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Downregulation of the Na^+-D-glucose Cotransporter SGLT1 by Protein RS1 (RSC1A1) is Dependent on Dynamin and Protein Kinase C

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Abstract. We have previously shown that the regulatory protein RS1, cloned from pig, rabbit and human (RSC1A1), is localized intracellularly and inhibits the transcription of the Na^+-D-glucose cotransporter SGLT1 in LLC-PK_1 cells. We also reported that transport activities of human SGLT1 (hSGLT1) and human organic cation transporter hOCT2 expressed in *Xenopus* oocytes were decreased upon co-expression of human RS1 (hRS1). The present paper indicates that the glucose transporter GLUT1 and the peptide transporter PEPT1 are not influenced by hRS1. Voltage-clamp experiments in oocytes expressing hSGLT1 demonstrated that hRS1 reduced the maximal substrate-induced currents but did not change substrate activation, membrane potential dependence, Na^+ dependence or substrate selectivity of hSGLT1. Co-expression experiments with a dominant-negative dynamin mutant showed that the posttranslational inhibition of hSGLT1 by hRS1 was dependent on the function of dynamin. Finally, we observed that hRS1 changed the short-term effect of protein kinase C (PKC) on hSGLT1. Whereas the PKC activators phorbol-12-myristate-13-acetate (PMA) and *sn*-1,2-dioctanoyl glycerol (DOG) increased α-methyl glucose (AMG) uptake expressed by hSGLT1 alone as described earlier, PMA and DOG decreased AMG uptake mediated by hSGLT1 when hRS1 was co-expressed. Taken together, these data indicate that hRS1 modulates dynamin-dependent trafficking of intracellular vesicles containing hSGLT1 in *Xenopus* oocytes, and modulates PKC-dependent short-term regulation of this transporter.

Key words: Sodium-glucose cotransport — Organic cation transport — Regulation — Regulator RS1 — Dynamin — Protein kinase C

Introduction

After many solute transporters in the plasma membrane have been cloned and functionally characterized, the factors regulating their expression and functional activities have become a main topic in transporter research. In addition to transcription, mRNA stability and translation, posttranslational processes may be regulated. These posttranslational regulations include changes of transporter activity within the plasma membrane, transfer from the Golgi apparatus to the plasma membrane, retrieval from the plasma membrane, recycling and degradation. Posttranslational regulation may be mediated by modifications of the transporter itself, through interacting proteins or by modification of proteins that are involved in transporter turnover or recycling. In this paper, we investigate the function of the intracellular protein RS1 (RSC1A1) which is encoded by an intronless single-copy gene, and is involved in the regulation of the Na^+-D-glucose cotransporter SGLT1 (Veyhl et al., 1993; Lambotte et al., 1996; Reinhardt et al., 1999). RS1 is a 67–68 kDa polypeptide that was cloned from pig, rabbit and human, with ~70% amino-acid identity between these three species (Veyhl et al., 1993; Lambotte et al., 1996; Reinhardt et al., 1999). RS1 of all three species contains consensus sequences for protein kinase C, casein kinase II, and a ubiquitin-associated (UBA) domain (Reinhardt et al., 1999). Originally, we

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had suggested that RS1 might be a regulatory subunit (RS) of SGLT1. That hypothesis was based on tracer-flux measurements in *Xenopus laevis* oocytes co-expressing RS1 and SGLT1, which suggested that RS1 acted on SGLT1 by affecting both the maximal velocity V_{max} and the apparent K_m value for glucose (Veyhl et al., 1993; Lambotte et al., 1996). However, this interpretation was abandoned because we found that RS1 is localized intracellularly and that human RS1 (hRS1) also changed the expression of the human organic cation transporter hOCT2, which has no structural similarity to SGLT1 (Reinhardt et al., 1999). Further experiments in *Xenopus* oocytes showed that co-expression of hRS1 with human SGLT1 (hRS1) decreased the V_{max} of glucose transport in parallel with the amount of hSGLT1 protein present in the plasma membrane (Valentin et al., 2000). In that study, we also observed that expression of hRS1 decreased plasma membrane surface area. These data suggested that hRS1 alters the equilibrium between endo- and exocytosis of membrane vesicles, including those which contain SGLT1 (Valentin et al., 2000). This finding could not explain, however, the previous finding that co-expression of RS1 changed the apparent K_m of SGLT1 for glucose (Veyhl et al., 1993; Lambotte et al., 1996). Recently, we found that a fusion protein of green fluorescent protein (GFP) and pRS1 migrated into the nucleus, and that nuclei of LLC-PK_1 cells contained degradation products of pRS1 (*unpublished data*). In agreement with the nuclear localization of pRS1, we observed that RS1 inhibited the transcription of SGLT1 in LLC-PK_1 cells (Korn et al., 2001).

The present paper shows that the effect of hRS1 is selective inasmuch hRS1 inhibits some structurally different plasma membrane transporters, whereas several other transporters are not affected. It further indicates that the effects of hRS1 on the expression of hSGLT1 in *Xenopus* oocytes do not require transcription of additional proteins. We also found that hRS1 does not affect the kinetic properties of hSGLT1; the previously observed effect of hRS1 on the apparent K_m values of hSGLT1 for α-methyl glucose (AMG) could be attributed to a glucose- and time-dependent increase of the inhibition by hRS1. Most importantly, we show that downregulation of hSGLT1 by co-expression of hRS1 is dependent on dynamin, and that protein kinase C potentiates the effect of hRS1 within minutes.

Materials and Methods

Plasmids

DNA of rat wild-type dynamin (DyWt) and dominant-negative mutant of dynamin (DyMu) (Obar et al., 1990) were digested with *Ssp*I and *Kpn*I and cloned into the oocyte expression vector pOG2 cut with *Sma*I and *Kpn*I (Arndt et al., 2001).

In Vitro Synthesis of cRNA

For injections into *Xenopus* oocytes, m7G(5′)ppp(5′)G-capped cRNA was prepared, purified, evaluated and stored as described earlier (Veyhl et al., 1993). To prepare sense cRNA from hRS1 (Lambotte et al., 1996), rbSGLT1 (Hediger et al., 1987), hSGLT1 (Hediger, Turk & Wright, 1989), rOCT1 (Gründemann et al., 1994), rOCT2 (Okuda et al., 1996), hOCT2 (Gorboulev et al., 1997), hGLUT1 (Mueckler & Lodisch, 1986), hPEPT1 (Liang et al., 1995), wild-type (DyWT) and mutant dynamin 1 from rat (DyMu) (Damke et al., 1994; Herskovits et al., 1993; Obar et al., 1990), the respective purified plasmids were linearized with *Not*I (rbSGLT1, hOCT2, hPEPT1, DyWt, DyMu), *Eco*RI (hSGLT1), *Xba*I (hRS1), and *Hind*III (hGLUT1). cRNA was synthesized using T3 polymerase (rbSGLT1, hSGLT1), T7 polymerase (hOCT2, hRS1, hPEPT1, DyWT, DyMu) or SP6 polymerase (rOCT1, rOCT2, hOCT2, hGLUT1) as described earlier (Veyhl et al., 1993). cRNAs were prepared employing the "mMESSAGE mMACHINE" kit (Ambion, Austin, TX), using ammonium acetate precipitation. cRNA concentrations were estimated from ethidium bromide-stained agarose gels using polynucleotide marker as standards (Gründemann & Koepsell, 1994).

Expression of Transporters in *Xenopus* Oocytes

Stage V–VI oocytes were obtained by partial ovariectomy from female *Xenopus laevis*, selected and injected with cRNAs or water as described earlier (Veyhl et al., 1993). Per oocyte, we injected 50 nl of water (control) or 50 nl of water containing transporter cRNAs alone (the indicated amounts in Fig. 1*a*, 2.5 ng in Figs. 1*b*–7) or together with hRS1-cRNA (the indicated amounts in Fig. 1*b*, 7.5 ng in Figs. 2–7). For protein expression, the injected oocytes were incubated for 2–3 days at 16°C in ORi buffer (in mM: 5 HEPES-Tris, pH 7.4, 100 NaCl, 3 KCl, 2 $CaCl_2$ and 1 $MgCl_2$) containing 50 mg/l gentamycin. In part of the experiments, 50 mg/l of the transcription inhibitor actinomycin D were added to the incubation medium. For protein kinase C (PKC) stimulation, oocytes were injected with the respective cRNAs and incubated for 3 days in the presence of actinomycin D. Then, one group of oocytes was incubated for 2 min with 1 μM phorbol-12-myristate-13-acetate (PMA) and washed with ORi buffer. Thereafter, tracer uptake of [^{14}C]AMG was measured over a time period of 15 min. Alternatively, after 3 days incubation, oocytes were injected with 50 nl of I-ORi buffer (in mM: 5 HEPES-Tris, pH 7.4, 100 KCl, 3 NaCl, 2 $CaCl_2$ and 1 $MgCl_2$) or with I-ORi buffer containing 20 μM *sn*-1,2-dioctanoylglycerol (DOG). These oocytes were incubated for 30 min and uptake of [^{14}C]AMG was measured over 15 min.

Tracer-flux Experiments

Uptake of [^{14}C]AMG mediated by SGLT1 from human and rabbit was measured as described earlier (Veyhl et al., 1993). Oocytes expressing SGLT1 and water-injected or non-injected control oocytes were incubated for 15 min (Figs. 1–4) or for the indicated time periods (Fig. 7) in ORi buffer supplemented with 50 μM [^{14}C]AMG without or with 200 μM phlorizin. After washing, the oocytes were solubilized in 5% SDS, and analyzed by scintillation counting. In the presence of phlorizin, tracer uptake in oocytes expressing SGLT1 was similar to tracer uptake in water-injected oocytes and less than 5% of uptake in the absence of phlorizin. The AMG uptake indicated in Fig. 1 represents phlorizin-inhibitable uptake, whereas uptake in Figs. 2–4 and 7 represents the uptake in SGLT1 expressing oocytes minus the uptake measured in water-injected control oocytes. Uptake of [^{14}C]tetraethylammonium (TEA) in oocytes expressing the organic cation transporters

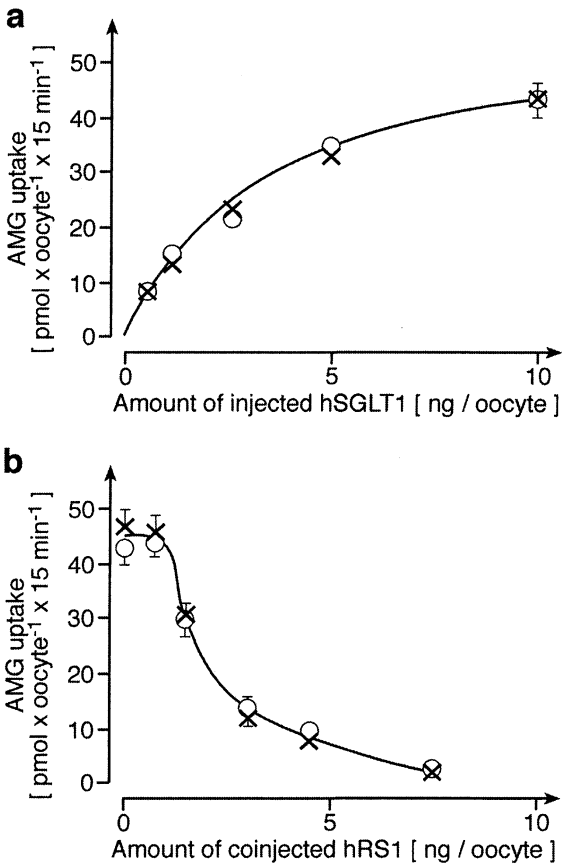

Fig. 1. Inhibition of hSGLT1 by co-expression of hRS1 in *Xenopus* oocytes does not depend on transcription. Oocytes were injected with the indicated concentrations of hSGLT1-cRNA (*a*) or with 2.5 ng of hSGLT1-cRNA plus various amounts of hRS1-cRNA (*b*) and incubated for 3 days either in ORi (◯) or ORi containing 50 μg/ml actinomycin D (×). Then, we measured the uptake of [^{14}C]AMG (50 μM for 15 min) in the absence or presence of 200 μM phlorizin. Typical experiments out of two (*a*) or three (*b*) are presented. Data points are means ± SEM of phlorizin-inhibitable uptake without and with phlorizin.

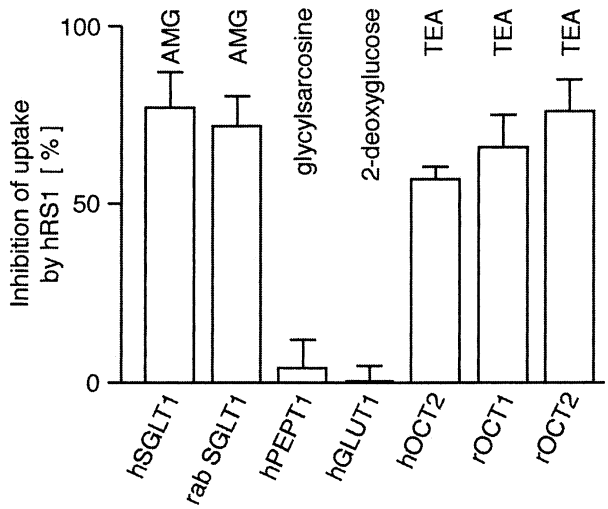

Fig. 2. hRS1 selectively inhibits some, but not all plasma membrane transporters by posttranslational mechanisms. Oocytes were injected with 2.5 ng of the respective transporter-cRNAs (*bottom*) with or without 7.5 ng of hRS1-cRNA or with water, and incubated for 3 days in the presence of actinomycin D. Then, we measured uptake of suitable substrates over 15 min (hSGLT1, rbSGLT1: 50 μM [^{14}C]AMG; hPEPT1: 500 μM [^{3}H]glycyl-sarcosine at pH 6.5; hGLUT1: 1 mM 2-deoxy-D-[^{3}H]glucose in the absence and presence of 0.5 mM phloretin; rOCT1, rOCT2, hOCT2: 100 μM [^{14}C]TEA). Bars indicate the inhibition of uptake by hRS1 that was computed after correction for endogenous uptake in water-injected control oocytes (hSGLT1, rbSGLT1, hPEPT1, rOCT1, rOCT2, hOCT2) or after correction for endogenous uptake measured in the presence of 0.5 mM phloretin (hGLUT1). A typical experiment out of three is shown. In this experiment, the following transport rates were obtained after correction for endogenous uptake (in pmol × oocyte^{-1} × 15 min^{-1}): 70 ± 7 for hSGLT1, 120 ± 11 for rbSGLT1, 126 ± 11 for hPEPT1, 89 ± 7 for hGLUT1, 88 ± 7 for hOCT2, 25 ± 3 for rOCT1, and 60 ± 5 for rOCT2.

rOCT1, rOCT2 or hOCT2 was measured as described (Gorboulev et al., 1997; Arndt et al., 2001). For these measurements, oocytes were incubated for 15 min with 100 μM [^{14}C]TEA in the absence or presence of 100 μM cyanine863. The cyanine863 inhibitable uptake was calculated. It was identical to the TEA uptake in oocytes expressing rOCT1, rOCT2 or hOCT2 minus TEA uptake in water-injected oocytes. Uptake of 2-deoxy-D-[^{3}H]-glucose by the Na^+-independent human glucose transporter GLUT1 (hGLUT1) (Mueckler & Lodish, 1986) was measured by incubating oocytes expressing hGLUT1 or water-injected oocytes for 15 min with HT-buffer (in mM: 5 HEPES-Tris, pH 7.5, 85 tetramethylammonium chloride, 3 KCl, 2 $CaCl_2$, and 1 $MgCl_2$) containing 1 mM of 2-deoxy-D-[^{3}H]-glucose plus 49 mM mannitol, 1 mM 2-deoxy-D-[^{3}H]-glucose plus 48.5 mM mannitol and 0.5 mM phloretin, or 1 mM 2-deoxy-D-[^{3}H]-glucose plus 49 mM unlabeled 2-deoxy-D-glucose. In hGLUT1-expressing oocytes, 2-deoxy-D-[^{3}H]-glucose uptake measured in the presence of 0.5 mM phloretin or in the presence of 49 mM 2-deoxy-D-glucose was similar to the uptake measured in water-injected oocytes. Uptake of glycylsarcosine by the human peptide transporter hPEPT1 was measured by incubating oocytes expressing hPEPT1 and water-injected oocytes for 15 min in ORi buffer adjusted to pH 6.5 supplemented with 500 μM [^{3}H]glycyl-sarcosine (Liang et al., 1995). The difference between uptake in oocytes injected with hPEPT1 cRNA and uptake in water-injected oocytes was calculated. Control experiments showed that the glycylsarcosine uptake at pH 6.5 was 2-fold higher compared to the uptake at pH 7.5. The presented uptake in individual experiments represents mean ± SEM values computed from measurements in 8–10 oocytes of each group. All experiments were performed with 2 to 4 different batches of oocytes.

Measurement of Substrate-induced Currents

Two-electrode voltage-clamp measurements in oocytes expressing hSGLT1 were performed 3–5 days after cRNA injection, as described in detail previously (Arndt et al., 2001). For the electrophysiological experiments, oocytes were bathed in ORi, and membrane voltage was held at −50 mV. Current/voltage (*I*/*V*) curves were obtained in the absence and presence of 10 mM AMG by stepping membrane voltage from −50 mV to various voltages ranging from −150 to +50 mV. For measurements involving solutions with Na^+ concentrations below 100 mM, NaCl was replaced isosmotically by choline chloride. To determine the affinity of hSGLT1 for the substrate AMG, AMG was added to ORi buffer at various concentrations up to 10 mM. To determine the affinity of hSGLT1 for the inhibitor phlorizin, we added

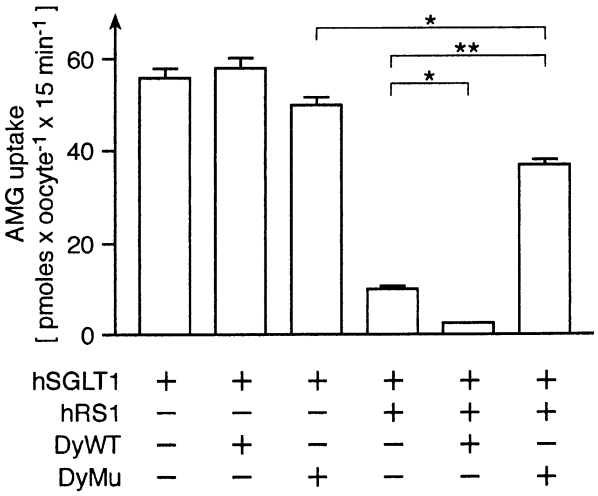

Fig. 3. Effect of hRS1 on hSGLT1 in *Xenopus* oocytes depends on the presence of functionally intact dynamin. Oocytes were injected with water, with cRNA of hSGLT1 (2.5 ng), hRS1 (7.5 ng), dynamin wildtype (DyWt, 10 ng) or dynamin mutant (DyMu, 10 ng) as indicated (*bottom labels*) and incubated for 3 days in the presence of actinomycin D. Then, we measured uptake of 50 μM AMG for 15 min. Bars indicate uptake computed after subtraction of endogenous uptake in water-injected oocytes. A typical experiment out of four is presented. $*P < 0.05$, $**P < 0.001$. The inhibition of AMG uptake by hRS1 was largely reduced after co-expression of dominant-negative dynamin mutant.

phlorizin at various concentrations up to 3 μM to ORi buffer containing a fixed concentration of AMG (1 mM). Substrate-induced currents were obtained by subtracting the currents in the absence of substrate from the currents observed in the presence of substrate.

Biochemicals

Methyl-α-D-[^{14}C]glucopyranoside (AMG; 11.7 GBq/mmol) and 2-Deoxy-1-[^{3}H]glucose (407 GBq/mmol) were obtained from Amersham Biosciences (Freiburg, Germany); glycyl-2-[^{3}H]-sarcosine (2.22 TBq/mmol) and [^{14}C]tetraethylammonium (TEA; 2.0 GBq/mmol) from Biotrend (Köln, Germany); actinomycin D, phorbol-12-myristate-13-acetate (PMA), *sn*-1,2-dioctanoyl-glycerol (DOG), and phloretin from Sigma (Deisenhofen, Germany). The other chemicals and enzymes were obtained as described earlier (Veyhl et al., 1993; Arndt et al., 2001).

Statistics

Results are presented as arithmetic means ± SEM. In tracer-flux measurements, each data point represents the difference between the mean uptake in 8–10 oocytes expressing the respective transporter and the mean uptake in 8–10 control oocytes from the same oocyte batch that were injected with water or with hSGLT1-cRNA and measured in the presence of phlorizin. In Figs. 1–4 and in Fig. 7, one representative experiment out of three or four are shown. In the presented voltage-clamp experiments, each data point indicates the mean current ± SEM from 4–6 individual oocytes. Unpaired Student's *t*-test was used to test for a difference between mean values. The Hill equation was fitted to the substrate activation and inhibition curves of AMG induced currents.

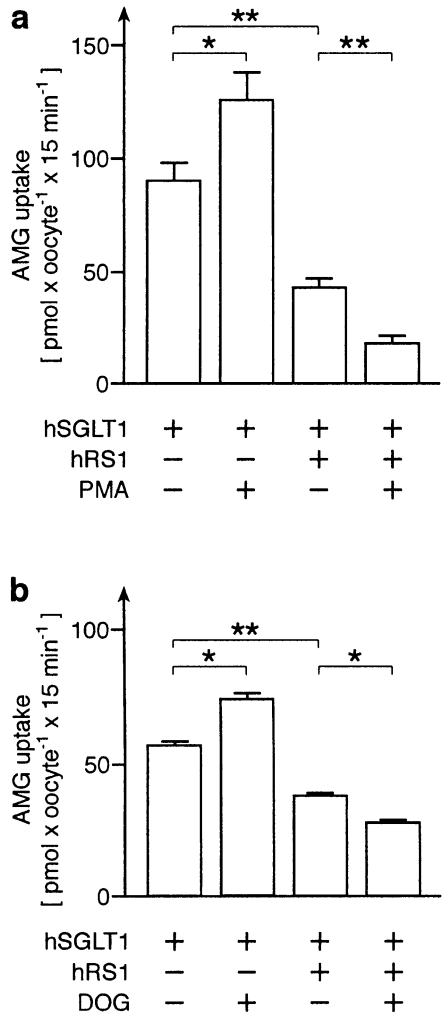

Fig. 4. Co-expression of hRS1 modulates the effect of protein kinase C on hSGLT1. Oocytes expressing hSGLT1 with or without hRS1 for 3 days in the presence of actinomycin D were incubated for 2 min with 1 μM PMA (*a*) or injected with 1 pmole of DOG per oocyte, and then incubated for 30 min (*b*). Then, we measured uptake of 50 μM [^{14}C]AMG over 15 min. Typical experiments are shown. Bars indicate AMG uptake computed after correction for endogenous uptake in water-injected oocytes. $*P < 0.05$, $**P < 0.01$.

Results

Effect of hRS1 on hSGLT1 Is not Blocked by Actinomycin D

We have previously demonstrated that RS1 can act as modulator of transcription in LLC-PK$_1$ cells (Korn et al., 2001). Analogously, the observed effects of hRS1 on the expression of heterologous hSGLT1 cRNA in *Xenopus* oocytes might depend on hRS1-induced transcriptional changes of endogenous proteins that are involved in post-transcriptional regulation of hSGLT1. For this reason, we investigated whether the expression of Na^+-D-glucose cotransport

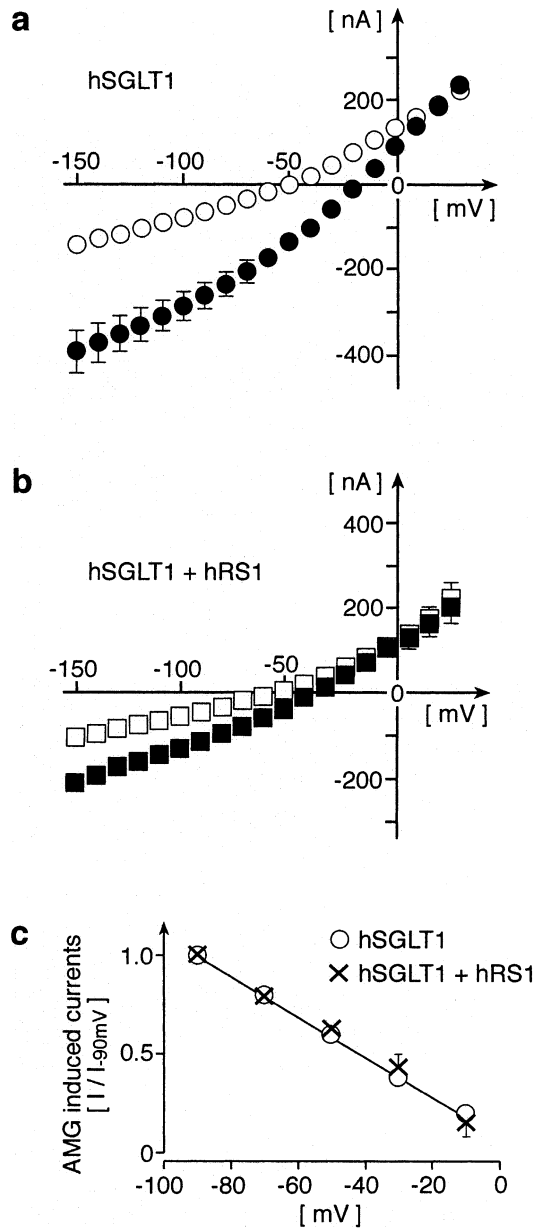

Fig. 5. hRS1 does not alter membrane potential-dependence of substrate-induced currents in oocytes expressing hSGLT1. Steady-state current-voltage relations of currents induced by 10 mM AMG were determined in oocytes expressing either hSGLT1 alone (*a*) or together with hRS1 (*b*) in the absence of actinomycin D. Open and closed symbols in *a* and *b* represent currents in the absence and presence of AMG, respectively. (*c*) The data of *a* and *b* in normalized form (expressed as fractions of $I_{-90\ mV}$, i.e., the current induced by 10 mM at −90 mV). The data points represent mean ± SEM from 4–6 oocytes.

by hSGLT1, without and with co-expression of hRS1, was changed when 50 mg/l of actinomycin D was added while incubating the cRNA-injected oocytes for expression. Actinomycin D is a potent inhibitor of transcription (Buller & White, 1990; Scheer, 1987; Krishek, Moss & Smart, 1996). The presence of actinomycin D did neither influence the expression of Na^+-D-glucose cotransport by hSGLT1 alone (Fig. 1*a*) nor the inhibition of hSGLT1-expressed Na^+-D-glucose cotransport by hRS1 (Fig. 1*b*). In the absence and presence of actinomycin, co-expression of hRS1 and hSGLT1 led to the typical reduction of Na^+-dependent D-glucose uptake (Lambotte et al., 1996). Similarly, actinomycin D did not affect the inhibition by hRS1 of organic cation transport by hOCT2 (*data not shown*). These data indicate that the effects of hRS1 on the expression of the transport activities of hSGLT1 and hOCT2 are independent of effects of hRS1 on the transcription of endogenous proteins.

Effect of Human RS1 on Other Plasma Membrane Transporters

Previously, we showed that hRS1 inhibits the expression not only of hSGLT, but also of the organic cation transporter hOCT2 (Reinhardt et al., 1999). Because hSGLT1 and hOCT2 have no overall structural similarity, we examined the selectivity of hRS1 in several further transporters (Fig. 2). To detect with higher sensitivity any putative effects of hRS1 on expressed transport activities, we injected relatively small amounts of transporter cRNAs (2.5 ng per oocyte) that were in a range where expressed transport was correlated to the amount of injected cRNAs (*see* e.g., Fig. 1*a*). The amount chosen for co-injected hRS1-cRNA (7.5 ng per oocyte) largely inhibited AMG transport expressed by hSGLT1 (Fig. 1*b*). To detect hRS1-induced changes independently, whether they reflect changes in maximal transport rates or K_m values, the uptake measurements were performed using substrate concentrations below or around the apparent K_m values of the respective transporters.

Fig. 2 shows that co-expression of hRS1 led to a drastic inhibition of AMG uptake via hSGLT1 or rbSGLT1, and of TEA uptake via rOCT2, hOCT2 or rOCT1. In contrast, glycylsarcosine uptake via hPEPT1 and uptake of 2-deoxy-D-glucose via hGLUT1 were not significantly inhibited by hRS1. These data show that the effect of hRS1 exhibits, similar to that of pRS1, some selectivity for plasma membrane transporters, and that this selectivity does not correlate with the degree of overall structural similarity between the transporters.

Coexpression of a Dominant-Negative Dynamin Mutant Blocks the Effect of hRS1 on hSGLT1

We observed previously in *Xenopus* oocytes that the inhibition of hSGLT1-mediated AMG uptake upon co-expression of hRS1 was correlated with decreased amounts of hSGLT1 protein in the plasma membrane and with a decrease of total plasma membrane area (Lambotte et al., 1996; Valentin et al., 2000). These

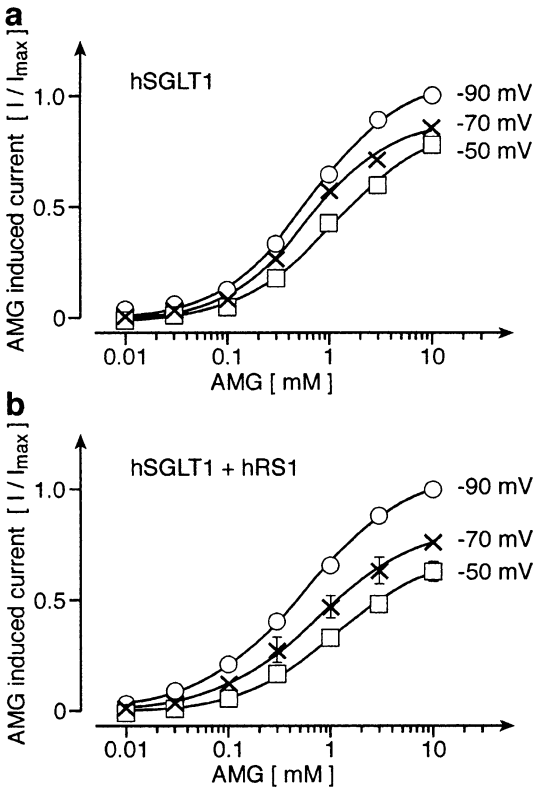

Fig. 6. hRS1 does not alter the concentration dependence of AMG-induced currents in oocytes expressing hSGLT1. Oocytes expressing hSGLT1 (*a*) or hSGLT1 plus hRS1 (*b*) in the absence of actinomycin D were clamped to −50 mV, −70 mV or −90 mV and superfused with increasing concentrations of AMG. AMG-induced currents (*I*) were normalized individually to the currents induced in the same oocyte by 10 mM AMG at −90 mV (I_{max}). The points represent mean values ± SEM from 4–6 oocytes.

data suggested that hRS1 induces changes in endo- or exocytotic processes that are involved in regulation and/or turnover of SGLT1. For this reason we investigated whether the effects of hRS1 are dependent on the presence of functionally intact dynamin. Dynamin is a member of a large family of proteins that are involved in membrane fission and vesicular trafficking; three isoforms and various splice variants of dynamin are known (van der Bliek et al., 1993). Dynamins are GTPases and are found at the plasma membrane, at early endosomes, and at the *trans*-Golgi network. They are involved in receptor-mediated endocytosis, synaptic vesicle recycling, caveolae internalization, and vesicle trafficking from the Golgi (Hinshaw, 2000). After binding GTP, several dynamin molecules form a ring around the neck of membrane in- or evaginations; upon GTP hydrolysis, this ring effects membrane fission and vesicle release (Hinshaw, 1999; McNiven et al., 2000). Overexpression of dynamin mutants with a disrupted GTP-binding domain such as Lys44Ala mutant of rat dynamin I (DyMu) inhibit internalization of plasma membrane vesicles during endocytosis and release of vesicles from the *trans*-Golgi network during exocytosis (Herskovits et al., 1993; Damke et al., 1994; Jones et al., 1998; van der Bliek, 1999; Hinshaw, 2000; Kreitzer et al., 2000).

To test whether dynamin is required for the action of hRS1 upon hSGLT1, we coinjected cRNA either of wild-type dynamin I (DyWt) or of the "dominant-negative" dynamin I mutant (DyMu) together with cRNA of hSGLT1 alone or of hSGLT1 and hRS1. Figure 3 shows one representative experiment out of four. In oocytes expressing hSGLT1, the uptake of AMG was not significantly changed by co-expression of either wild-type (DyWt) or dominant-negative dynamin (DyMu). Co-expressing hRS1 together with hSGLT1 resulted in the characteristic inhibition of AMG uptake. Notably, this inhibition was greatly reduced by co-expression of dominant-negative dynamin (inhibition without *vs.* with DyMu: 78 ± 12% *vs.* 39 ± 11%, mean ± SEM from four independent experiments, $P < 0.01$). Co-expression of DyWt-cRNA enhanced the inhibitory effect of hRS1 on AMG uptake via hSGLT1 in two out of four experiments (e.g., in the experiment shown in Fig. 3). These data indicate that dynamin is necessary for inhibition of hSGLT1 by hRS1. A requirement of dynamin, in turn, supports the conclusion that hRS1 acts by reducing the number of hSGLT1 molecules in the plasma membrane (Valentin et al., 2000).

Stimulation of Protein Kinase C Modulates the Effect of hRS1 on SGLT1

hRS1 contains four consensus sequences for phosphorylation by protein kinase C that are conserved in RS1 from pig and rabbit (Reinhardt et al., 1999). On the other hand, it is known that the activity of hSGLT1 expressed in *Xenopus laevis* oocytes was increased after stimulation of protein kinase C (Hirsch, Loo & Wright, 1996). Therefore, we investigated whether the effect of hRS1 on hSGLT1 can be influenced by PKC. In the experiment shown in Fig. 4, we examined whether the PKC activators phorbol-12-myristate-13-acetate (PMA) and *sn*-1,2-dioctanoyl glycerol (DOG) modulate the effect of hRS1 on hSGLT1. Three days after injection of the respective cRNAs, some oocytes were incubated for 2 min with 1 μM PMA, immediately followed by measurement of [^{14}C]AMG uptake as described above. Other oocytes were injected with 1 pmole of DOG each (corresponding to a final intracellular DOG concentration of ∼1 μM). In this case, [^{14}C]AMG uptake was measured after 30 min.

Fig. 4 shows that PMA or DOG each individually stimulated AMG uptake by hSGLT1 considerably by 40% and 32%, respectively, in keeping with a previous report (Hirsch et al., 1996). In oocytes co-expressing hSGLT1 and hRS1, PMA or DOG each

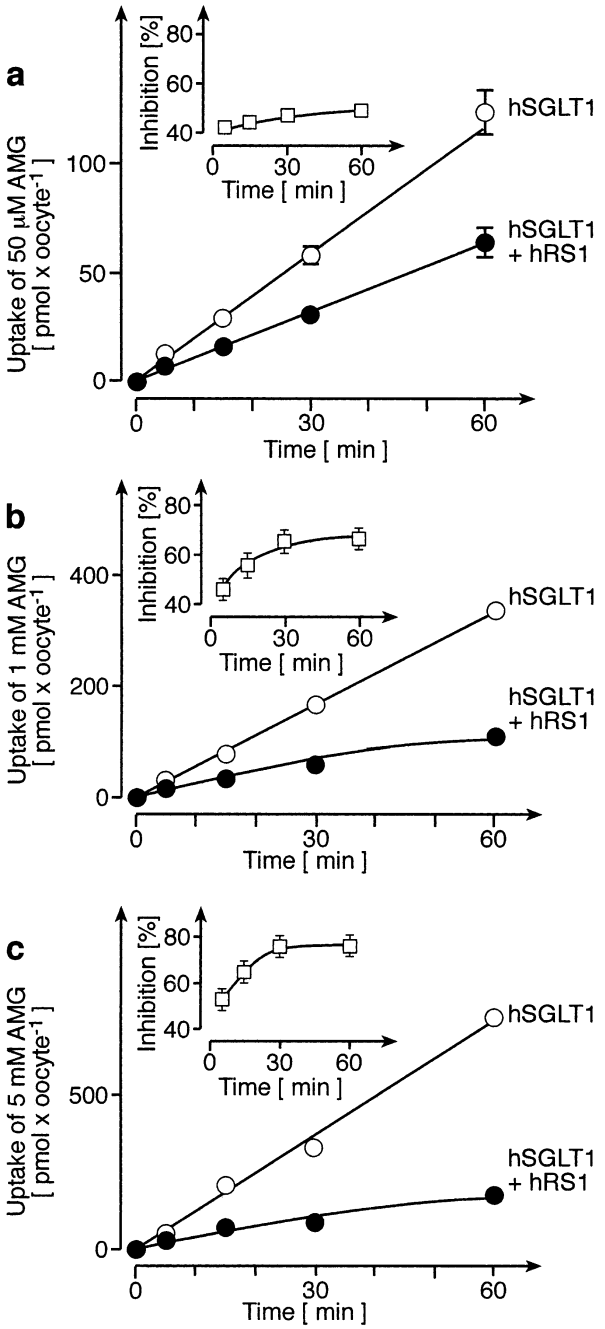

Fig. 7. Effects of hRS1 on the time dependence of expressed Na^+–glucose cotransport at low and high AMG concentration. Oocytes were injected with 2.5 ng hSGLT1-cRNA (○) or 2.5 ng hSGLT1-cRNA plus 2.5 ng hRS-cRNA (●) and incubated for 3 days in the absence of actinomycin D. The phlorizin inhibitable uptake of 50 µM (*a*), 1 mM (*b*) or 5 mM [^{14}C]AMG (*c*) was measured at different times of incubation and corrected for the endogenous uptake in water-injected oocytes. The insets represent the inhibition of expressed Na^+-D-glucose transport by hRS1.

individually decreased AMG uptake by SGLT1 considerably compared to unstimulated oocytes (*see* Fig. 4). In three experiments of expressing SGLT1 and hRS1, PMA and DOG reduced AMG uptake (by 44 ± 12% and 35 ± 12%, respectively; $P < 0.01$). Thus, PMA or DOG may affect hSGLT1 in opposite directions, depending on whether hRS1 is present or not. The data indicate that hRS1 is involved in protein kinase C-dependent short-term regulation of hSGLT1.

Reevaluation of the Effect of hRS1 on the Kinetic Properties of hSGLT1

Our finding that the inhibition of AMG uptake by hRS1 was dynamin-dependent suggested that hRS1 modulates endocytosis and/or exocytosis of SGLT1. This is in apparent conflict with the previously described observation that hRS1 decreased the K_m of hSGLT1 for AMG in uptake experiments because an effect on substrate affinity suggests a modification of SGLT1 within the plasma membrane rather than changes in the amount of SGLT1 (Veyhl et al., 1993; Lambotte et al., 1996).

To test for the possibility that the reported effect of hRS1 on the $K_{0.5}$ might be due to an effect of hRS1 on the transcription of an endogenous regulator, we compared the substrate-concentration dependence of AMG uptake in oocytes expressing either hSGLT1 alone, or together with hRS1 in the presence or absence of actinomycin D. We found again that uptake of [^{14}C]AMG for 1 h (Veyhl et al., 1993; Lambotte et al., 1996) exhibited significantly lower $K_{0.5}$ in oocytes co-expressing hSGLT1 and hRS1, as compared to oocytes expressing hSGLT1 alone; this effect was not affected by the absence or presence of actinomycin D in the 2–3 days between cRNA injection and uptake measurements (*data not shown*).

To determine whether the changes of the $K_{0.5}$ caused by the co-expression of hRS1 were due to an effect of hRS1 on the membrane potential, we compared substrate-induced currents (10 mM AMG for 30 sec) in oocytes expressing hSGLT1 without (Fig. 5*a*) and with co-expression of hRS1 (Fig. 5*b*), using the two-electrode voltage-clamp method. In water-injected control oocytes, substrate-induced currents were always smaller than 3 nA (*not shown*). In oocytes expressing hSGLT1, 10 mM AMG induced currents that were membrane potential-dependent as expected (Loo et al., 1993), i.e., more negative potentials were associated with larger absolute currents (−256.6 ± 47.1 nA at −150 mV *vs.* −135.2 ± 21.8 nA at −50 mV). Co-expression of hRS1 greatly reduced these currents (−108.1 ± 15.6 nA at −150 mV, and −68.2 nA ± 25.2 n at −50 mV). Figure 5*c* displays the currents induced by 10 mM AMG at various membrane potentials between −10 mV and −90 mV. These data show that hRS1 did not alter the potential dependence of AMG transport.

The concentration dependence of AMG-induced currents at −90 mV, −70 mV or −50 mV is shown for oocytes expressing hSGLT1 (Fig. 6*a*) or co-express-

ing hSGLT1 and hRS1 (Fig. 6*b*). In oocytes co-expressing hSGLT1 and hRS1, AMG-induced currents were decreased as compared to oocytes expressing hSGLT1 alone, in keeping with the effect of hRS1 on [^{14}C]AMG uptake (Fig. 5). In contrast to 1-hour uptake of [^{14}C]AMG, measurements of the AMG-induced currents showed no significant effect of hRS1 on the $K_{0.5}$ of hSGLT1 for AMG ($K_{0.5}$ for AMG without hRS1 *vs.* with hRS1: −90 mV: 0.65 ± 0.06 mM *vs.* 0.61 ± 0.04 mM; −70 mV: 0.61 ± 0.08 mM *vs.* 0.79 ± 0.09 mM; −50 mV: 1.1 ± 0.15 mM *vs.* 1.28 ± 0.19 mM). Hill coefficients ranged from 0.75 to 1.15. The apparent $K_{0.5}$ values decreased with hyperpolarizing membrane potentials as described earlier (Hirayama et al., 1996).

We also investigated whether hRS1 has an effect on the affinity of hSGLT1 for phlorizin. Co-expression of hSGLT1 and hRS1 resulted in inhibition of AMG-induced currents as for the experiment shown in Fig. 5. At −70 mV, 3 μM phlorizin inhibited the currents induced by 1 mM AMG by more than 95% in oocytes expressing hSGLT1 either alone or together with hRS1. The IC_{50} and Hill coefficients for phlorizin inhibition were not significantly different for oocytes expressing hSGLT1 alone as compared to oocytes co-expressing hSGLT1 and hRS1 (IC_{50}/Hill coefficient without *vs.* with hRS1 at −70 mV: 102.3 ± 6.8 nM/0.96 ± 0.05 *vs.* 125.3 ± 17.6 nM/0.75 ± 0.06). Na^+ activation of AMG-induced currents was determined at −70 mV by applying pulses of 1 mM AMG at various Na^+ concentrations replacing Na^+ by choline. We found that the Na^+ activation of AMG transport by hSGLT1 was not significantly changed by hRS1 ($K_{0.5}$/Hill coefficient without *vs.* with hRS1: 41.8 ± 1.9 mM/1.57 ± 0.11 *vs.* 44.4 mM ± 1.7 mM/1.64 ± 0.06).

Finally, we investigated whether the substrate specificity of hSGLT1, which is able to translocate a variety of sugar analogues (Hirayama et al., 1996), was changed by co-expression of hRS1. We expressed hSGLT1 alone and together with hRS1, as described for Fig. 5, and confirmed the presence of the characteristic inhibition of AMG-induced currents by hRS1. Individual oocytes clamped to −70 mV were successively superfused with 1 mM solutions each of D-glucose, AMG, D-galactose, 3-O-methyl-D-glucopyranoside, β-D-allose, D-mannitol and D-xylose. We found that hRS1 does not change the selectivity of hSGLT1 (substrate-induced currents relative to 1 mM D-glucose, without *vs.* with hRS1: AMG 0.96 ± 0.22 *vs.* 0.92 ± 0.08 > D-galactose 0.72 ± 0.22 *vs.* 0.80 ± 0.08 > 3-O-methyl-D-glucopyranoside 0.48 ± 0.14 *vs.* 0.50 ± 0.05 > β-D-allose 0.06 ± 0.02 *vs.* 0.08 ± 0.03 > D-mannitol 0.02 ± 0.03 *vs.* 0.01 ± 0.02).

Because the electrophysiological measurements showed virtually no effect of hRS1 on the kinetic properties of hSGLT1 other than I_{max}, we tried to identify the reason for the apparent affinity change observed in the tracer-flux measurements. Membrane potential was not significantly different when hRS1 was expressed alone or together with hSGLT1; a small transport-induced depolarization observed in oocytes co-expressing hRS1 and hSGLT1 did not explain the decrease in apparent $K_{0.5}$ (*data not shown*). However, the apparent affinity change after co-expression of hRS1 was no longer detectable when uptake time was reduced from 1 h to 5 min ($K_{0.5}$ without *vs.* with hRS1: 0.50 ± 0.08 mM *vs.* 0.45 ± 0.03 mM). The effect of hRS on V_{max} was still observed (V_{max} without *vs.* with hRS1: 25.2 ± 2.2 pmol × oocyte^{-1} × 5 min^{-1} *vs.* 9.8 ± 0.04 pmol × oocyte^{-1} × 5 min^{-1}).

In the experiment shown in Fig. 7, we determined the time course for [^{14}C]AMG uptake at three different concentrations of AMG in oocytes expressing either hSGLT1 alone or together with hRS1. In these experiments, we co-injected a smaller amount of hRS1-cRNA, which induced only an about 50% inhibition in order to obtain uptake rates that were sufficiently high to support accurate measurements even with short uptake periods. With 50 μM AMG, uptake was nearly linear over 60 min in oocytes expressing either hSGLT1 or hSGLT1 plus hRS1 (Fig. 7*a*). With 1 mM or 5 mM AMG, uptake was linear over 60 min in oocytes expressing hSGLT1 alone (Figs. 7*b*, *c*). In contrast, the uptake rate of 1 mM or 5 mM AMG significantly decreased with time when hSGLT1 and hRS1 were co-expressed (Figs. 7*b*, *c*). The data suggest that the degree of inhibition by hRS1 increased with time and AMG concentrations (*see* inserts in Figs. 7*a-c*). The increase of hRS1-induced inhibition between 5 min and 30 min of incubation was 5 ± 3%, 19 ± 6%, and 23 ± 6% for glucose uptake measured with 50 μM, 1 mM or 5 mM AMG, respectively ($P < 0.05$ for difference between 50 μM and 5 mM AMG). The glucose- and hRS1-dependent effect on the time course of AMG uptake explains our previously observed effect of hRS1 on the $K_{0.5}$ of hSGLT1 for AMG in which uptake was measured over a 1-h period.

Discussion

In this paper, we further investigate the role of hRS1 as a modulator of membrane transport. Our approach was to measure the effect of hRS1 on the activity of various membrane transporters in *Xenopus* oocytes in the presence of a dominant-negative dynamin mutant, a transcription blocker and activators of protein kinase C. Our results provide new insights regarding the post-transcriptional mechanisms by which RS1 modulates expression or activity of plasma membrane transporters. After the cloning and first characterization of RS1 from pig and human, several further properties of RS1 emerged that com-

pletely transformed our initial ideas as to the function of this protein.

For instance, we found that RS1 is an intracellular protein and potentially associated with the plasma membrane, rather than a membrane-associated extracellular protein as initially assumed (Valentin et al., 2000). Moreover, we observed that hRS1 may modulate not only SGLT1, but also other transporters without structural similarity to SGLT1 (Reinhardt et al., 1999). A physiologically important role of RS1 was suggested by the observation that the well-known upregulation of SGLT1 in LLC-PK_1 cells upon reaching confluency was prevented by overexpression of RS1, but increased by inhibition of endogenous RS1 expression via an antisense strategy (Korn et al., 2001). A further striking observation was that reduction of endogenous pRS1 increased the transcription of SGLT1 in LLC-PK_1 cells tenfold (Korn et al., 2001). This suggested a direct or indirect effect of RS1 in the nucleus. Finally, using fusion RS1 proteins with green fluorescent protein and immunocytochemistry, we recently found that RS1 is not only localized at the plasma membrane but is also present around and within the nucleus (*unpublished data*).

In the present study, we conducted further co-expression experiments in *Xenopus* oocytes because this experimental setting allows to analyze post-transcriptional effects of RS1. We used human RS1 because co-expression of hRS1 with hSGLT1 resulted in a concentration-dependent decrease of expressed glucose transport, whereas the co-expression of pRS1 or rbRS1 together with rbSGLT1 induced more complex effects that were concentration-dependent and biphasic (Veyhl et al., 1993; Lambotte et al., 1996; Reinhardt et al., 1999). Since pRS1 inhibits transcription of pSGLT1 in LLC-PK_1 cells (Korn et al., 2001) we tested whether the effects of hRS1 on the expression of transporters may be mediated by hRS1-induced changes in transcription of regulatory oocyte proteins. Our results, however, exclude this possibility: the effects of hRS1 on the expression of hSGLT1 or hOCT2 were similar in the presence or absence of the transcription inhibitor actinomycin D.

The observation that hRS1 inhibited transporters without structural similarity to SGLT1 raised the possibility that hRS1 changes the plasma membrane turnover and thereby inevitably alters the membrane concentration of all integral membrane proteins as well. For pRS1, this possibility had been excluded since pRS1 did not alter the expression of the glucose transporter hGLUT1 and the Na^+/Cl^- GABA-co-transporter GAT1 (Veyhl et al., 1993). However, membrane capacitance measurements showed that plasma membrane surface area of oocytes was decreased after expression of hRS1 (Valentin et al., 2000), suggesting a less specific effect of hRS1. We found that co-expression of hRS1 did not affect substrate uptake by the peptide transporter hPEPT1 or by the glucose transporter hGLUT1, but inhibited SGLT1 from two species and two subtypes of the rat organic cation transporter. This indicates selectivity of hRS1 for a group of transporters that have no overall structural similarity. So far, the selectivity of hRS1 is poorly characterized, and the molecular basis for the apparent selectivity of hRS1 is not understood. The association of RS1 with the plasma membrane and the dependence of hRS1-action on dynamin (*see* below) strongly suggest that post-transcriptional effects of hRS1 are mainly directed at proteins in or at the plasma membrane. The apparent selectivity could be explained by sorting of different groups of membrane proteins into different types of endocytotic vesicles or *trans*-Golgi vesicles. hRS1 may only modulate endocytosis or exocytosis of one type of vesicles that is dependent on the function of dynamin. Another possibility is that hRS1 interacts directly or indirectly with a protein domain that is common to various transporters such as phosphorylation or ubiquitinylation sites. The interaction of RS1 with ubiquitinated proteins or ubiquitination is an attractive possibility because the RS1 proteins cloned so far contain a conserved ubiquitin-associated (UBA) domain at the C-terminus (Valentin et al., 2000), and preliminary data suggest binding of ubiquitin to this domain (*unpublished data*). UBA domains have been described in proteins with various functions. These are proteins that are involved in pathways utilizing ubiquitination and phosphorylation, DNA repair proteins and suppressors of protein degradation in the proteasome (Hofmann & Bucher, 1996; Miao et al., 2000; Clarke et al., 2001). Interestingly, it has been reported that endocytosis of receptors, plasma membrane transporters and ion channels may be regulated by monoubiquitination (Hicke & Riezman, 1996; Strous et al., 1996; Hicke, 1997, 2001; Rotin, Staub & Haguenauer-Tsapis, 2000; Dunn & Hicke, 2001).

Considering the various, hitherto unclear post-transcriptional mechanisms that might underlie the observed effects of RS1, our finding in the present paper that the inhibition of hSGLT1 by hRS1 is dependent on the presence of functionally intact dynamin represents a significant step forward. Dynamin is required for pinching off the vesicles that are budding from the plasma membrane during endocytosis (Herskovits et al., 1993; Damke et al., 1994, 1996), for vesicle transport from endosomes to the Golgi apparatus (Llorente et al., 1998; Nicoziani et al., 2000), and probably also for vesicle trafficking from the Golgi apparatus to the plasma membrane (Jones et al., 1998; Hinshaw, 2000; Kreitzer et al., 2000). Because co-expression of dominant-negative dynamin with hSGLT1 per se had no effect on glucose transport in the absence of RS1, the amount of hSGLT1 in the plasma membrane might not be significantly changed by inhibition of dynamin-de-

pendent trafficking of hSGLT1. The observation that co-expression of wildtype dynamin I increased the inhibitory effect of hRS1 on the hSGLT1, whereas co-expression of the dominant-negative dynamin mutant decreased that effect, implies that the effect of hRS1 is either due to a stimulation of endocytic retrieval of hSGLT1 from the plasma membrane, or to an inhibition of hSGLT1 trafficking from the Golgi and insertion into the plasma membrane. It is less probable but cannot be excluded that hRS1 inhibits or stimulates several dynamin-dependent processes but that the stimulation of the endocytosis or the inhibition on trafficking from the Golgi dominates. Immunohistochemical investigation of the effects of RS1 on the intracellular distribution of SGLT1 may help to identify the site where hRS1 acts post-transcriptionally.

Electrophysiological measurements showed that co-expression of hRS1 inhibited the maximal velocity of hSGLT1, but had no significant effect on its apparent $K_{0.5}$, in contrast to a previous report using tracer flux measurements and an incubation time of 1 h. We observed that the inhibitory effect of hRS1 on AMG uptake by hSGLT1 increased with increasing AMG concentrations in the time course of the uptake measurements. This unexpected observation showed that hRS1 does not change the functional properties of hSGLT1 in the way we previously assumed. Rather, it is consistent with our interpretation that hRS1 changes the dynamin dependent equilibrium between SGLT1 associated with intracellular vesicles and SGLT1 within the plasma membrane. The AMG dependence of the regulation of SGLT1 expression by hRS1 deserves a further detailed investigation. It could be due to energy dependence of RS1 function and AMG-induced decrease of the intracellular ATP level.

Finally, we observed that the expression of hRS1 differentially affected the effect of PKC stimulation on AMG transport by hSGLT1. Without hRS1, glucose uptake by hSGLT1 was increased upon stimulation of PKC by PMA or DOG. In contrast, in the presence of hRS1, glucose uptake by hSGLT1 was decreased by PMA or DOG. Importantly, the inhibitory effect of the PKC activators in the presence of hRS1 was induced within minutes, showing for the first time that hRS1 participates in the short-term regulation of a plasma membrane transporter. Transport activities measured in all previous co-expression experiments reflect the dynamic equilibrium between hSGLT1 within the plasma membrane and intracellular vesicles that had been built up during the two or three days of expression. This equilibrium may have been mainly influenced by vesicle trafficking from the Golgi, degradation or other long-term intracellular regulations. The hRS1-dependent short-term effect of DOG or PMA on the Na^+-D-glucose cotransport is supposed to represent a PKC effect on the modulation of endocytosis by hRS1 rather than an effect on hRS1 acting on trafficking from the Golgi or on the stability of hRS1.

Taken together, our data show that hRS1 modulates the turnover of plasma membrane proteins via dynamin-dependent processes and participates in short-term regulation of plasma membrane transporters.

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