 in E. coli and antibody production

pRS1 was cloned into the *E. coli* expression vector pET22b(+) and introduced into the *E. coli* strain BL21[DE3] as described in Section 2. In this system pRS1 was expressed with six histidine residues attached to the C-terminus, and the expression could be induced by IPTG. Silver-stained SDS–polyacrylamide gels of *E. coli* cell extracts showed that IPTG induced a polypeptide with an apparent molecular mass of 100 kDa (data not shown). This polypeptide could be highly enriched by affinity chromatography on Ni^{2+} nitriloacetic acid agarose and was further purified by band elution from SDS–polyacrylamide gels (Fig. 1, lane a). The identity of the expressed 100 kDa polypeptide with pRS1 was verified by amino acid sequencing of gel-eluted protein (data not shown). After immunization of rabbits with purified pRS1 protein, polyclonal antibodies were raised (pRS1-ab). The pRS1-ab antiserum and the affinity purified antibody reacted specifically with the 100 kDa polypeptide that was expressed after IPTG induction of the pRS1 transfected *E. coli* cells. The reaction of the pRS1-ab antiserum with 50 pg of affinity purified pRS1 protein is shown in lane b of Fig. 1. The antibody binding to the 100 kDa polypeptide could be blocked by preincubating the pRS1-ab antiserum with expressed pRS1 protein (Fig. 1, lane c). We failed to detect any reaction of expressed

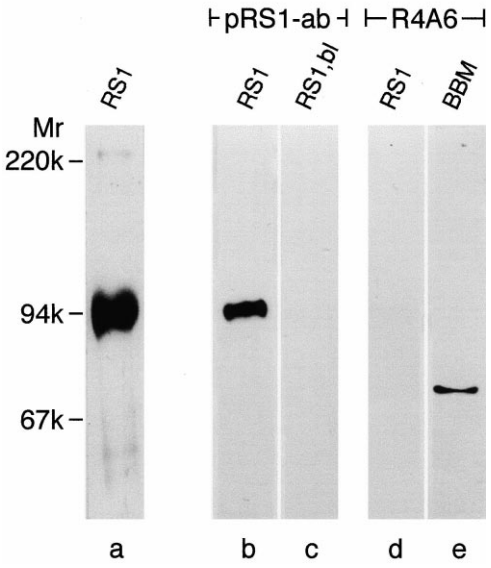

Fig. 1. Expression of pRS1 in *E. coli* and generation of an antibody against pRS1. pRS1 was expressed in *E. coli*, purified as described in Section 2 and separated by SDS–polyacrylamide gel electrophoresis. In lane a, 2.5 μg of purified pRS1 protein expressed in *E. coli* was applied to the SDS–gel and stained with silver. Lanes b and c show Western blots where 50 pg of purified pRS1 protein expressed in *E. coli* (RS1) were applied per lane and developed with the antiserum raised against the purified pRS1 protein (pRS1-ab). In lane c the specificity of the immunoreaction was tested by absorbing the antiserum with expressed pRS1 protein (RS1, bl). Lanes d and e show Western blots of expressed pRS1 protein (RS1) and porcine renal brush border membranes (BBM) that were developed with the monoclonal antibody R4A6. 1 ng of protein was applied to lane d, and 20 μg of protein to lane e.

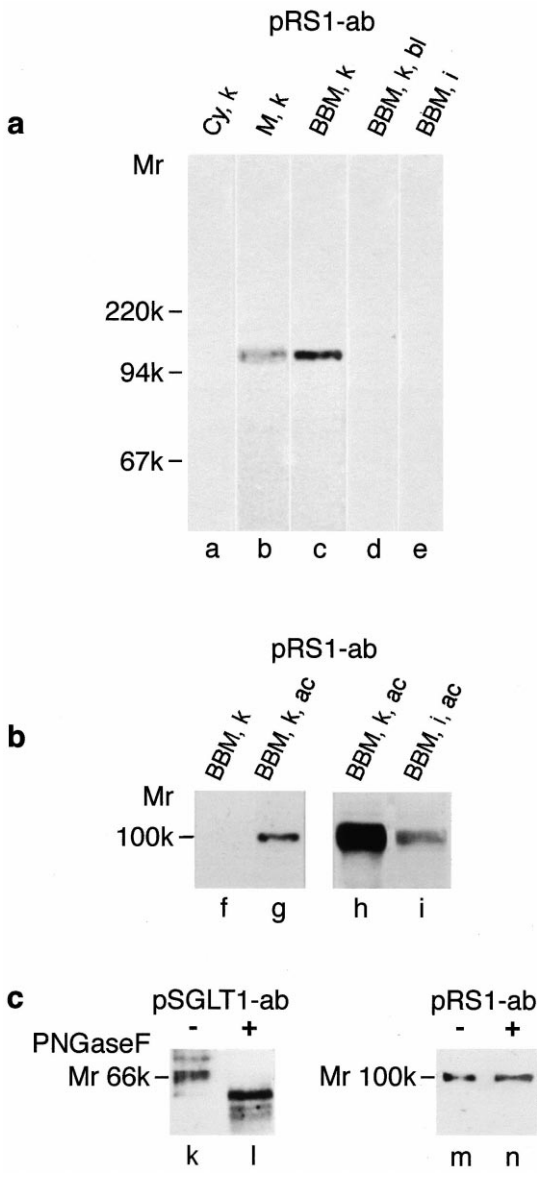

Fig. 2. Demonstration of pRS1 in brush border membranes by Western blotting. Different protein fractions from porcine renal cortex and porcine small intestine were separated by SDS–polyacrylamide gel electrophoresis, blotted to nitrocellulose membranes and developed with different antibodies. In (a) and (b) Western blots with different fractions from renal cortex (the cytosolic fraction Cy,k in lane a; the crude membrane fraction M,k in lane b; the brush border membrane fraction BBM,k in lanes c, d, f, g, h), and from small intestine (the brush border membrane fraction BBM,i in lanes e and i) are shown. Per lane 20 μg (lanes a–e, h, i) or 2 μg (lanes f, g) of protein was applied. Before electrophoresis the samples were solubilized with SDS in the presence of β-mercaptoethanol and urea. Those in lanes g, h, i were additionally acetylated. The blots were developed with affinity purified pRS1-ab (lanes a–c, e, f–i) or with affinity purified pRS1-ab which was blocked with excess of pRS1 (lane d). In (c) pRS1 in renal brush border membranes was tested for *N*-glycosylation. 22 μg of brush border membrane protein was incubated without (−) and with (+) PGNase F, fractionated by SDS–PAGE and tested for reaction with affinity purified peptide antibodies against rbSGLT1 (lanes k, l) and pRS1 (lanes m, n).

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pRS1 with the monoclonal antibody R4A6 which had been used to identify pRS1 from a lambda gt11 library [1] although various experimental conditions for Western blotting were tested (one example is shown in lane d of Fig. 1). The antibody R4A6 increased high affinity phlorizin binding to renal and intestinal brush border membranes and showed in Western blots of renal and intestinal brush border membranes selective binding to a 75 kDa polypeptide (see [2,3] and lane e in Fig. 1). Because R4A6 does not bind the expressed pRS1 in Western blots, the 75 kDa polypeptide in renal and intestinal brush border membranes must be considered as a cross-reacting polypeptide. Consequently the previously described immunohistochemical localization of R4A6 binding to renal and intestinal brush border membranes [2,4] may not represent the localization of pRS1.

3.2. *Localization and quantification of pRS1 in renal brush border membranes*

The polyclonal antibody against pRS1 (pRS1-ab) was employed to probe pRS1 in different protein fractions from kidney cortex, small intestine and muscle. Using our standard sample buffers (which contain β-mercaptoethanol and urea) for SDS–polyacrylamide gel electrophoresis no specific binding to pRS1 was observed with the crude antiserum (data not shown). However, after affinity purification of pRS1-ab, RS1 protein could be detected: A significant signal with a 100 kDa polypeptide was observed in crude plasma membranes isolated from renal kidney which increased after purification of brush border membranes (Fig. 2, lanes b, c) and was blocked when the antibody was preincubated with excess of pRS1 (Fig. 2, lane d). No reaction was observed in the renal cytosol (Fig. 2, lane a) and in small intestinal brush border membranes (Fig. 2, lane e). With pRS1 protein expressed in *E. coli* the antibody reaction in Western blots observed under standard con-

ditions (with β-mercaptoethanol and urea in the sample buffer for SDS–polyacrylamide electrophoresis) was not altered when the proteins were acetylated by iodoacetic acid (data not shown). In contrast to the recombinant protein, the antibody binding to renal brush border membranes was drastically increased by acetylation of the proteins applied to SDS–gel electrophoresis (compare lane f with lane g, or lane c with lane h in Fig. 2). After acetylation antibody binding to a 100 kDa polypeptide could be also observed with small intestinal brush border membranes (Fig. 2, lane i). In Fig. 3 we estimated the amount of pRS1 protein that was associated with renal brush border membranes using different amounts of the recombinant pRS1 protein for calibration. Here the protein samples were acetylated before SDS–polyacrylamide gel electrophoresis. 40 ± 6 ng of pRS1 per mg of brush border membrane protein ($n = 3$) was estimated. This represents 0.6 ± 0.08 pmol pRS1 protein per mg brush border membrane protein and is about 200-fold smaller than the number of high affinity phlorizin binding sites in porcine renal brush border membrane which may represent the number of SGLT1 transporters [27]. Because of different posttranslational modifications of pRS1 expressed in *E. coli* and in renal brush border membranes, pRS1-ab may have a different sensitivity to pRS1 in both preparations. Notwithstanding this uncertainty our data suggest that the number of pRS1 molecules in the brush border membrane is too small for RS1 to be a subunit of SGLT1 as previously hypothesized [1,5].

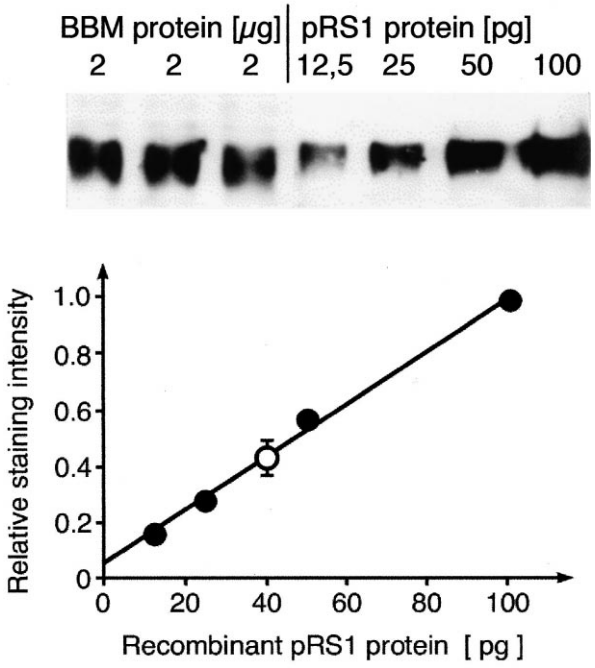

Fig. 3. Estimation of pRS1 protein amount in renal brush border membranes. Renal brush border membranes and purified pRS1 protein expressed in *E. coli* were acetylated with iodoacetic acid and the indicated protein amounts were applied to SDS–gel electrophoresis, blotted to nitrocellulose and developed with affinity purified antibody against pRS1. The top panel shows a Western blot from one experiment where 2 μg of brush border membrane protein (BBM protein) were applied to the first three lanes, and increasing concentrations of recombinant pRS1 protein to the other lanes. The bottom panel shows that the signal intensity increased linearly with the amount of recombinant pRS1 protein ($r^2 > 0.99$). The mean signal intensity ± S.D. of the 100 kDa band determined in the first three lanes is also indicated. The data suggest that 2 μg of brush border membrane protein contain about 40 ± 6 pg of pRS1 protein.

pRS1 contains several consensus sequences for *N*-glycosylation which are not conserved between RS1 from pig, rabbit and man (unpublished data). To investigate whether RS1 in the kidney is glycosylated, we treated porcine renal brush border membranes with PGNase F and performed Western blotting with the affinity purified pRS1-ab and with a peptide antibody against SGLT1. Whereas a significant mobility shift was observed for SGLT1 as described previously [28] the mobility for pRS1 was not changed (Fig. 2c). This suggests that pRS1 at the brush border membrane is not *N*-glycosylated.

3.3. Expressed RS1 in subcellular fractions of Xenopus oocytes

Xenopus oocytes were injected with water, *pRS1*-cRNA, *rbRS1*-cRNA, *rbSGLT1*-cRNA and/or *hSGLT1*-cRNA, incubated for 3 days, homogenized, fractionated by differential centrifugation, and characterized with antibodies against RS1 and SGLT1. The following subcellular fractions were characterized: a homogenate fraction where the nucleus and high density particles had been removed by a $1\,000 \times g$ centrifugation, a washed $10\,000 \times g$ pellet of this homogenate fraction (P1) which contained most of the plasma membranes, a washed $200\,000 \times g$ pellet (P2) of the $10\,000 \times g$ supernatant which was enriched in microsomal vesicles [29], and the supernatant of the $200\,000 \times g$ centrifugation (S2)

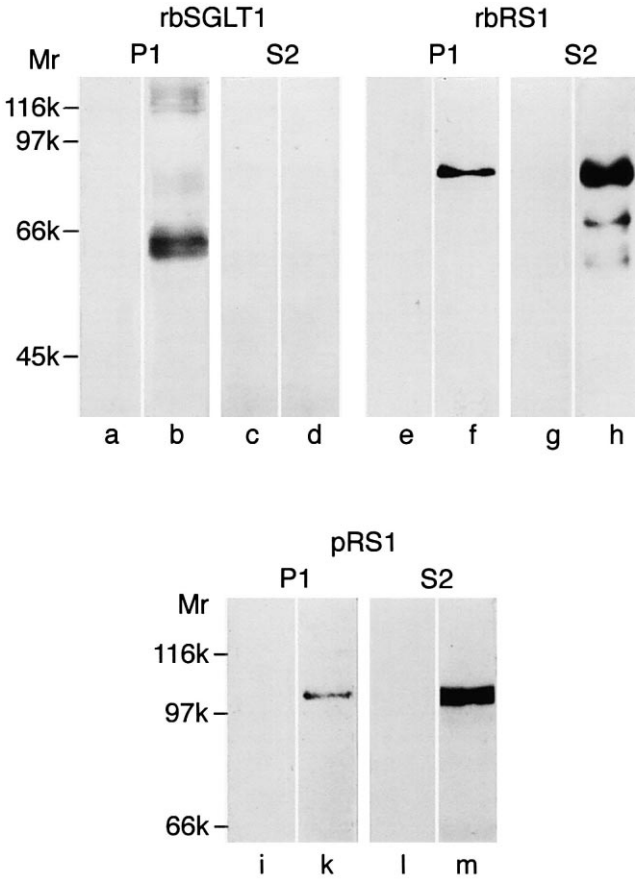

Fig. 4. Detection of rbSGLT1, rbRS1 and pRS1 after expression in *Xenopus* oocytes and differential centrifugation. *Xenopus* oocytes were injected with water (lanes a, c, e, g, i, l), 10 ng rbSGLT1-cRNA (lane b), 25 ng rbRS1-cRNA (lanes f, h) or 25 ng pRS1-cRNA (lanes k, m) and incubated for 3 days. Thereafter the oocytes were homogenized and differential centrifugation was performed. Samples (5 μg protein per lane) of the $10\,000\times g$ pellet containing the plasma membranes (P1) and of the $200\,000\times g$ supernatant (S2) were applied to SDS–polyacrylamide gel electrophoresis and Western blotting. The Western blots were developed with peptide antibody against rbSGLT1 (lanes a–d) or with the affinity purified antibody pRS1-ab which cross-reacts with rbRS1 (lanes e–m).

which only contained soluble proteins. The enrichment of plasma membranes in P1 had been previously demonstrated with the help of marker enzymes [29]. We verified the plasma membrane enrichment in P1 by incubating intact oocytes with membrane-impermeable biotin (w-biotin) and demonstrating that labeled proteins were almost exclusively found in P1 (data not shown). The ultrastructure of protein and lipid components in P1, P2 and S2 was analyzed by transmission electron microscopy. P1 contained mainly monolamellar vesicles with diameters between 200 and 500 nm whereas in P2 mainly smaller vesicles (diameters between 50 and 100 nm) were found together with some larger vesicles (data not shown). S2 was free of vesicles and membranes and contained aggregates with diameters smaller than 30 nm (data not shown).

Fig. 4 shows Western blots with different fractions of oocytes which had been injected with water and different cRNAs. They were developed with affinity purified pRS1-ab or a peptide antibody against rbSGLT1. In rbSGLT1-cRNA injected oocytes rbSGLT1 protein was found only in the plasma membrane fraction P1 (Fig. 4, lane b) but not in the microsomal fraction P2 (data not shown) and in the $200\,000\times g$ supernatant S2 (Fig. 4, lane d). Large amounts of rbRS1 and pRS1 were detected in the S2 fraction which only contains soluble proteins (Fig. 4, lanes h and m). Smaller amounts of pRS1 and rbRS1 were apparently associated with the plasma membranes since a significant antibody reaction was observed in P1 which was routinely washed two times (Fig. 4, lanes f and k). This membrane associated fraction of RS1 even remained constant when P1 was washed for another three times (data not shown). These data indicate that after overexpression of RS1 in *Xenopus* oocytes most RS1 protein is found as soluble protein in the cytosol, however, a small fraction of RS1 is associated with the plasma membrane. In contrast, in porcine kidney pRS1 protein was only observed in the brush border membrane fraction and could not be detected in the cytosol.

3.4. Intracellular localization of membrane associated pRS1

To determine the intra- or extracellular localization of membrane associated pRS1 intact oocytes were labeled either with the lipid soluble *N*-hydroxysuccinimido-biotin (l-biotin) or with water soluble sulfosuccinimidyl-6-(biotinamido)-hexanoate (w-biotin). In a control experiment we showed that the intracellular proteins in the S2 fraction were labeled by l-biotin but not by w-biotin (data not shown). Oocytes injected with water or 20 ng of pRS1-cRNA/oocyte were incubated for 3 days, labeled by addition of l-biotin or w-biotin to the bath, washed,

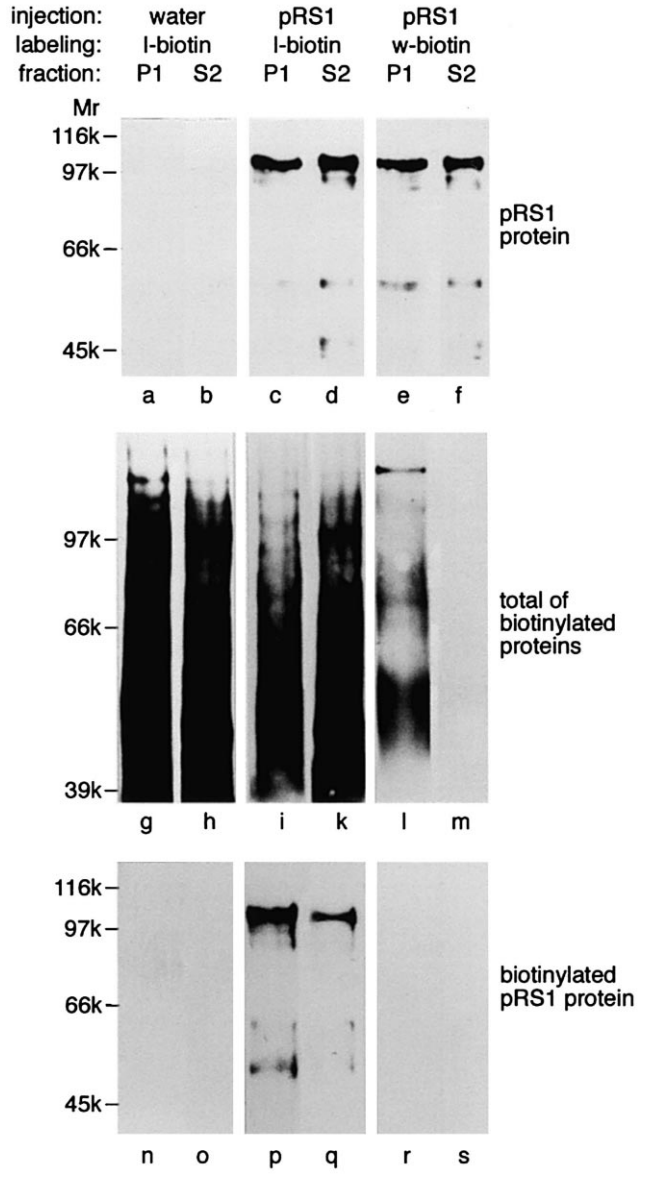

Fig. 5. Labeling of pRS1 protein in *Xenopus* oocytes with membrane-permeable and -impermeable reagents. *Xenopus* oocytes were injected with water or with 20 ng pRS1-cRNA and incubated for 3 days. Then the oocytes were labeled with lipid soluble *N*-hydroxysuccinimido-biotin (l-biotin) or with water soluble sulfosuccinimidyl-6-(biotinamido)-hexanoate (w-biotin), washed, homogenized and fractionated by differential centrifugation. The different fractions were tested for the presence of pRS1 protein by Western blotting with affinity purified antibody against pRS1 (pRS1 protein). Then the biotinylated proteins in the different fractions were affinity purified on a streptavidin column and detected by Western blotting with an streptavidin peroxidase conjugate (total of biotinylated proteins). Finally the affinity purified biotinylated proteins were investigated for pRS1 protein by Western blotting with the affinity purified antibody against pRS1 (biotinylated pRS1 protein).

homogenized and fractionated. In lanes a–f of Fig. 5 the expression of pRS1 was tested by Western blotting and it was verified that the P1 and S2 fractions isolated after labeling with l-biotin and w-biotin contained similar amounts of pRS1. In a further step we purified the biotin labeled proteins of the different fractions by affinity chromatography on streptavidin agarose, separated the proteins by SDS–polyacrylamide gel electrophoresis and visualized the biotin labeled proteins with peroxidase coupled streptavidin (Fig. 5, lanes g–m). Only after labeling with l-biotin were proteins in the S2 fraction labeled (compare lanes h and k with lane m). Finally the biotin labeled proteins that had been affinity purified over streptavidin were tested for the presence of pRS1 by Western blotting. After labeling with l-biotin, the biotinylated pRS1 protein was detected in the P1 and S2 fraction (lanes p, q). In contrast no biotinylated pRS1 protein was detected in P1 or S2 when the oocytes had been labeled with w-biotin (Fig. 5, lanes r, s). The data indicate that pRS1 protein is localized at the intracellular side of the plasma membrane.

3.5. Attempts to characterize the effects of RS1

Previously we demonstrated that the V_{max} of Na^+-D-glucose cotransport activity expressed by hSGLT1 was largely reduced when hRS1 was coexpressed and that the apparent K_m of the remaining transport activity was also reduced [6]. To determine whether RS1 alters the transporter concentration in the plasma membrane we estimated the amount of hSGLT1 protein in the plasma membrane fraction P1 after expression of *hSGLT1* alone or together with *hRS1*. Fig. 6 shows a representative experiment out of four in which 5 ng *hSGLT1*-cRNA or 5 ng *hSGLT1*-cRNA plus 20 ng *hRS1*-cRNA per oocyte were injected. Transport activity was measured with one part of the oocytes. From the other part the plasma membrane fraction P1 was isolated and Western blots were performed which were developed with a specific antibody against hSGLT1. By the coinjection of *hRS1*-cRNA the expressed phlorizin inhibitable uptake of 50 μM [^{14}C]AMG was decreased 50 to 90%. In parallel the concentration of hSGLT1 protein in the P1 fraction was reduced between 64 and 90% as estimated by densitometry of the West-

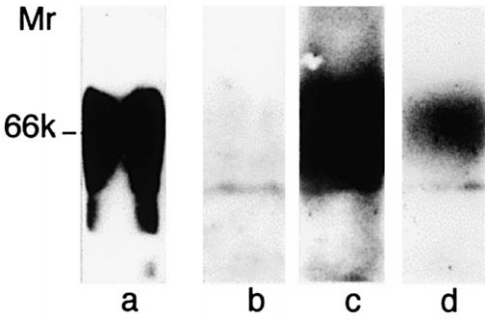

Fig. 6. Effects of coexpression of RS1 with SGLT1 in *Xenopus* oocytes on the amount of SGLT1 in the plasma membrane. *Xenopus* oocytes were injected with water (lane b), 5 ng *hSGLT1*-cRNA (lane c) or 5 ng of *hSGLT1*-cRNA plus 20 ng *hRS1*-cRNA (lane d). After 3 days incubation the plasma membrane fraction P1 was isolated from the different batches of oocytes, separated by SDS–polyacrylamide gel electrophoresis, blotted to nitrocellulose and developed with the affinity purified antibody *hSGLT1*-ab. Lane a shows a positive control with small intestinal brush border membranes. 5 μg of protein were applied on lanes b–d and 20 μg of protein to lane a.

ern blots. The data show that the amount of hSGLT1 protein in the plasma membrane is reduced when hRS1 is coexpressed with hSGLT1.

Since the transport activities of both SGLT1 and the human cation transporter OCT2 were decreased by coexpression of *hRS1* [7] we wondered whether RS1 may have a general effect on the plasma membrane. We injected *Xenopus* oocytes with water, 5 ng of *hSGLT1*-cRNA, 10 ng of *hRS1*-cRNA, or with 5 ng of hRS1-cRNA plus 10 ng of *hRS1*-cRNA and incubated the oocytes for 3 days. Then we verified the expression of SGLT1 and RS1 by measuring the uptake of AMG and determined the capacitance of the oocytes trying to detect changes of the plasma membrane. In these experiments which were performed with four different oocyte batches, the phlorizin inhibitable uptake of 50 μM [^{14}C]AMG expressed by *hSGLT1* was reduced between 60 and 80% when *hRS1* was coexpressed. After the capacitance measurement some of the oocytes were fixed and investigated by electron microscopy. Fig. 7 shows a summary of capacitance measurements. The expression of SGLT1 did not significantly change the capacitance of the oocytes. However, the capacitance was decreased by 35 ± 5 (S.E.M.) % when hRS1 was expressed alone ($P < 0.01$). When hRS1 was coexpressed with hSGLT1 the effect of hRS1 on the membrane capacitance was smaller, however still significant (16 ± 4 (S.E.M.) %, $P < 0.01$). To verify that the observed differences in capacitance represent changes in the membrane surface we investigated water injected control oocytes and hRS1 expressing oocytes by electron microscopy. Fig. 8 shows plasma membrane segments from the animal poles of a water injected oocyte and a *hRS1*-cRNA injected oocyte with 39% differences in membrane capacitance. It can be seen that heights and the amount of the microvilli were strongly reduced when hRS1 was expressed. From two water injected control oocytes and two hRS1-cRNA injected oocytes sections from the animal pole were used to estimate the effect of hRS1 on the surface area. The length of the sectioned plasma membrane was measured in relation to the circle arcs of the analyzed oocyte surface (Table 1). By expression of hRS1 in these oocytes the capacitance was reduced by 36.5%. The length of the cross-sectioned plasma membrane per

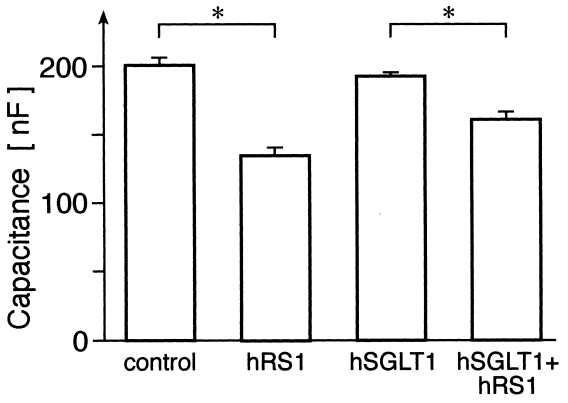

Fig. 7. Effects of the expression of RS1 on the plasma membrane capacitance in *Xenopus* oocytes. The experiments were performed with three batches of *Xenopus* oocytes. The oocytes were injected with water (control), 10 ng *hRS1*-cRNA (hRS1), 5 ng *hSGLT1*-cRNA (hSGLT1) or 5 ng *hSGLT1*-cRNA plus 10 ng *hRS1*-cRNA (hSGLT1+hRS1) and incubated for 3 days. In part of the oocytes the capacitance was measured (control 14 oocytes, hRS1 16 oocytes, hSGLT1 42 oocytes, hSGLT1 plus hRS1 11 oocytes). Some of the control oocytes and of the *hRS1*-cRNA injected oocytes were further proceeded for electron microscopy (see Fig. 8). In part of the oocytes injected with *hSGLT1*-cRNA or with *hSGLT1*-cRNA plus *hRS1*-cRNA the expressed phlorizin inhibitable uptake of 50 μM [^{14}C]AMG was measured. In the three oocyte batches the expressed AMG uptake was 60–80% smaller when hRS1 was coexpressed. Mean values ± S.E.M. and the significance of hRS1 effects on the capacitance (*$P < 0.01$) are indicated.

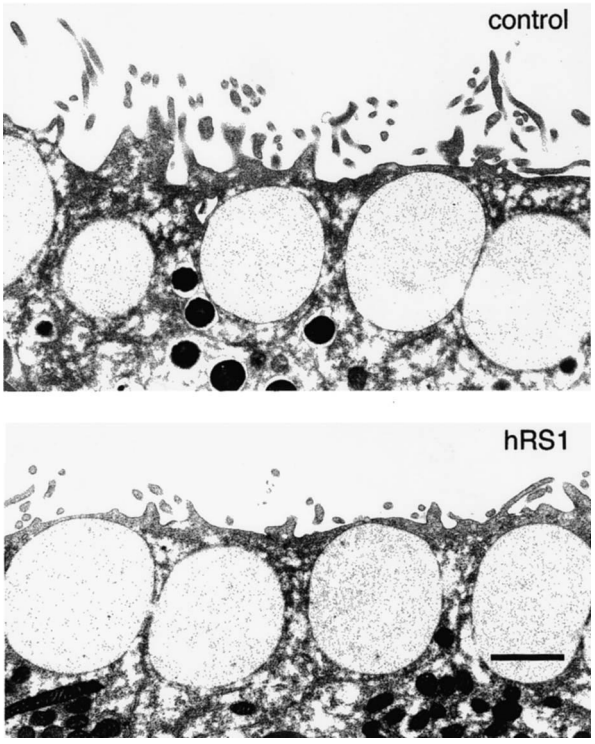

Fig. 8. Effect of the expression of RS1 on the ultrastructure of the plasma membrane in *Xenopus* oocytes. *Xenopus* oocytes were injected with water (control) or 10 ng of *hRS*-cRNA (hRS1), incubated for 3 days and analyzed for membrane capacitance as in Fig. 7. After the capacitance measurements the oocytes were fixed and prepared for transmission electron microscopy. Representative plasma membrane segments from the animal poles are shown. The capacitance of control oocyte was 236 nF and the capacitance of hRS1 expressing oocyte 145 nF. Bar 1 μm.

length of oocyte surface arcs was reduced by 37%. Although a detailed statistical evaluation was not performed the data suggest that the reduction of the oocyte capacitance after expression of hRS1 mainly reflects a reduction of the plasma membrane surface.

4. Discussion

This paper shows that RS1 is localized at the intracellular side of the plasma membrane and that the concentration of RS1 in renal brush border membranes is more than one order of magnitude smaller than the concentration of SGLT1. These data contradict our previous hypothesis that RS1 may be a subunit of SGLT1 [1,5]. This hypothesis was based on the demonstration of functional effects of RS1 on the expression of glucose transport by SGLT1 in combination with immunohistochemical data with our monoclonal antibody R4A6 [2,4] which suggested that RS1 was localized at the extracellular side of the plasma membrane. Functional interactions between RS1 and SGLT1 were concluded from coexpression experiments that were performed in *Xenopus* oocytes [1,6]. They indicated changes in V_{max} and apparent K_m values for Na^+-D-glucose cotransport expressed by SGLT1 and appeared to be specific for SGLT1 or SGLT1 homologous transporters.

Because our present data show that R4A6 does not react with RS1 in Western blots but cross-reacts with an unrelated 75 kDa polypeptide, the previously described immunohistochemical localization of R4A6 cannot be supposed to represent the localization of RS1. R4A6 probably interacts with a conformational epitope of RS1 which is not accessible after Western blotting. R4A6 increased high affinity phlorizin binding to brush border membranes from pig kidney and

Table 1
Effect of the expression of hRS1 on the plasma membrane surface in *Xenopus* oocytes

Oocyte	Capacitance (nF)	Analyzed arcs of oocyte surface (μm)	Length of sectioned plasma membrane (μm)	Length of sectioned plasma membrane per arcs of oocyte surface
Control	236	67.6	180.3	2.67
Control	227	64.9	231.5	3.57
hRS1	145	58.0	101.8	1.76
hRS1	149	69.2	149.3	2.16

X. laevis oocytes were injected with water (control) or 10 ng of hRS1-cRNA (hRS1) and incubated for 3 days. The capacitance of the oocytes was measured. Then the oocytes were fixed for electron microscopy embedded in plastic, ultrathin sectioned and stained. Photographs from the animal pole of oocytes were taken. The length of the sectioned plasma membrane was measured and normalized to the length of the analyzed arcs of the oocyte surface.

rat small intestine [2,3] and porcine RS1 protein had been isolated by screening a renal expression library with R4A6 [1].

Another important observation of the present paper is the reduction of the membrane capacitance after expression of hRS1 in *Xenopus* oocytes which was combined with a reduction of the plasma membrane surface. The effect of hRS1 on the membrane capacitance was more pronounced when hRS1 was expressed without hSGLT1, however, it was still significant after coexpression with hSGLT1. Since the amount of hSGLT1 protein per total protein in the oocyte plasma membrane fraction and the V_{max} of the expressed glucose transport were decreased by coexpression of hRS1 changes of the plasma membrane surface by endocytosis and/or membrane recycling may be involved in the effects of hRS1 on hSGLT1 in the plasma membrane. At the present state of our investigations we cannot exclude that the observed changes in the oocyte plasma membrane result from intracellular effects of RS1, for example from effects on the synthesis of plasma membrane components. However, the hypothesis that RS1 is involved in endocytosis and/or membrane recycling is tempting since RS1 contains a C-terminal domain of 42 amino acids which is conserved between the human, pig, rabbit and mouse and bears a consensus sequence for a ubiquitin associated (UBA) domain [30,31]. This UBA domain is part of the ubiquitin–proteasome system and is supposed to take part in the regulation of endocytosis [32].

Our recent observations show that RS1 has a much more general function as previously assumed. This is consistent with our previous finding that rbRS1 has a broad tissue distribution which is different from that of SGLT1. The effects of hRS1 on the plasma membrane may explain our previous observation that coexpression of hRS1 inhibited the expression of transport activity by structurally different transporters like hSGLT1 and the organic cation transporter hOCT2 [7]. However, the situation is more complex since RS1 from rabbit (rbRS1) inhibited glucose transport expressed by hSGLT1 but stimulated glucose transport expressed by rbSGLT1, and rbRS1 inhibited glucose transport expressed by hSGLT1 but did not change cation transport expressed by hOCT2 [7]. The reason for this apparent species dependent selectivity of RS1 is not understood. It could be explained by the involvement of interacting proteins that mediate or modulate the effects of the RS1. The effects of RS1 could for example be mediated via protein–protein interactions or by an unknown catalytic activity of RS1. The activity of RS1 may be modulated by phosphorylation of RS1 which contains consensus sequences for protein kinase C and casein kinase II that are conserved in man, pig, rabbit and mouse ([7] and unpublished data). Another not understood observation is our previous finding that coexpression of hRS1 with hSGLT1 or of pRS1 with rbSGLT1 changed the substrate dependence of the expressed uptake of [^{14}C]-α-methylglucoside [1,6]. Future experiments have to elucidate whether this effect was due to a protein–protein interaction between RS1 and SGLT1, to an effect of RS1 on a post-translational modification of SGLT1, or to an even more indirect effect, like an RS1 induced change of the plasma membrane composition which may influence the driving force for glucose transport or functional properties of the transporter.

Our recent data provide a basis for further investigations. For example, we have started to investigate the mechanism by which RS1 changes the membrane surface and to identify protein domains of RS1 which are required for the observed effect on the capacitance. In addition we shall try to identify proteins that interact with RS1 and may mediate the effects on plasma membrane transporters. Future investigation on RS1 may help to better understand the regulation of plasma membrane transporters [33,34].

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