The Plasma Membrane-associated Protein RS1 Decreases Transcription of the Transporter SGLT1 in Confluent LLC-PK₁ Cells*

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Previously we cloned RS1, a 67-kDa polypeptide that is associated with the intracellular side of the plasma membrane. Upon co-expression in Xenopus laevis oocytes, human RS1 decreased the concentration of the Na⁺/d-glucose co-transporter hSGLT1 in the plasma membrane (Valentin, M., Kühlkamp, T., Wagner, K., Krohne, G., Arndt, P., Baumgarten, K., Weber, W.-M., Segal, A., Veyhl, M., and Koepsell, H. (2000) Biochim. Biophys. Acta 1468, 367–380). Here, the porcine renal epithelial cell line LLC-PK₁ was used to investigate whether porcine RS1 (pRS1) plays a role in transcriptional up-regulation of SGLT1 after confluence and in down-regulation of SGLT1 by high extracellular d-glucose concentrations. Western blots indicated a dramatic decrease of endogenous pRS1 protein at the plasma membrane after confluence but no significant effect of d-glucose. In confluent LLC-PK₁ cells overexpressing pRS1, SGLT1 mRNA, protein, and methyl-α-d-glucopyranoside uptakes were drastically decreased; however, the reduction of methyl-α-d-glucopyranoside uptake after cultivation with 25 mM d-glucose remained. In confluent pRS1 antisense cells, the expression of SGLT1 mRNA and protein was strongly increased, whereas the reduction of SGLT1 expression during cultivation with high d-glucose was not influenced. Nuclear run-on assays showed that the transcription of SGLT1 was 10-fold increased in the pRS1 antisense cells. The data suggest that RS1 participates in transcriptional up-regulation of SGLT1 after confluence but not in down-regulation by d-glucose.

Because d-glucose has a key role in cellular metabolism, organisms dispose of highly efficient mechanisms to control transepithelial absorption of d-glucose in the small intestine and kidney and the d-glucose uptake into glucose-metabolizing cells. In the kidney and small intestine, d-glucose is translocated across epithelial cells by the consecutive operation of glucose transporters that belong to the following two gene families: secondary active Na⁺/d-glucose co-transporters of the SGLT family in the brush-border membrane, and Na⁺-independent glucose transporters of the GLUT family in the basolateral membrane (1, 2). The regulation of glucose transport affects transporters of both families, is cell type-specific, and is brought about by various mechanisms (1, 3). The regulation of glucose transport has pivotal physiological and biomedical importance. For example (i) one of the biological effects of insulin is the stimulation of d-glucose uptake into fat cells by GLUT4 (4, 5). (ii) The d-glucose absorption in small intestine is adapted to diet by glucose-dependent regulation of the high affinity Na⁺/d-glucose co-transporter SGLT1 (6, 7). (iii) The capacity of the d-glucose reabsorption in the proximal tubule is one of the factors that determined d-glucose plasma levels in diabetic patients (8). (iv) The glomerulosclerosis observed in diabetes may be explained by the up-regulation of GLUT1 in mesangium cells, which triggers the overproduction of matrix proteins (9, 10).

Regulation of SGLT1 has been studied in small intestine (7, 11–13), in Xenopus oocytes (14), and in the renal epithelial cell line LLC-PK₁ which is derived from porcine kidney proximal tubule cells (15–22). One of the factors that affect the expression of SGLT1 in small intestine and LLC-PK₁ cells is extracellular d-glucose concentration (7, 17, 18). However, with increasing d-glucose the expression of SGLT1 in small intestine was increased, whereas it was decreased in LLC-PK₁ cells, and the regulatory specificity for SGLT1 remained ambiguous. Another factor observed in cell culture is cell confluence. During confluence of LLC-PK₁ cells, up-regulation of SGLT1 was observed, together with other differentiation-specific gene products of kidney proximal tubules such as tight junctions and brush-border enzymes (16, 19, 22–24). The confluence-dependent regulation of SGLT1 in LLC-PK₁ cells appears to be mediated by protein kinases. Before confluence, activity of protein kinase C is high, and the mRNA level of SGLT1 is very low. After confluence, protein kinase C is down-regulated, whereas protein kinase A is up-regulated, and the level of SGLT1 mRNA is largely increased (19, 22, 24). One of the effects of protein kinase A is to enhance message stability, mediated by a specific RNA-binding protein (20, 21, 25).

Some years ago, we cloned RS1, a hydrophilic 67-kDa polypeptide from porcine kidney cortex that changed the expression of SGLT1 in Xenopus oocytes (26). Preliminary immunohistochemical experiments suggested that RS1 is localized at the extracellular side of the brush-border membrane and interacts specifically with SGLT1 (26, 27). Based on these findings, we put forward the hypothesis that RS1 is a subunit of SGLT1. This hypothesis was recently abandoned because we found that RS1 is actually localized at the intracellular face of the plasma membrane and that overexpression of human RS1 in Xenopus oocytes not only decreased the expression of human SGLT1 but also that of the human organic cation transporter hOCT2 (28, 29). In the present study, LLC-PK₁ cells were used to investigate the putative role of RS1 in the regulation of SGLT1 ex-
pression and activity by cell confluence and extracellular glucose concentration. Evidence is provided that RS1 is involved in the confluence-dependent regulation of SGLT1 by decreasing the expression of SGLT1 in nonconfluent LLC-PK1 cells via down-regulation of mRNA transcription. A role of RS1 in the glucose-dependent regulation of SGLT1 could not be detected.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The porcine renal epithelial cell line LLC-PK1 (15) was maintained in Dulbecco's modified Eagle's medium (DMEM) that contained 5 mM t-glucose and was supplemented with 10% (v/v) fetal bovine serum, 5 mM t-glutamine, 0.1 mM streptomycin sulfate, and 100 units/ml penicillin. In some experiments, cells were grown in medium with 20% dialyzed bovine serum. LLC-PK1 cells were transduced with an adenovirus containing the gag-pol encoding region of adenovirus. The virus was grown in large batches, concentrated and dialyzed against DMEM medium. An adenovirus containing the entire genome of adenovirus was used as transduction control. For transfection, the cells were transfected with pCMV6 empty plasmid, pCMV6 anti-oxytocin, or pIND anti-RS1 plasmid, which expresses a truncated form of RS1 under control of the minimal heat shock promoter (Pmhsp). The pIND plasmid is under control of the minimal heat shock promoter (Pmhsp) and confers the neomycin resistance gene. pIND anti-pRS1 was co-transfected with the pVgRXR plasmid (Invitrogen) that encodes receptors for ecdysone and retinoid X and contains a ZeocinTM resistance gene. After transfection, the cells were grown in DMEM medium that contained 20% dialyzed bovine serum, 5 mM t-glutamine, 0.1 mg/ml streptomycin sulfate, and 100 units/ml penicillin. The pRS1-specific antisense primer. In LLC-PK1 cells (sense, 5'-GCT TGG TAC CGA GCT CGG-3'), reverse transcriptase-PCR, a primer downstream from the T7 promoter was added. The reverse transcriptase was ligated to BstXI adapters, and inserted at the BstXI site of the eukaryotic expression vector pRCMV from Invitrogen (Leek, Netherlands). The corrected orientation of pRS1 was verified by restriction analysis and DNA sequencing (pRCMV-pRS1). For the overexpression of antisense RNA, a 1481 bp fragment of pRS1 cDNA (nucleotides (nt) 1–1481, GenBankTM accession number X64315), which encompassed the ATG translation initiation codon, was ligated to BstXI adapters and inserted into the pRCMV vector. A correct RS1 antisense clone (pRCMV anti-pRS1) was identified by restriction analysis and DNA sequencing. Analogously, an 1845-bp cDNA fragment of pRS1 (nt 1–1845) was cloned in reverse orientation into the pIND vector from Invitrogen (pIND anti-pRS1). The pIND plasmid is under control of the minimal heat shock promoter (Pmhsp) of a modified edysyn response element. It also confers the neomycin resistance gene. pIND anti-pRS1 was co-transfected with the pVgRXR plasmid (Invitrogen) that encodes receptors for edysyne and retinoid X and contains a ZeocinTM resistance gene. After co-transfection of pIND anti-RS1 with pVgRXR, the Pmhsp promoter is induced when the cells are treated with muristerone A (31). Muristerone A binds to edysyne/retinoid X receptor heterodimers that subsequently interact with a response element on the pIND vector, thereby activating the transcription of antisense pRS1 RNA from the minimal heat shock promoter (32). As another control for RS1 antisense experiments, the porcine oxytocin receptor (nt 1–1184, GenBankTM accession number X71796) was cloned in reverse orientation into the pRCMV vector (pRCMV anti-oxytoxin). Stable Transfection of LLC-PK1 Cells—Native LLC-PK1 cells grown in DMEM were transfected with pRCMV-pRS1, pRCMV anti-pRS1, the empty pRCMV plasmid, pRCMV anti-oxytoxin, or pIND anti-RS1 plus pVgEcr using Lipofectin reagent from Life Technologies, Inc. After transfection, the cells were kept in DMEM for 2 days. Selection was initiated by adding geneticin (G418) to the culture medium (0.4 mg/ml for 1 week and 0.8 mg/ml later). Single clones were isolated after 3 weeks and amplified in DMEM containing 0.8 mg/ml G418. Genomic integration of the pRCMV antisense pRS1 constructs and their transcription were tested by polymerase chain reactions (PCR) without and with reverse transcription, respectively. The employed primers were derived from the T7 region of the CMV promoter (sense, 5'-TAA TAC GAC TCA TAT TAG G-3') and from the cDNA sequence of pRS1 (antisense, 5'-CTT AAT TCA GCA GGC GGC-3', nt 790–809). For reverse transcriptase-PCR, a primer downstream from the T7 promoter (sense, 5'-GCT TGG TAG TCA CGA CTT GCG-3') was used together with the above described pRS1-specific antisense primer. In LLC-PK1 cells transfected with pIND anti-RS1 plus pVgEcr, transcription of pRS1 antisense mRNA was induced by cultivating the cells for 2 days in the presence of 5 μM muristerone A.

**Northern Analysis**—For Northern blots mRNA was isolated from LLC-PK1 cells (33). In some experiments the transcription was blocked by addition of 5,6-dichloro-2'-deoxyuridine (DRB) to culture medium. 5 μg of mRNA per lane were resolved on glyoxalagarose gels, blotted to Hybond N membrane (Amersham Pharmacia Biotech), cross-linked by UV irradiation, and hybridized to double-stranded cDNA probes of various genes that were labeled with [32P]dATP (34). The blots were stripped and reprobed several times. The blots were developed with a phosphorimager. The bands were quantified by digitizing the stained bands and subtracting the background staining (Sigmascan program from Sigma, Deisenhofen, Germany).

**Nuclear Run-on Transcription Assay**—The employed experimental protocol was adapted from procedures described previously (38, 39). LLC-PK1 cells were grown with 5 mM t-glucose in the medium until 5 days after confluence, washed with phosphate-buffered saline (0.1 mM Na2HPO4, 0.15 mM NaCl, pH 7.4), harvested, and lysed with lysis buffer that consisted of 0.01 M Tris-HCl, pH 7.4, 3 mM MglCl, 0.5% (v/v) Nonidet P-40, and 1 mM phenylmethylsulfonfluoride (PMSF). The nuclei were spun down by a 5 min centrifugation at 4000 × g (4 °C), washed three times with lysis buffer, and stored at −70 °C in 0.02 M Tris-HCl, pH 7.9, 0.075 mM NaCl, 0.5 mM EDTA, 0.85 mM diithiothreitol, 0.125 mM PMSF, 50% (v/v) glycerol, and 32 units/ml ribonuclease inhibitor. Transcription was initiated by adding 1010 nuclei to 100 μl of assay buffer that contained 0.1 M Tris-HCl, pH 7.9, 0.05 mM NaCl, 0.4 μM EDTA, 0.34 mM (NH4)2SO4, 4 mM MgCl2, 0.05 mM PMSF, 0.6 mM dithiothreitol, 2 mM ATP, 2 mM GTP, 2 mM CTP, 250 μCi of [α-32P]UTP, 0.25 μM [α-32P]methyl Heidelberg, Germany), and human ubiquitin (260-bp fragment from CLONTECH). For porcine cDNA probes, the membranes were washed at a final stringency of 0.25× SSPE (0.18 mM NaCl, 10 mM sodium phosphate, pH 7.7, 1.0 mM EDTA), 0.1% (w/v) SDS at 42 °C. For the human probe, 1× SSPE was used in the final stringency buffer. In some experiments, the autoradiographic signals detected on x-ray films were quantified by digitizing the stained bands and subtracting the background staining (Signmac program from Sigma, Deisenhofer, Germany).

The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; DBS, Dulbecco’s phosphate-buffered saline; PCR, polymerase chain reaction; RNase, ribonuclease; T7, T7 RNA polymerase; gelatin; sodium glyceraldehyde-phosphate dehydrogenase; hGAPDH, human GAPDH; hGAPDH, human GAPDH; FME, plasma membrane-enriched; V2R, vasopressin receptor 2; CMV, cytomegalovirus; BstXI, BstXI; Hin, Hin; Bgl, Bgl; XI, XI; DRB, 5,6-dichloro-2'-deoxyuridine (DRB).
40,000 × g supernatant for 2 h at 200 000 × g at 8 °C. For SDS-polyacrylamide gel electrophoresis, protein samples were incubated for 1 h at 37 °C in 50 mm NaHPO4, pH 6.8, 4 μl urea, 0.25 × β-mercaptoethanol, 1% (w/v) SDS, and 0.0005% (w/v) bromphenol blue. If immunodetection of pRS1 was intended, the SDS-treated protein samples were subsequently acetylated (29). After SDS-polyacrylamide gel electrophoresis performed according to Laemmli (41), the proteins were electrophoretically transferred to nitrocellulose membranes by semi-drying blotting (42). The nitrocellulose membranes were blocked for 1 h at 25 °C in blocking buffer (50 μl 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). For antibody reaction, the blots were incubated for 2 h at 25 °C with affinity-purified polyclonal rabbit antibodies against porcine SGLT1 (pSGLT1-ab) or porcine RS1 (pRS1-ab) that were diluted 1:10 or 1:250, respectively, in blocking buffer, respectively. The antibodies have been described earlier (29, 43). pSGLT1-ab are raised against the amino acids 525–542 of porcine SGLT1 and affinity-purified on the antigenic peptide fixed to Sepharose, whereas pRS1-ab was raised against recombinant pRS1 protein expressed in E. coli and affinity-purified on the recombinant protein fixed to enzyme-linked immunosorbent assay plates. After washing the blots with the blocking buffer they were incubated for 1 h at 25 °C with peroxidase-conjugated goat anti-rabbit IgG antiserum that was diluted 1:5000 in blocking buffer. Blots were then washed with blocking buffer, and bound label was visualized by enhanced chemiluminescence (ECL System from Amersham Pharmacia Biotech).

Measurements of Na+-D-Glucose Co-Transport Activity—The procedure was adapted from Kimmich et al. (30). LLC-PK1 cells were detached from culture dishes by incubation for 20 min (37 °C, 5% CO2) in Ca2+- and Mg2+-free DPBS (37°C). After 3 min of centrifugation at 250 × g, the pelleted cells were suspended at a protein concentration between 5 and 10 mg/ml in DPBS (37°C). Protein concentration was measured according to Bradford (44). For transport measurements, this stock suspension was kept at 37 °C. Measurements were started by adding 50 μl of the stock suspension into 200 μl of incubation medium that was shaken at 37 °C water bath. The incubation medium consisted of DPBS containing nonradioactive methyl-α-D-glucopyranoside (AMG) plus 1.9 kBq of [14C]AMG. Nonspecific AMG uptake was measured in the presence of 100 μM phlorizin. Because control experiments showed that the uptake rate of 40 μM or 2 mM AMG was linear during an incubation period of more than 5 min, the cells were routinely incubated for 2.5 min at 37 °C. The transport measurement was stopped by removing 200 μl of transport assay mixture and adding it to 1 ml of ice-cold stop solution (DPBS containing 1 mM phlorizin). The uptake time was determined by adding the ice-cold stop solution together with the respective amount of AMG. After 1 min of centrifugation at 8000 × g, the supernatant was removed; 1 ml of stop solution was added, and the sample was centrifuged again. The supernatant was carefully removed, and 100 μl of 0.5% (w/v) Triton X-100 were added. The tubes were shaken for 1 h at room temperature; 1 ml of Quickszem (Lumasafe™ Plus, Groningen, Netherlands) was added; the vial was closed, shaken vigorously, and counted in a β-counter.

Statistics—Means ± S.D. of phlorizin-inhibitable uptake rates were calculated from uptake measurements that were performed after 0 and 2.5 min of incubation of LLC-PK1 cells. For each individual uptake measurement, four independent determinations were performed in the absence of phlorizin plus four determinations in the presence of phlorizin to measure the Michaelis-Menten equation with an estimated time to equilibrium was fitted to uptake rates determined for different substrate concentrations. For densitometric quantifications of Northern blots and nuclear run-on assays, three to four independent hybridizations were used per each experimental condition, employing samples from two or more independent cultivations. The unpaired Student’s t test was used to evaluate the significance of differences between uptake rates or hybridization intensities.

Materials—[α-32P]dATP (111 TBq/mmol) and [α-32P]dUTP (29.6 TBq/mmol) were obtained from ICN (Eschwege, Germany), [14C]AMG (11.25 GBq/mmol) from Amersham Pharmacia Biotech, and ribonuclease inhibitor and RNase-free DNase from Fermentas (St. Leon-Rot, Germany). Peroxidase-conjugated anti-rabbit IgG from goat, RNase A, DRB, Dulbecco’s phosphate-buffered saline (DPBS), and polyadenylic acid were supplied by Sigma. Lipofectin and prestarmed marker proteins for SDS-polyacrylamide gel electrophoresis (BenchMark™) were supplied by Life Technologies, Inc., geneticin (G418) by Calbiochem, and Bradford protein assay by Bio-Rad. All other chemicals were obtained as described earlier (26, 43).

RESULTS

Endogenous Expression of SGLT1 and RS1 in LLC-PK1 Cells—LLC-PK1 cells were derived from porcine kidney and exhibited properties similar to the S3 segment of the proximal tubule (15). They normally expressed both SGLT1 and RS1 (17, 26). Previously, it has been described (16–19) that the expression of SGLT1 in LLC-PK1 cells is up-regulated after confluence and glucose concentrations. By confluence (subconfluent) and 5 days after confluence (confluent), cells were harvested, and mRNA was isolated. Northern blots were prepared and hybridized with a cDNA probe for pSGLT1 (SGLT1), stripped, hybridized with a cDNA probe for pRS1 (RS1), stripped again, and hybridized with cDNA probe for hGAPDH (GAPDH). The upper panel shows hybridization from one of three independent experiments. The densitometric quantification of the three experiments is summarized in the lower panel. For RS1, staining at 7.5 and 4.2 kb is summarized. Signals that differ significantly from those obtained with confluent cells grown with 5 mM D-glucose are indicated (* p < 0.01; ** p < 0.001).
of confluent cells grown in 25 mM d-glucose, SGLT1 protein levels were below the detection limit of our Western blot protocol (Fig. 2b). This confirms the above-described glucose-dependent down-regulation of SGLT1 and parallels the transport data.

At variance with porcine renal brush-border membranes where pRS1-ab stained a single polypeptide band at 100 kDa (29), in the PME fraction of LLC-PK1 cells, polypeptide bands were detected at about 100, 60, and in some cases also at about 90 kDa (Fig. 2, a and c). In LLC-PK1 cells grown in 5 or 25 mM d-glucose, the concentration of RS1 protein in the PME fraction was much higher before than after confluence (Fig. 2a, lower panel, and c). When the cells were grown in 5 or 25 mM d-glucose, no difference in pRS1 protein was apparent before versus after confluence (Fig. 2c). The data show that pRS1 protein concentration at the plasma membrane changes dramatically and opposite to the SGLT1 protein when the cells have reached confluence. In contrast to SGLT1, the expression level of RS1 protein at the plasma membrane is not affected by the p-glucose concentration in the medium.

Overexpression of pRS1 in LLC-PK1 Cells—To investigate whether RS1 affects the expression of SGLT1, pRS1 was overexpressed in LLC-PK1 cells. The cells were stably transfected with the pRcCMV vector containing the complete open reading frame of pRS1 downstream to the CMV promoter (cell lines S10–S13). LLC-PK1 cells stably transfected with the empty vector pRcCMV vector served as control (cell line CON3). Overexpression of pRS1 protein was verified in Western blots on homogenates of LLC-PK1 cells that were grown for 5 days after confluence in the presence of 5 mM d-glucose. The 1st lane of Fig. 3a shows a control reaction with purified brush-border membrane of porcine renal cortex. Whereas endogenous pRS1 protein was not detected in the homogenate of the control cell line (CON3 in Fig. 3a), in cell lines stably transfected with pRS1, overexpressed pRS1 was stained as a 100-kDa band (S10–S13 in Fig. 3a). The cells overexpressing RS1 exhibited a slightly higher proliferation rate as cell line CON3, reaching confluence between 6 and 7 days after seeding (Fig. 3b). After confluence, the cells overexpressing RS1 exhibited a little higher cell density than the control cells (Fig. 3b). Eleven days after seeding, total protein concentrations per cell were 0.65 ± 0.04 ng (CON3), 0.57 ± 0.04 ng (S11), and 0.53 ± 0.03 ng (S12). In Fig. 3c, the activity of Na+-d-glucose co-transport was compared in cell lines overexpressing pRS1 (S10–S13), in native LLC-PK1 cells (native), and in LLC-PK1 cells that had been stably transfected with the empty vector (CON3). The cells were grown with 5 mM d-glucose and harvested 5 days after confluence, and initial uptake of 10 μM AMG was measured after 2.5 min of incubation in the presence and absence of 100 μM phlorizin. Fig. 3c shows that the phlorizin-inhibitable AMG uptake rates in cell lines S10–S13 were 5–15 times smaller than those measured in native LLC-PK1 cells and in cell line CON3. To determine whether the reduced uptake in RS1 overexpressing cells indicates a decrease of transporter affinity or maximal transport rate, the substrate dependence was measured with cell line S11. Fig. 3d shows that the maximal transport rate in cell line S11 (0.17 ± 0.01 nmol mg−1 × min−1) was about 10 times smaller than the maximal transport rate in native LLC-PK1 cells that were grown under the same conditions (upper panel of Fig. 7, Vmax = 2.03 ± 0.05 nmol mg−1 × min−1). The Kms value for AMG uptake by cell line S11 was 0.36 ± 0.07 mM. This is half the apparent Kms value of native LLC-PK1 cell lines grown and harvested under the same conditions (upper panel of Fig. 7, Kms = 0.79 ± 0.04 mM). Because the plasma membrane depolarizes during Na+-d-glucose co-transport and the Kms value of SGLT1 for glucose increases with...
The uptake measurements were performed with cells that were grown in 5 mM D-glucose and harvested 5 days after confluence. Western blot in the upper panel was performed with plasma membrane-enriched membrane fractions (PME) that were isolated from the different cell lines. It was developed with the affinity-purified antibody against pSGLT1 (pSGLT1-ab). The Northern blots in the lower panel were hybridized with cDNA probes specific for pSGLT1, glyceraldehydephosphate dehydrogenase (GAPDH) or the vasopressin receptor type 2 (V2R).

Next, we compared the amount of SGLT1 protein in PME fractions isolated from cell lines CON3, S11, and S12 and the amount of cellular SGLT1 mRNA in these cell lines. For these experiments, the cells were grown in 5 mM D-glucose and harvested 5 days after confluence. The Western blot in the upper panel of Fig. 4 shows that SGLT1 protein could be detected in the PME fraction of line CON3 but was not detected in the RS1-overexpressing cell lines S11 and S12. The Northern blots in the lower panel of Fig. 4 show that the decrease of SGLT1 protein in cell lines S11 and S12 mainly reflects a difference in mRNA concentrations. Compared with line CON3, the concentration of SGLT1 mRNA was dramatically decreased in cell lines S11 and S12. In contrast, the mRNA concentrations of another membrane-spanning protein (the vasopressin receptor 2, V2R) and of a cytosolic protein (glyceraldehydephosphate dehydrogenase, GAPDH) were not changed.

Decrease of SGLT1 mRNA and SGLT1 protein in LLC-PK1 cell lines that were stably transfected with pRS1. The amounts of pSGLT1 protein (upper panel) and pSGLT1 mRNA (lower panel) were compared in the control cell line CON3 and the pRS1-overexpressing cell lines S11 and S12. The cells were grown in the presence of 5 mM D-glucose and harvested 5 days after confluence. The Western blot in the upper panel was performed with plasma membrane-enriched membrane fractions (PME) that were isolated from the different cell lines. It was developed with the affinity-purified antibody against pSGLT1 (pSGLT1-ab). The Northern blots in the lower panel were hybridized with cDNA probes specific for pSGLT1, glyceraldehydephosphate dehydrogenase (GAPDH) or the vasopressin receptor type 2 (V2R).

Transcriptional Regulation of SGLT1 by RS1...
Generation of LLC-PK1 Cell Lines Where the Expression of Endogenous pRS1 Is Reduced—To characterize further the effect of pRS1 on the expression of SGLT1 related to confluence and extracellular d-glucose concentration, an antisense strategy was employed. We generated stably transfected LLC-PK1 cell lines that transcribe a 1.5-kb antisense pRS1-cRNA fragment that covers the ATG start codon. Most measurements were performed with cell lines containing antisense RS1 introduced with the pRcCMV vector (cell lines AS6 and AS8). Control experiments were carried out with an LLC-PK1 cell line that was stably transfected with the empty vector (cell line CON3), with native LLC-PK1 cells, and with a LLC-PK1 cell line in which a 1.2-kb antisense construct of the porcine oxytocin receptor was stably expressed. Some experiments were also performed with an LLC-PK1 cell line where antisense RS1 was stably transfected with the pIND vector from Invitrogen (cell line AS2, see Fig. 6). In this system the transcription of RS1 can be induced by muristerone A (31). The characterization of the RS1 antisense cell lines is shown in Fig. 5. PCR and reverse transcriptase-PCR experiments showed that antisense pRS1 DNA was incorporated in the genome of the transfected LLC-PK1 cell lines (lanes d and e) and that it was transcribed into antisense RS1 RNA (lanes l and m, respectively). No reaction products were observed with native LLC-PK1 cells (lanes c and i) or with cell line CON3 that had been stably transfected with the empty pRcCMV vector (lane k). To determine the amount of pRS1 protein associated with the plasma membrane, LLC-PK1 cells were grown in the presence of 5 mM d-glucose until 2 days before reaching confluence. Then the respective PME fractions were isolated, and pRS1 protein was analyzed by Western blots using affinity-purified pRS1-ab. In line CON3, intact pRS1 protein and additional proteolytic fragments were observed at ~60 and ~55 kDa (lane o). In contrast, the antisense RS1 cell lines AS6 and AS8 contained no intact pRS1 protein and largely reduced amounts of the proteolytic fragments (lanes p and q).

To test whether proliferation and/or cell size was altered in the antisense pRS1 cell lines, we determined the cell density and protein content 5 days after confluence. The antisense pRS1 cell lines, native LLC-PK1 cells, and the control cell line CON3 all reached confluence between 6 and 8 days after seeding. The cell densities determined 5 days after reaching confluence were not significantly different: native LLC-PK1 cells 1.3 ± 0.2, CON3 1.3 ± 0.2, AS6 0.9 ± 0.1, and AS8 1.0 ± 0.1 (× 10^5 cells/cm², means ± S.D. from three independent experiments). Similarly, the protein contents per cell were not significantly different between cell lines: native LLC-PK1 cells 0.71 ± 0.15, CON3 0.70 ± 0.15, AS6 0.64 ± 0.03, and AS8 0.84 ± 0.03 (mg/cell, means ± S.D., n = 3).

Na⁺-d-Glucose Co-transport Activity in RS1 Antisense Cell Lines—First, we examined the initial rates of phlorizin-inhibitable uptake of AMG in the pRS1 antisense cell lines as compared with various control cell lines. As the expression of SGLT1 in LLC-PK1 cells is lower when the cells are grown in 25 mM d-glucose as compared with 5 mM d-glucose (17, 18), both conditions were tested. The initial uptake rates of 40 μM [¹⁴C]AMG were measured with suspended cells that had been grown until 5 days after confluence. Fig. 6 (upper panel) shows similar initial phlorizin-inhibitable glucose uptake rates in three different control cell lines, i.e. in native LLC-PK1 cells, in cell line CON3, which was stably transfected with the empty pRcCMV vector, and in the uninduced pRS1 antisense cell line AS2. Five to ten times higher initial uptake rates were observed in all three cell lines when cells were grown in the presence of 5 mM d-glucose instead of 25 mM d-glucose. With an LLC-PK1 cell line stably transfected with a 1.2-kb antisense construct of the porcine oxytocin receptor, AMG uptake rates were obtained that were in the range of the other control cell lines (data not shown). In the pRS1 antisense cell lines up-regulation of Na⁺-d-glucose co-transport as compared with controls was observed (lower panel). This effect was dramatic in cells that were grown with 25 mM d-glucose in the medium. It was smaller when the basal Na⁺-d-glucose co-transport activity was increased during cultivation with 5 mM d-glucose. Under this condition the up-regulation in cell line AS6 was not statistically significant (Fig. 6). The smaller up-regulation in the RS1 antisense cell lines after cultivation with 5 mM d-glucose compared with 25 mM d-glucose may be due to a limited
capacity of LLC-PK₁ cells to express Na⁺-d-glucose co-transport activity.

To distinguish whether the increase of AMG uptake in the pRS1 antisense cell lines reflects an increase in Vₘₐₓ or a decrease of Kₘ, the concentration dependence of AMG uptake was compared in the antisense pRS1 cell line AS8 and native LLC-PK₁ cells. Fig. 7 shows the concentration dependence of phlorizin-inhibitable uptake rates are presented as mean values ± S.E. of triplicate experiments. Native LLC-PK₁ cells (native), control cell line CON3, non-induced cell line AS2 (AS2 uninduced), pRS1 antisense cell lines AS6 and AS8, and the muristerone-induced RS1 antisense cell line AS2 (AS2 induced) were compared.

Effect of RS1 on the expression of Na⁺-d-glucose co-transport in LLC-PK₁ cells grown in high or low glucose. Control LLC-PK₁ cells (upper panel) and pRS1 antisense LLC-PK₁ cells (lower panel) were grown with 25 mM d-glucose (shaded bars) or 5 mM d-glucose in the medium (open bars). Five days after confluence, the cells were harvested and washed in transport medium, and the initial uptake rates of 40 μM [¹⁴C]AMG were determined after 2.5 min of incubation in the absence and presence of 100 μM phlorizin. Phlorizin-inhibitable uptake rates are presented as mean values ± S.E. of triplicate experiments. Native LLC-PK₁ cells (native), control cell line CON3, non-induced cell line AS2 (AS2 uninduced), pRS1 antisense cell lines AS6 and AS8, and the muristerone-induced RS1 antisense cell line AS2 (AS2 induced) were compared.

FIG. 6. Effects of RS1 on the expression of Na⁺-d-glucose co-transport in LLC-PK₁ cells grown in high or low glucose. Control LLC-PK₁ cells (upper panel) and pRS1 antisense LLC-PK₁ cells (lower panel) were grown with 25 mM d-glucose (shaded bars) or 5 mM d-glucose in the medium (open bars). Five days after confluence, the cells were harvested and washed in transport medium, and the initial uptake rates of 40 μM [¹⁴C]AMG were determined after 2.5 min of incubation in the absence and presence of 100 μM phlorizin. Phlorizin-inhibitable uptake rates are presented as mean values ± S.E. of triplicate experiments. Native LLC-PK₁ cells (native), control cell line CON3, non-induced cell line AS2 (AS2 uninduced), pRS1 antisense cell lines AS6 and AS8, and the muristerone-induced RS1 antisense cell line AS2 (AS2 induced) were compared.

FIG. 7. Effect of RS1 on the substrate dependence of Na⁺-d-glucose co-transport in LLC-PK₁ cells. Native LLC-PK₁ cells (upper panel) and cells of the pRS1 antisense cell line AS8 (lower panel) were grown in 25 mM d-glucose (closed symbols) or with 5 mM d-glucose (open symbols). Five days after confluence, the cells were harvested and analyzed for initial rates of [¹⁴C]AMG uptake at different AMG concentrations in the absence or presence of 100 μM phlorizin as in Fig. 6. Mean values ± S.D. (n = 3) of phlorizin-inhibitable uptake rates are plotted versus the AMG concentrations. The Michaelis-Menten equation was fit to the data.
and for the vasopressin receptor V2R. In the RS1 antisense cell lines AS6 and AS8, the staining of both SGLT1 transcripts was increased in contrast to GAPDH and V2R. The increase of GLUT1 mRNA in the RS1 antisense cell lines was significantly different from the hybridization obtained with line CON3 grown with the respective glucose concentration (CON3, p < 0.01; AS6, p < 0.05). Comparing the cultivation with 5 and 25 mM glucose, the staining differences in each of the two SGLT1 transcripts in cell line CON3 were significantly different (*p < 0.001). In cell lines AS6 and AS8, the staining differences after cultivation in 5 and 25 mM glucose were significantly different when the stainings of both SGLT1 transcripts were summarized (p < 0.05).

To determine whether the effect of RS1 on SGLT1 expression is related to confluence of the cells, we next investigated whether in the RS1 antisense cell lines SGLT1 mRNA was already increased before confluence. In four independent experiments, cell lines CON3, AS6, and AS8 were grown to about 60% confluence in the presence of 5 mM D-glucose. The mRNAs were separated on agarose gels and hybridized with cDNA probes specific for pSGLT1 and GAPDH. Fig. 10 shows that before confluence SGLT1 mRNA was not significantly increased in the RS1 antisense cell lines. We also compared the chlorozin-inhibitable uptake rates of 40 μM AMG in cell lines CON3, AS6, and AS8 that were grown to 60% confluence in the presence of 5 mM D-glucose or 25 mM D-glucose. From three independent experiments the following uptake rates (in pmol × mg protein⁻¹ × min⁻¹) were obtained: 5 mM D-glucose 0.44 ± 0.08 (CON3), 0.48 ± 0.04 (AS6), 0.40 ± 0.09 (AS8); 25 mM D-glucose 0.36 ± 0.04 (CON3), 0.32 ± 0.04 (AS6), 0.32 ± 0.04 (AS8). The data show that the expression of SGLT1 is not significantly different in subconfluent RS1 antisense and control LLC-PK₁ cells irrespective as to whether they have been grown with 5 or 25 mM D-glucose. This indicates that the effect of pRS1 only applies after polarization of LLC-PK₁ cells. A preliminary set of experiments was performed to evaluate whether the RS1-dependent regulation of SGLT1 observed in confluent LLC-PK₁ cells is specific for this plasma membrane transporter. In Northern blots, the cDNA probes of two other plasma membrane transporters, the Na⁺-independent glucose transporter GLUT1 (35) and the polyspecific organic cation transporter OCT2 (36), and a cDNA probe of the cytosolic protein ubiquitin were hybridized to mRNAs isolated from cell lines CON3, AS6, and AS8. The cell lines were grown 5 days after reaching confluence in the presence of 5 mM D-glucose. The hybridization signal of the Northern blots was quantified and normalized to the signal of cell line CON3. From two independent experiments, the data obtained with both RS1 antisense cell lines (AS6 and AS8) were combined. The relative signal intensities of RS1 antisense cell lines were 1.80 ± 0.17 for GLUT1, 1.19 ± 0.54 for OCT2 and 1.18 ± 0.22 for ubiquitin. The increase of GLUT1 mRNA in the RS1 antisense cell lines was statistically significant (p < 0.01); however, it was much less pronounced than the mRNA increase of SGLT1. To investigate whether the effects of RS1 on SGLT1 mRNA concentration represent changes in the rate of transcription or in mRNA stability, we compared the degradation of the two SGLT1 mRNA transcripts in native LLC-PK₁ cells and in RS1 antisense cell lines AS6 or AS8. The cell lines were grown with 5 mM D-glucose.
tion of SGLT1 mRNA increases. In contrast, SGLT1 mRNA

After 30 min of incubation of the nuclei with radioactively

...2-kb SGLT1 and gapdh as in Fig. 9. The hybridizations were performed on four

...expression of SGLT1 mRNA. It was also found that the

...bodies were washed. Northern blots were performed and hybridized with
dNA of pSGLT1 and hGAPDH as in Fig. 9. The hybridizations were performed on four

...1-kb SGLT1 and gapdh as in Fig. 9. The hybridizations were performed on four

...expression of SGLT1 mRNA. It was also found that the

...bodies were washed. Northern blots were performed and hybridized with

gaptrophosphate, the newly synthesized mRNA was isolated and hybridized to membrane-bound cDNAs of hGLUT1, hOCT2, pSGLT1, and of a fragment of the lacI gene which served as a control for nonspecific binding. Identical results were obtained from two independent experiments showing that the synthesis of SGLT1 mRNA in the RS1 antisense cell lines AS6 and AS8 was significantly (p < 0.01) increased by a factor of about 10 (Fig. 11). The synthesis of GLUT1 mRNA was not changed significantly, whereas the synthesis of hOCT2 mRNA was increased by a factor of 2(p < 0.05).

**DISCUSSION**

The amount of the Na\(^{+}-\)D-glucose co-transporter molecules in the plasma membrane may be changed within minutes via endo- and exocytosis (14). In contrast, the transcriptional regulation of SGLT1 is known to occur more slowly. The expression of SGLT1 is altered via changes of intracellular mRNA concentrations that were reported to be mediated by factors either directly affecting the expression (11) or the stability of the mRNA (21, 25). When LLC-PK\(_1\) cells grown in 5 mM D-glucose differentiate during confluence, the cytosolic concentration of SGLT1 mRNA increases. In contrast, SGLT1 mRNA decreases when the cells de-differentiate after being detached mechanically or by trypsin treatment (19, 22). In confluent LLC-PK\(_1\) cells, the concentration of cytosolic SGLT1 mRNA is also dependent on the concentration of D-glucose in the incubation medium (17). In the past, many studies have been performed in LLC-PK\(_1\) cells to resolve the mechanisms that underlie these mRNA changes. They identified a correlation between the increase of SGLT1 mRNA during confluence with an increase of the intracellular cAMP and showed that SGLT1 mRNA was increased by cAMP-elevating agents (22). Furthermore, it was revealed that the cAMP-dependent increase of SGLT1 mRNA relied on a decelerated message degradation that was mediated by binding of a cytosolic protein to a defined 3′ region of mRNA (21, 25). In addition, it was found that the decrease of SGLT1 mRNA during de-differentiation can be blocked or accelerated by inhibition or stimulation of protein kinase C, respectively (19). Concerning the regulation of SGLT1 in LLC-PK\(_1\) cells, many questions have remained unsolved. For example, it is not known which signaling mechanisms underlie the up-regulation of SGLT1 when LLC-PK\(_1\) cells are gaining confluence, which cellular events induce the decrease of SGLT1 message during de-differentiation of the LLC-PK\(_1\) cells, and how protein kinase C affects the concentration of SGLT1 mRNA.
In this paper, we present data that show that the previously cloned 67-kDa polypeptide RS1 (26) is involved in transcriptional regulation of the Na\(^+\)-d-glucose co-transporter SGLT1 in LLC-PK\(_1\) cells. We generated stably transfected LLC-PK\(_1\) cell lines that either overexpress RS1 or transcribe antisense mRNA of RS1 and thereby suppress the translation of endogenous RS1. Opposite effects were obtained by both maneuvers. In the cell lines overexpressing RS1, SGLT1 mRNA, SGLT1 protein, and the Na\(^+\)-d-glucose co-transport activities were decreased, whereas in RS1 antisense cell lines message, protein, and Na\(^+\)-d-glucose transport activities were dramatically increased. Because the stability of SGLT1 mRNA was not significantly changed in RS1 antisense cells and the transcription of SGLT1 was largely increased in a nuclear run-on assay, our data suggest that RS1 is involved in the repression of SGLT1 transcription. Some selectivity for SGLT1 of this RS1 effect is proposed because the transcription of GAPDH and GLUT1 was not changed in the RS1 antisense cell lines, and the observed increase in transcription of the organic cation transporter OCT2 was much smaller than that of SGLT1. Interestingly, in the RS1 antisense cell lines, the message of SGLT1 was only increased when the cells were grown until after confluence. This indicates that RS1-mediated effects upon SGLT1 expression only apply in polarized cells forming a closed monolayer. Depletion of RS1 in subconfluent cells is not sufficient to initiate the up-regulation of SGLT1 but RS1 may act in concert with other factors that are involved in the confluence-dependent up-regulation of SGLT1. Because the concentration of RS1 at the plasma membrane was drastically reduced after confluence, RS1 may play an important role during this regulation.

Our data suggest that RS1 does not participate in the glucose-dependent down-regulation of SGLT1 in LLC-PK\(_1\) cells. After cultivation of LLC-PK\(_1\) cells with 25 mM \(\Delta\)-glucose versus 5 mM \(\Delta\)-glucose, glucose transport by SGLT1 was reduced to a similar extent in controls and pRS1-overexpressing cells. Also, after cultivation with 25 mM \(\Delta\)-glucose versus 5 mM \(\Delta\)-glucose, SGLT1 mRNA was reduced to a similar extent in controls and RS1 antisense LLC-PK\(_1\) cells. In controls and RS1 antisense cell lines, the decrease of SGLT1 mRNA was correlated with a decrease of SGLT1 protein in a plasma membrane-enriched membrane fraction. The finding that the glucose-induced decrease of SGLT1 protein in the RS1 antisense cells was not combined with a significant decrease of the maximal \(\Delta\)-glucose transport rate, as observed in control cells, is probably due to a limited capacity of the plasma membrane for functional active transporter molecules.

To avoid over-interpretation of the data, it must be kept in mind that stable overexpression or reduction of RS1 by antisense strategies may lead to complex changes in the cells. For example, concentration and activity of intracellular regulatory proteins and second messengers may change. Even the increase in the transcription of SGLT1 in isolated nuclei can rather be an effect that is mediated by the up-regulation or activation of cytosolic proteins that are translocated into the nucleus and activate transcription. However, since opposite effects were obtained after overexpressing RS1 and reducing endogenous RS1 in our stably transfected cell lines, and since we have recently been able to demonstrate that RS1 actually migrates into the nucleus,\(^2\) we hypothesize that RS1 is a plasma membrane-associated protein that migrates into the nucleus to act as an inhibitory transcription factor for SGLT1.

To discuss further the function of RS1, previous data must be recalled and re-evaluated. We identified porcine RS1 (pRS1) after screening an expression library with a monoclonal antibody raised against renal brush-border membrane proteins (26), and we cloned proteins with amino acid identities of 74 and 65% from human (hRS1) and rabbit (rRS1), respectively (28, 45). RS1 is a rather hydrophilic protein with two conserved consensus sequences for protein kinase C, three conserved consensus sequences for casein kinase II, and a conserved C terminus of 42 amino acids that contains a consensus sequence for a ubiquitin-associated domain (47, 48) and a short hydrophobic \(\alpha\)-helix at the C-terminal end (26). We showed that hRS1 is an intronless single copy gene that localizes to chromosome 1p36.1 (45). Performing labeling experiments with membrane-impermeant reagents on oocytes of Xenopus laevis expressing RS1, we recently localized RS1 to the intracellular side of the plasma membrane (29). Functional activity of RS1 was originally concluded from effects that were observed when RS1 was expressed after cRNA injection into Xenopus oocytes (26, 28, 45). When RS1 was expressed alone, the membrane surface area was reduced (29), and after co-expression of hRS1 with human SGLT1, the \(V_{\text{max}}\) of expressed glucose co-transport and the amount of expressed SGLT1 protein in the plasma membrane were decreased (28, 45). The effect of RS1 on the expression of SGLT1 in X. oocytes is not specific for this plasma membrane transporter as originally assumed (26) because the transport activity expressed by the human cation transporter OCT2 was also reduced upon co-expression with RS1 (28). However, it apparently does not concern all plasma membrane proteins because the expression of some other plasma membrane transporters was not changed (26). The effects of RS1 observed in Xenopus oocytes probably represent functional activity of RS1 protein at the plasma membrane that is independent of transcription. First, in the employed oocyte expression system, RS1 and SGLT1 were expressed by the injection of their cRNAs. Second, identical results were obtained when the experiments were performed in the absence or presence of the transcription blocker actinomycin D.\(^3\) Possible functions of RS1 at the plasma membrane are an involvement in endo- or exocytosis or an involvement in the degradation of a subset of plasma membrane transporters.

Further experiments are required to determine how RS1 is activated at the plasma membrane and by what mechanism RS1 exhibits the described effects on the transcription of SGLT1. The disappearance of RS1 during confluence of LLC-PK\(_1\) cells suggests an activation of RS1 that may occur at the plasma membrane. A similar type of dual function has been reported for \(\beta\)-catenin. On the one hand, \(\beta\)-catenin links cadherin in adhesion receptors to the actin cytoskeleton, and on the other hand it stimulates gene expression by binding to transcription factors (49). The proposed function of RS1 has also similarities to the sterol regulatory element-binding protein pathway where the metabolism of cholesterol is regulated by a sterol-dependent release of transcription factors called sterol regulatory element-binding proteins from the endoplasmic reticulum (50). It will be a challenge to characterize further the regulatory pathway of RS1 and to identify physiological regulations that involve RS1.

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\(^3\) M. Veyhl and H. Koepsell, unpublished data.
The Plasma Membrane-associated Protein RS1 Decreases Transcription of the Transporter SGLT1 in Confluent LLC-PK1 Cells

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