Protein RS1 (RSC1A1) Downregulates the Exocytotic Pathway of Glucose Transporter SGLT1 at Low Intracellular Glucose via Inhibition of Ornithine Decarboxylase


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ABSTRACT

Na\(^+\)-d-glucose cotransporter 1 (SGLT1) is rate-limiting for glucose absorption in the small intestine. Shortly after intake of glucose-rich food, SGLT1 abundance in the luminal membrane of the small intestine is increased. This upregulation occurs via glucose-induced acceleration of the release of SGLT1-containing vesicles from the trans-Golgi network (TGN), which is regulated by a domain of protein RS1 (RSC1A1) named RS1-Reg. Dependent on phosphorylation, RS1-Reg blocks re-uptake of vesicles containing SGLT1 or concentrative nucleoside transporter 1. The hypothesis has been raised that RS1-Reg binds to different receptor proteins at the TGN, which trigger release of vesicles with different transporters. To identify the presumed receptor proteins, two-hybrid screening was performed. Interaction with ornithine decarboxylase 1 (ODC1), the rate-limiting enzyme of polyamine synthesis, was observed and verified by immunoprecipitation. Binding of RS1-Reg mutants to ODC1 was characterized using surface plasmon resonance. Inhibition of ODC1 activity by RS1-Reg mutants and the ODC1 inhibitor difluoromethylornithine (DFMO) was measured in the absence and presence of glucose. In addition, short-term effects of DFMO, RS1-Reg mutants, the ODC1 product putrescine, and/or glucose on SGLT1 expressed in oocytes of Xenopus laevis were investigated. High-affinity binding of RS1-Reg to ODC1 was demonstrated, and evidence for a glucose binding site in ODC1 was provided. Binding of RS1-Reg to ODC1 inhibits the enzymatic activity at low intracellular glucose, which is blunted at high intracellular glucose. The data suggest that generation of putrescine by ODC1 at the TGN stimulates release of SGLT1-containing vesicles. This indicates a biomedically important role of ODC1 in regulation of glucose homeostasis.

Introduction

For rapid adaption of cellular uptake to physiologic demands, the concentrations of transporters in the plasma membrane can be changed by regulation of endocytosis and/or exocytosis of transporter-containing vesicles. Endocytic vesicles are delivered to endosomes, where they are sorted for degradation or recycling to the plasma membrane. Transporter-containing vesicles delivered to the plasma membrane may also be derived from the Golgi as has been described for upregulation of Na\(^+\)-d-glucose cotransporter 1 (SGLT1) in the small intestine after uptake of glucose-rich food (Gorboulev et al., 2012; Veyhl-Wichmann et al., 2016). The increased concentration of intracellular glucose in the enterocytes activates the release of SGLT1-containing vesicles from the Golgi (Veyhl et al., 2003, 2006; Kroiss et al., 2006; Veyhl-Wichmann et al., 2016). Due to rapid turnover of SGLT1 in the plasma membrane, including endocytosis and degradation (Wright et al., 1997), glucose-dependent release of SGLT1-containing vesicles from the Golgi may lead to a 2- to 4-fold upregulation of SGLT1 in the brush-border membrane within minutes (Veyhl-Wichmann et al., 2016). The intracellular 67–68-kDa protein RS1 (gene RSC1A1) is critically involved in glucose-dependent post-translational

ABBR EVIAT I ONS: Ab, antibody; AMG, a-methyl-d-glucopyranoside; AZ, antizyme; AZIN, antizyme inhibitor; BFA, brefeldin A; CNT1, concentrative nucleoside transporter 1; DFMO, difluoromethylornithine; DTT, dithiothreitol; GFP, green fluorescent protein; GFP-S-hRS1(2-312); GFP and S-Tag linked to the N terminus of hRS1(2-312); GFP-S-hRS1(2-98); GFP and S-Tag linked to the N terminus of hRS1(2-98); GST, glutathione-S-transferase; hCNT1, human CNT1; hOCN, human ODN; hRS1, human RS1; hRS1-Reg, human RS1-Reg; hRS1-Reg-P-Ab, antibody against a peptide motif within human RS1-Reg; hSGLT1, human SGLT1; K\(_D\), equilibrium binding dissociation constant; NHS, sulfo-N-hydroxysuccinimide; ODC, ornithine decarboxylase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RS1-Reg, NH2-terminal regulatory domain of RS1; SGLT1, Na\(^+\)-d-glucose cotransporter 1; S-hRS1-H, hRS1 with N-terminal S-tag and C-terminal His-tag; SPR, surface plasmon resonance; TGN, trans-Golgi network; YFP, yellow fluorescent protein.
regulation of SGLT1 at the Golgi. RS1 is encoded by a single-copy gene that first emerged in mammals (Veyhl et al., 1993; Lambotte et al., 1996; Reinhardt et al., 1999; Osswald et al., 2005). In LLC-PK1 cells, RS1 was detected at the trans-Golgi network (TGN), where it was colocalized with SGLT1 and dynamin (Kroiss et al., 2006). When studying the effects of RS1 on mammalian transporters expressed in oocytes of Xenopus laevis, post-translational regulation of Na⁺–D-glucose cotransporters, Na⁺–nucleoside cotransporters, and organic cation transporters was observed (Reinhardt et al., 1999; Veyhl et al., 2003; Errasti-Murugarren et al., 2012). We analyzed post-translational regulation of human SGLT1 (hSGLT1) and human concentrative nucleoside transporter 1 (hCNT1) by expressing the transporters in oocytes and measuring short-term effects of injected human RS1 (hRS1) fragments on transport activities and transporter concentrations in the plasma membrane (Veyhl et al., 2003, 2006; Vernaleken et al., 2007; Errasti-Murugarren et al., 2012). We observed that downregulation of hSGLT1 and hCNTs by hRS1 and/or hRS1 fragments was prevented when the Golgi was dissociated with brefeldin A (BFA), or fusion of exocytotic vesicles with the plasma membrane was blocked with botulinum toxin B but was not changed by blockers of endocytosis. We therefore concluded that RS1 blocks the exocytotic release of the transporters from the TGN. Recently, we showed that post-translational regulation of hSGLT1 and hCNT1 is mediated by an N-terminal domain of hRS1 termed hRS1-Reg, which contains many predicted phosphorylation sites of protein kinases (Veyhl-Wichmann et al., 2016). Since it was observed that hRS1-Reg downregulates hSGLT1 expression in a glucose-dependent manner, whereas downregulation of hCNT1 expression is independent of glucose, and because the efficacy of hRS1-Reg for downregulation of hSGLT1 versus hCNT1 was differentially dependent on phosphorylation of hRS1-Reg, we concluded that hRS1-Reg downregulates different exocytotic transporter pathways. We raised the hypothesis that differentially phosphorylated forms of hRS1-Reg bind to different receptor proteins at the TGN that are involved in regulation of different exocytic pathways for plasma membrane transporters. Evidence was provided that short-term upregulation of SGLT1 the in small intestine is due to a glucose-induced blunting of RS1-Reg–mediated blockage of the exocytic pathway of SGLT1 (Veyhl-Wichmann et al., 2016).

The aim of the present study was to identify one of the hypothesized receptor proteins of hRS1-Reg. We performed two-hybrid screening with an N-terminal domain of hRS1 and characterized one of the identified interacting proteins, the human ornithine decarboxylase 1 (hODC1). Here, we present evidence that hODC1 is a receptor protein for hRS1-Reg that is involved in glucose-dependent short-term regulation of hSGLT1.

Materials and Methods

Materials. α-Methyl-d-[14]Cglucopyranoside (14C-AMG) (11.1 GBq/mmol) and [5-3H]thidine (0.91 TBq/mmol) were obtained from American Labeled Chemical Inc. (St. Louis, MO), and d,l-[1-14C]ornithine (2.07 TBq/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany). S-protein agarose (agarose beads with coupled S-protein) was purchased from Merck (Darmstadt, Germany), and glutathione-sepharose 4B beads were from GE Healthcare (Munch, Germany). DL-α-difluoromethylornithine (DFMO), bovine thymobrin, Acetate solution, anti–c-myc agarose (agarose beads with linked anti-myc antibodies), and antinimous IgG agarse beads (agarose beads with covalently linked antibodies against mouse IgG raised in goat) (A6531) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Protease inhibitor cocktail set III was provided by Calbiochem (Darmstadt, Germany), and pretausted weight markers (BenchMark) were from Life Technologies (Karlruhe, Germany). A human cDNA library of embryonic kidney cells expressed in vector pPUR-N was obtained from Dualesystem Biotech (Schlieren, Switzerland). Bait vector pMetYcgate for the split ubiquitin assay, the linkers B1 and B2, as well as yeast strain THY.AP4 were provided by C. Ciarimboli (Medizinische Klinik und Poliklinik D, Universitätsklinikum, Münster, Germany) (Brast et al., 2012). Expression vector pcDNA3 encoding for full-length human ODC fused to a C-terminal myc tag was supplied by S. Matsuzawa (Burnham Institute for Medical Research, La Jolla, CA) (Matsuzawa et al., 2005). ProteOn GLC sensor chip, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, sulfo-N-hydroxysuccinimide (NHS), and ethanolamine were purchased from Bio-Rad (München, Germany), and LC-NHS-biotin was from Fisher Scientific GmbH (Schwerte, Germany). Other reagents were purchased as previously described (Keller et al., 2005; Vernaleken et al., 2007; Veyhl-Wichmann et al., 2016).

Antibodies. Mouse monoclonal antibody against myc (anti-myc-Ab) (OP10) was obtained from Calbiochem (Darmstadt, Germany), and mouse monoclonal antibody against green fluorescent protein (anti-GFP-Ab) (MMS-118P) was from Covance (Freiburg, Germany). An antibody against hODC1 raised in mouse (anti-hODC1-Ab) was purchased via Antibodies-online (ABIN518505; Aachen, Germany). Polyclonal antibodies against full-length hRS1 (anti-hRS1-Ab) containing an N-terminal S-tag and a C-terminal His-tag (S-hRS1-H) were raised in rabbits. Cloning and purification of S-hRS1-H is described later. Polyclonal immune serum against a peptide within hRS1-Reg (amino acids 40–57 of hRS1) containing a C-terminal cysteine (IKPSDSRIEPKAVKLC–C) was raised in rabbits (anti-hRS1-Reg-P-Ab). The antibody was purified using antigenic peptide coupled via the C-terminal cysteine to polyacrylamide particles.

Cloning. To clone the bait for the split ubiquitin analysis, we used hRS1 (Lambotte et al., 1996) as template. An N-terminal hRS1 fragment comprising amino acids 1–312 with linkers termed B1 and B2 for insertion into the bait vector pMetYcgate (Obrdlik et al., 2004) was amplified by polymerase chain reaction (PCR). A forward primer with B1 linker (underlined) 5′-ACA AGT TTG TAG AAG AAA AAG GTA GCC ACC TTC CCA ACC ACC ATG TCA TCA TTA CCA ACT TCA GAT GGG-3′ and a reverse primer with B2 linker (underlined) 5′-TCC GGC ACC ACC CAC TTT GTA CAA GAA AGC TGG GTA GGG CTT AAG ATC TGG AGT GGA AAT GG-3′ were used. The amplified constructs were cloned into the pMetYcgate vector (Obrdlik et al., 2004) using the PsiI and HindIII restriction sites. The constructs were expressed in yeast strain THY.AP4 (Obrdlik et al., 2004).

For immunoprecipitation, hRS1 fragments expressed in pEGFP-TEV-S-Tag vector (Filatova et al., 2009) were used and constructs were generated in which GFP followed by an S-Tag was linked to the N-terminus of hRS1(2-312) (GFP-S-hRS1(2-312)) or hRS1(2-98) (GFP-S-hRS1(2-98)). To clone GFP-S-hRS1(2-312) and GFP-S-hRS1(2-98), RS1 fragments framed with linkers containing restriction sites were prepared by PCR. In the case of hRS1(2-312), the forward primer 5′-GCTCGAGGCTCAAGTCATTACCCACCTGGGGAACG3′ was inserted into the S-Tag recognition site (underlined), and the reverse primer 5′-GGCTACTGCTCTGAAGCGCCGCTGTAATACTCTGATTTGAG-3′ contained an AscI recognition site (underlined). hRS1-S was used as template for the PCR. Using the PsiI and AscI restriction sites, the PCR product was inserted into vector pEGFP-TEV-S-Tag using the open reading frame of GFP and the S-Tag. For PCR amplification of the hRS1(2-98) fragment with linkers, GFP-S-hRS1(2-312) was used as template. The forward primer 5′-CCTGCTGACCTGATCGGGCTGTTAACTTCTGAGTGG-3′ located in the C-terminal of the GFP coding region and the reverse primer 5′-GGGACCCAGCTTCTGGAGGCTGTTAACTTCTGAGTGG-3′ containing an
and uracil. Plasmid DNAs from positive colonies were isolated, synthetic complete medium lacking leucine, tryptophan, histidine, and tryptophanomycin. Colonies cotransformed with a human cDNA library in vector pPR3-N. Colonies containing hRS1(1-312) were transfected with empty vector pMetYCgate vector or pMetYCgate containing hRS1(1-312) were selected on plates containing oocyte expression vector [see vector pNB1uYFP in Nour-Eldin et al. (2006)] using an advanced uracil-excision-based cloning technique (Nour-Eldin et al., 2006) to identify proteins that interact with membrane proteins (Brast et al., 2006). As a control peptide for interaction analysis using surface plasmon resonance (SPR), we cloned a peptide containing the amino acids 150–312 of hRS1. For PCR amplification, vector pET21a containing hRS1 (hRS1/pET21a) was used as template. A forward primer with a BglII site and a reverse primer with an XhoI site were used. The PCR product was digested with BglII and XhoI and cloned into vector pET21a vector with BglII and XhoI.

For overexpression of hODC1 in Escherichia coli and purification on glutathione-sepharose 4B, the sequence encoding for hODC1 was obtained by PCR using hODC1 in pcDNA3 plasmid (Matsuzawa et al., 2005) as template. A forward primer with an Acc65I recognition site and a reverse primer with an XhoI site were used. The PCR product was cut with Acc65I and XhoI and ligated to the pET42b vector, which was treated with the same restriction enzymes. The resulting construct was transformed into the E. coli BL21(DE3) strain (Invitrogen, Carlsbad, CA) for protein expression. The expression yields a fusion protein consisting of glutathione-s-transferase (GST) followed by a His-tag, a thrombin cleavage site, the S-tag, hODC1 protein, and a second His-tag (hODC1/pET42b).

Full-length hRS1 was expressed in Sf9 insect cells. Therefore, hRS1 containing an SH-tag (hRS1-H) was cloned into vector pVL1392 (BD Biosciences, Emergnegem, Belgium) as described [see vector pVL1392 in Veyhl et al. (2006)].

For expression of hODC1 in oocytes, the hODC1 coding sequence was cloned into vector pRSSP (Busch et al., 1996) using EcoRI as 5' and XhoI as 3' restriction sites of the vector (hODC1/pRSSP).

To visualize localization of hSGLT1 in oocytes, yellow fluorescence protein (YFP) was fused to the C terminus of hSGLT1 (hSGLT1-YFP). Therefore, the complementary DNA of hSGLT1 was cloned into a YFP-containing oocyte expression vector [see vector pNB1uYFP in Nour-Eldin et al. (2006)] using an advanced uracil-excision-based cloning technique (Nour-Eldin et al., 2006).

Two-Hybrid Screening by the Split Ubiquitin Assay. To identify proteins that interact with RS1 at the Golgi, we used the two-hybrid split ubiquitin assay, which has been developed to identify proteins that interact with membrane proteins (Brast et al., 2012). The yeast reporter THY.AP4 strains transfected with empty pMetYCgate vector or pMetYCgate containing hRS1(1-312) were cotransformed with a human cDNA library in vector pPR3-N. Colonies reacting positive in the β-galactosidase assay were selected on synthetic complete medium lacking leucine, tryptophan, histidine, and uracil. Plasmid DNAs from positive colonies were isolated, transformed into E. coli DH10B (Grant et al., 1990), and amplified. DNA sequences were determined after PCR amplification using the primer 5'-GTC GAA AAT TCA AGA CAA GG-3'.

Expression and Purification of hODC1 and hRS1. hODC1 was expressed in E. coli. Strain BL21(DE3)Star (Live Technologies, Darmstadt, Germany) harboring the pET42b plasmid encoding a GST-hODC1 fusion protein with a thrombin site between the N-terminal GST-tag and hODC1 was cultivated at 30°C until an optical density at 600 nM of 0.6–0.8 = 0.6–0.8 was obtained. After induction of protein expression with 0.2 mM isopropyl-1-thio-β-d-galactopyranoside, bacteria were grown for 24 hours. Thereafter, bacteria were pelleted by 15-minute centrifugation at 6000 × g, washed, and resuspended in 20 mM Tris (pH 8.3), 150 mM NaCl, 10 mM EDTA, 2 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. For lysis, bacteria were sonified at 4°C, and cellular debris was sedimented by ultracentrifugation for 1 hour at 100,000 × g. Protein purification was performed by adding 0.5 ml of glutathione-sepharose 4B beads to 20 ml of lysate and incubating the suspension for 1 hour at 4°C. The affinity resin was pelleted by centrifugation for 5 minutes at 5000 × g, washed with 20 mM Tris (pH 8.3), 150 mM NaCl, and 2 mM DTT; and incubated in 1 ml of washing buffer supplemented with bovine thionine (2 units) for 2 hours at 37°C to release hODC1. After centrifugation for 5 minutes at 5000 × g, purified hODC1 was obtained in the supernatant and dialyzed against 10 mM HEPES (pH 7.5), 150 mM NaCl, 0.005% (v/v) Tween 20 (for SPR measurements), or against 50 mM Tris-HCl (pH 7.2) for assays of ODC activity. The purification was monitored using SDS-PAGE and Coomassie staining (Fig. 3A).

Tagged full-length hRS1 protein (S-hRS1-H) was expressed in Sf9 insect cells using vector S-tag-hRS1-His/pVL1392 and purified on nickel charged agarose, as described (Veyhl et al., 2006).

Expression and Purification of hRS1-Reg, hRS1-Reg Variants, and hRS1(150-312). E. coli bacteria (strain BL21 Star) were transformed with pET21a plasmids containing His-tagged hRS1-Reg, hRS1-Reg mutants, or hRS1(150-312). Protein expression was induced by adding 1 mM isopropyl-1-thio-β-d-galactopyranoside, and bacteria were subsequently grown for 5 hours at 30°C. After 15-minute centrifugation at 6000 × g, bacteria were washed and suspended in 20 mM Tris-HCl (pH 8.0) containing 500 mM NaCl and 50 mM imidazole. The cells were lysed by sonication at 4°C, and cellular debris was removed by 1-hour centrifugation at 100,000 × g. For protein purification, the supernatants were mixed with nickel charged agarose, and the beads were incubated for 1 hour under rotation and poured into an empty gravity flow column. After washing with 20 mM Tris (pH 8.0) containing 500 mM NaCl and 50 mM imidazole, protein was eluted with 20 mM Tris (pH 8.0) containing 500 mM NaCl and 500 mM imidazole. Fractions containing purified protein were pooled and dialyzed against 10 mM HEPES (pH 7.5), 150 mM NaCl, and 0.005% (v/v) Tween 20 for SPR measurements or against 50 mM Tris-HCl (pH 7.2) for enzymatic assays.

SDS-PAGE and Western Blotting. SDS-PAGE and Western blotting were performed as described (Keller et al., 2005). For SDS-PAGE, protein samples were treated for 30 minutes at 37°C in 60 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS and 7% (v/v) glycerol (SDS-PAGE sample buffer). With the exception of one gel in Fig. 3B, 100 mM DTT was added to the SDS-PAGE sample buffer. The gels were stained with Coomassie Brilliant Blue. Separated proteins were transferred to polyvinyl difluoride membrane and stained with antibodies raised in rabbits (anti-hRS1-Ab 1:10,000, anti-hRS1-Reg-P-Ab 1:5000) or mice (anti-myc-Ab, anti-GFP-Ab, anti-hODC1-Ab) using peroxidase-conjugated secondary antibodies against rabbit or mouse IgG, respectively. Bound secondary antibodies were visualized by enhanced chemiluminescence. For determination of apparent molecular masses (M_r), prestained weight markers were used.

Cultivation and Harvesting of Caco-2 Cells. Caco-2 cells were cultivated until forming a confluent polarized monolayer as described (Kipp et al., 2003). Cells were grown for 18 days at 37°C on Petri dishes in minimal essential medium containing 1 mM D-glucose and supplemented with 10% fetal calf serum, 1% nonessential amino acids, and 1% glutamine. The cells were detached using Accutase solution, collected by low-speed centrifugation, washed with phosphate-buffered saline (PBS), and suspended in ice-cold PBS containing protease inhibitor cocktail set III from Calbiochem.

Coprecipitation Experiments. For coprecipitation of overexpressed proteins, HEK293 cells were transiently transfected with vectors encoding for GFP-S, GFP-S-hRS1(2-312), or GFP-S-hRS1(2-98) and hODC1 containing an myc tag (hODC-myc). The four proteins were expressed to similar levels. After washing at 0°C with PBS...
buffer, cells were detached from culture dishes and suspended at 0°C in 10 mM HEPES (pH 7.2), 142 mM KCl, 5 mM MgCl₂, 2 mM EGTA, and 0.5% (v/v) NP40 containing 0.1 μM phenylmethylsulfonyl fluoride and 10 μM/ml protease inhibitor cocktail from Calbiochem (lysis buffer). The suspensions were sonicated at 0°C, and nonsoluble material was removed by 1-hour centrifugation at 100,000 × g. The pull-down assays with S-protein agarose beads or anti e-myc agarose beads were performed at 4°C. Thirty microliters of suspended agarose beads was added to 0.5 ml of the 100,000 × g supernatant, which contained 0.5 mg of protein. The suspension was mixed for 1 hour by rotation. The beads were collected by centrifugation at 6000 × g and washed thrice with lysis buffer. Proteins bound to the S-protein agarose beads or the anti e-myc agarose beads were released from the beads by incubation for 5 minutes at 95°C in the presence of 1% (w/v) SDS. The proteins were analyzed by SDS-PAGE and Western blotting.

Coprecipitation of endogenously expressed hRS1-Reg with hODC1 was performed with membranes isolated from differentiated Caco-2 cells. Caco-2 cells in PBS containing protease inhibitors were disrupted by sonication on ice, and nuclei and cell organelles were removed by centrifugation at 1000 × g and 10,000 × g, respectively. The 10,000 × g supernatant (Fig. 2A, cleared lysate) was centrifuged for 1 hour at 100,000 × g (4°C). The 100,000 × g supernatant (Fig. 2A, cytosol) was removed, whereas the pellet (Fig. 2A, total cell membranes) was washed with PBS. After solubilization of the membranes in PBS containing 2% CHAPS (solubilization buffer), the solubilized membranes (Fig. 2B) were incubated for 1 hour at room temperature with an antibody against hODC1 raised in mouse (anti-hODC1-Ab, 1:5000). For precipitation of hODC1-protein complexes, anti-mouse IgG-agarose beads were added, and the suspension was incubated for 1 hour at room temperature. As a control, solubilization buffer containing anti-hODC1-Ab was incubated with anti-mouse IgG-agarose beads. The beads were isolated by low-speed centrifugation and washed with PBS. Bound proteins (Fig. 2B, Ag-eluate, Ag-eluate supernatant, which was centrifuged 15 minutes at 1000 × g) was washed thrice with lysis buffer. After solubilization of the membranes containing lipids, a middle phase mainly containing cytosolic material, and a lower phase mainly containing egg yolk. The middle phase was removed, diluted with 20 mM Tris-HCl (pH 7.5) and 10 mM EDTA, and centrifuged 15 minutes at 1000 × g. After this centrifugation, the middle phase was again removed, diluted, and centrifuged at 1000 × g. The middle phase obtained from this third centrifugation (called lipid-depleted homogenates) was used for determination of endogenous ODC activity.

Measurement of Enzymatic Activity of Ornithine Decarboxylase. ODC activity was determined by measuring the amount of 14CO₂ liberated from L-[1-14C]ornithine, as described (Milovic et al., 1998). The reaction was performed in closed reaction containers containing a needle with pierced filter papers inside the lid. The filter papers were soaked with 20 μl of 1 M benzenthionium hydroxide solution. One hundred microliters of 50 mM Tris-HCl (pH 7.2) containing 0.7 μM pyridoxal-5-phosphate and 10 ng of purified hODC1 or different amounts of lipid-depleted oocyte homogenate plus different concentrations of hRS1-Reg(S20A), hRS1-Reg(S20E), DFMO, and/or D-glucose was added to the bottom of the reaction container. To start the reaction, 16 μl of 50 mM Tris-HCl (pH 7.2) containing l-ornithine traced with 14C-labeled l-ornithine, 2.5 mM DTT, and 0.1 mM EGTA was added, and the container was closed. After 1-hour incubation at 37°C, the reaction was stopped by adding 200 μl of 0.6 N perchloric acid. After 30-minute incubation at 37°C, the filter papers of the reaction containers were transferred into scintillation vials containing 3 ml of Lumasafe scintillation cocktail (Luma LSC, Groningen, The Netherlands) plus 75 μl of 10% (v/v) acetic acid.

cRNA Synthesis. For cRNA synthesis, hSGTL1 in vector pBSSi SK (Vernaleken et al., 2007), hCNT1 in vector pBSSH KS (Errasti-Murugarren et al., 2012), hODC1 in vector pBSsp, and hSGTL1-YFP in vector pNB22 were used. To prepare sense cRNAs, the purified plasmids were linearized with EcoRI (hSGTL1), XbaI (hCNT1), MluI (hODC1), or NheI (hSGTL1-YFP). mG6/‘G-capped sense cRNAs were synthesized using T3 polymerase (hSGTL1, hCNT1), T7 polymerase (hSGTL1-YFP), or SP6 polymerase (hODC1). cRNAs were prepared using the mMESSAGE mMachine kit (Life Technologies).
Expression of hSGLT1, hSGLT1-YFP, hCNT1, and hODC1 and in Oocytes of *X. laevis*. Mature female *X. laevis* were anesthetized by immersion in water containing 0.1% 3-aminobenzoinoic acid ethyl ester. After partial ovarioectomy, stage V or VI oocytes were treated overnight with 1 mg ml⁻¹ collagenase I in Ori buffer [5 mM HEPES (pH 7.6), 110 mM NaCl, 3 mM KC1, 1 mM MgCl₂, and 2 mM CaCl₂]. The oocytes were washed with Ca²⁺-free Ori buffer and kept at 16°C in modified Barth’s solution [15 mM HEPES (pH 7.6), 88 mM NaCl, 1 mM KC1, 0.3 mM Ca(NO₃)₂, 0.4 mM CaCl₂, and 0.8 mM MgSO₄] containing 12.5 μM gatmycin. Selected oocytes were injected with 50 nl of water containing cRNAs (10 ng of hSGLT1, 25 ng hSGLT1-YFP, 10 ng of hCNT1, and/or 5 ng of hODC1). For expression, oocytes were incubated for 2 or 3 days at 16°C in modified Barth’s solution with gentamycin. Noninjected oocytes (tracer uptake measurements) or water-injected oocytes (electrical measurements) were incubated in parallel.

### Injection of Peptides, AMG, and Biochemicals into Oocytes

Two or 3 days after injection of transporter cRNAs into oocytes, DFP0, putrescine, AMG, hRS1-Reg, and/or BFA were injected. We injected 40 nl of K-Ori buffer containing 1.2 nmol DFP0, 0.4 pmol putrescine, 1.4 pmol hRS1-Reg, 1.25 nmol AMG, and/or 5 pmol BFA. hSGLT1-mediated uptake of [¹⁴C]AMG, hCNT1-mediated uptake of [³H]uridine, and hSGLT1-YFP-mediated glucose-induced currents were measured 1 hour later. Intracellular concentrations of injected compounds were estimated by assuming an internal distribution volume of 0.4 μl (Zeuthen et al., 2002).

### Measurements of AM or Uridine Uptake in Oocytes

hSGLT1-mediated AM uptake was determined by correcting AM uptake in hSGLT1-expressing oocytes for AM uptake measured in non-cRNA-injected oocytes, which were handled in parallel. Oocytes were incubated for 20 minutes at room temperature in Ori buffer containing 25 μM AMG traced with [¹⁴C]AMG. Thereafter, the oocytes were washed 4 times with ice-cold Ori buffer containing 1 mM phlorizin. hCNT1-mediated uridine uptake was determined by measuring the difference in uridine uptake between oocytes in which hCNT1 was expressed by cRNA injection and non-cRNA-injected oocytes. Oocytes were incubated for 20 minutes at room temperature with Ori buffer containing 5 μM uridine traced with [³H]uridine and washed 4 times with ice-cold Ori buffer. Single oocytes were solubilized in 5% (w/v) SDS and analyzed for radioactivity by scintillation counting.

### Measurement of hSGLT1-YFP-Mediated Glucose-Induced Short-Circuit Current in Oocytes

For measurement of glucose-induced short-circuit currents in the two-electrode voltage-clamp mode, non-cRNA-injected control oocytes and hSGLT1-YFP-expressing oocytes were superfused at room temperature with 10 mM citrate-Tris (pH 5.0) containing 30 mM potassium gluconate, 1 mM LaCl₃, 1 mM CaCl₂ plus 170 mM sorbitol (buffer without glucose); the membrane potential was clamped to −50 mV, and the steady-state short-circuit current was measured. Superfusion was switched to buffer with glucose in which 100 mM sorbitol was replaced by 100 mM D-glucose, and steady-state short-circuit current at −50 mV was measured again. Glucose-induced currents were determined by subtracting steady-state current in the absence glucose from steady-state current in the presence of 100 mM D-glucose. In non-cRNA-injected control oocytes, no significant glucose-induced currents were observed.

### Quantification of Fluorescence Associated with Oocyte Plasma Membranes

Fluorescence intensity associated with the plasma membrane of hSGLT1-YFP-expressing oocytes was measured with a confocal laser-scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) equipped with a Leica HCX IRAPO L25x/095W objective (excitation 514 nm, detection 528–580 nm). The optical plane was set to the equator of the oocyte, and the settings of YFP fluorescence acquisition (laser intensity and gain of photomultiplier tubes) were kept constant for all tested oocytes. YFP fluorescence intensity of a quarter circular segment per oocyte was measured using the LAS AF software from Leica.

### Incubation of Caco-2 Cells with DFO and/or hRS1-Reg(S20E) Coupled to Nanohydrogel

Monolayers of differentiated Caco-2 cells were incubated for 30 minutes at 37°C with fetal calf serum-free minimal essential medium with or without the addition of 5 mM DFO, 0.25 mg/ml nanohydrogel, 2.5 ng hRS1-Reg(S20E) linked to 0.25 mg/ml nanohydrogel or DFO plus hRS1-Reg(S20E) linked to nanohydrogel. Nanohydrogels were synthesized, and hRS1-Reg(S20E) was coupled as described (Veyhl-Wichmann et al., 2016) with the exception that alloxan was used for oxidation and hydroxethylacrylate for quenching of free thiol groups (Singh et al., 2013).

### Transport Measurements in Caco-2 Cells

Phlorizin-inhibited AMG uptake into Caco-2 cells was measured as described (Kipp et al., 2003). Confluent monolayers of Caco-2 cells were washed three times at 37°C with Heps buffer [10 mM Hepes (pH 7.2), 137 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂] and incubated for 10 minutes at 37°C with Hepes buffer containing 0.7 μM [¹⁴C]AMG or 0.7 μM [¹³C]AMG plus 1 mM phlorizin. AMG uptake was stopped with ice-cold Hepes buffer containing 1 mM phlorizin, and the monolayers were washed with the same buffer. Cells were solubilized with 2% (w/v) SDS, and radioactivity was determined by scintillation counting. The difference between AMG uptake in the absence and presence of phlorizin was calculated.

### Statistics and Fitting Procedures

In the SPR experiments, *kₐ* and *kₘₐₐ₅₉* rate constants were obtained by fitting the experimental sensograms of individual experiments using a simple 1:1 Langmuir-type interaction model. All chi square values for the fitted data were less than 10% of the maximal responses. Half-maximal concentration values for inhibition of ODC activities (IC₅₀) (Fig. 5, A and C) were determined by fitting the Hill equation to the data. *Kₐ* values (Fig. 5B) were calculated by fitting the Michaelis-Menten equation to the data. The half-maximal effective concentration (EC₅₀) for D-glucose–induced protection of ODC activity from inhibition by DFO (Fig. 5D) was determined by fitting a one-site binding model to the data. Binding constants (*Kₐ*, *Kₐ*, *Kₐ*), *Kₐ*, values, IC₅₀, and EC₅₀ for glucose activation are presented as means ± S.E., which were obtained by fitting data from individual experiments. In experiments with oocytes (Figs. 6–8), mean values ± S.E. are indicated. When three or more experimental conditions were compared, the significance of differences was determined by analysis of variance using post hoc Tukey comparison. Significance of differences of two experimental conditions was determined by Student’s *t* test. *P* < 0.05 was considered significant.

### Results

**Ornithine Decarboxylase Binds to an N-terminal Regulatory Domain of hRS1 (RSC1AI).** We screened a cDNA library from human embryonic kidney for proteins that interact with a polypeptide comprising amino acids 2–312 of hRS1 [hRS1(2-312)] using the two-hybrid split ubiquitin system (Johnsson and Varshavsky, 1994). The identified proteins are indicated in Supplemental Table 1. We characterized the interaction of hRS1(2-312) with hODC1 (GenBank, NP_002530.1) (Kahana and Nathans, 1984) in detail because effects of ODC on SGLT1 activity have been reported (Johnson et al., 1995; Uda et al., 2002).

For coprecipitation of overexpressed proteins, GFP containing an S-tag fused to the N terminus of hRS1(2-312) [GFP-hRS1(2-312)] was expressed in HEK293 cells together with hODC1 containing a C-terminal myc-tag (hODC1-myc). In cell lysates, either GFP-S-hRS1(2-312) was precipitated by S-tag binding to S-protein agarose and coprecipitation of hODC1-myc was demonstrated using an antibody against myc (Fig. 1A), or...
vesicles at the TGN, and that this effect is mediated by hRS1-Reg comprising amino acids 16–98 of hRS1 (Veyhl-Wichmann et al., 2016). To determine whether hODC1 binds to hRS1-Reg, we expressed either GFP-S-hRS1(2-98) or GFP-S together with hODC1-myc, precipitated ODC1-myc with agarsose-coupled anti-myc antibodies, and investigated whether GFP-S or GFP-S-hRS1(2-98) was coprecipitated (Fig. 1C). In contrast to GFP-S, GFP-S-hRS1(2-98) was coprecipitated, suggesting that hODC1 binds to hRS1-Reg.

Differentiated Caco-2 cells, which represent an established model for human enterocytes and express SGLT1 (Kipp et al., 2003), were used to demonstrate the association between endogenously expressed, membrane-bound hODC1 and hRS1. To validate hRS1 expression in Caco-2 cells, we performed Western blots with a nuclei- and mitochondria-free cell lysate, a cytosolic fraction, and a total cell membrane fraction using antibody against full-length hRS1 (anti-hRS1-Ab) (Fig. 2A). In the cell lysate and the cytosol, protein bands with apparent Mr values of 50,000, 31,000, 20,000, 17,000 were stained in addition to a 68,000 Mr band representing full-length hRS1 (Fig. 2A). It is noteworthy that anti-hRS1-Ab only stained the 31,000 Mr protein in the total cell membrane fraction. When the Western blots were stained with an antibody directed against a peptide within hRS1-Reg (hRS1-Reg-P-Ag), the 68,000, 31,000, and 20,000 bands were stained in the cell lysate and the cytosol, whereas in the total cell membrane fraction, only the 31,000 band was stained (Fig. 2A). The data support our interpretation that RS1-Reg is associated with Golgi membranes (Veyhl-Wichmann et al., 2016). To investigate whether the 31,000 hRS1 fragment forms a complex with hODC1, we incubated solubilized total cell membranes with a monoclonal antibody against hODC1 and precipitated hODC1 with agarsose beads containing a covalently linked antibody against mouse IgG (Fig. 2B). We observed coprecipitation of the 31,000 Mr hRS1-Reg fragment comprising hRS1-Reg with hODC1, indicating in vivo association of membrane-bound hODC1 with hRS1-Reg.

**Demonstration and Characterization of hRS1-Reg Binding to Ornithine Decarboxylase by Surface Plasmon Resonance.** To verify and characterize the interaction of hRS1-Reg with hODC1, we performed SPR using purified recombinant hODC1 as immobilized ligand and the purified recombinant hRS1-Reg variant proteins hRS1-Reg(S20E) and hRS1-Reg(S20A) as analytes. In these variants, phosphorylation at a predicted phosphorylation site is mimicked or prevented by replacement of serine with glutamate or alanine. The peptide hRS1-Reg(S20E) downregulated hSGLT1 expressed in X. laevis oocytes with a 3500-fold lower EC₅₀ than hRS1-Reg(S20A) (Veyhl-Wichmann et al., 2016). We also investigated whether the glucose-induced 19,000-fold decrease of the EC₅₀ of hRS1-Reg(S20E) observed for downregulation of hSGLT1 in oocytes (Veyhl-Wichmann et al., 2016) is reflected by a decreased equilibrium dissociation constant (Kₑ) value for binding of hRS1-Reg(S20E) to hODC1. Finally, we determined whether covalent modification of hODC1 within the substrate binding site by DFMO, which blocks ODC activity (Grishin et al., 1999), alters binding of hRS1-Reg(S20E).

Human ODC1 (Matsuzawa et al., 2005), the RS1-Reg peptides, and a control peptide comprising amino acids 150–312 of hRS1 were expressed in E. coli and purified by affinity chromatography. SDS-PAGE analysis showed that purified hODC1 migrates as a single band with an expected molecular weight.

**Fig. 1.** Coprecipitation of N-terminal fragments of hRS1 with human ODC1 in HEK293 cells with overexpressed proteins. GFP-S, GFP-S-hRS1(2-312), and GFP-S-hRS1(2-98) were expressed in HEK293 cells together with hODC1-myc. Cells were lysed, cell debris was removed, and either GFP-S was precipitated with S-protein agarose (S-protein-Ag) or hODC1-myc was precipitated with an anti-myc antibodies coupled to agarose (anti-myc-Ab-Ag). The precipitated agarose beads were washed, and bound proteins were released by SDS and analyzed in Western blots using an antibody against myc (anti-myc-Ab) or an antibody against GFP (anti-GFP-Ab). (A) Coprecipitation of hODC1-myc with GFP-S-hRS1(2-312) using GFP-S as control. hODC1-myc in the SDS-eluted proteins is stained. (B) Coprecipitation of GFP-S-hRS1(2-312) with hODC1-myc using GFP-S as control. GFP-S-hRS1(2-312) in SDS-eluted proteins is stained. (C) Coprecipitation of GFP-S-hRS1(2-98) with hODC1-myc using GFP-S as control. GFP-S-hRS1(2-98) in SDS-eluted proteins is stained.
mass of 54 kDa (Pritchard et al., 1982) (Fig. 3A). For the specific enzymatic activity of purified recombinant hODC1 measured at 37°C in the presence of 8 μM ornithine and 0.7 μM cosubstrate pyridoxal-5-phosphate and 0.34 mM DTT, a value of 2.9 ± 0.5 mol CO₂/mg protein/h (mean value ± S.D., n = 3) was obtained. During SDS-PAGE performed in the presence of DTT, the purified hRS1-Reg mutants migrate at about 10 kDa, representing monomers, whereas the control peptide migrates at about 25 kDa (Fig. 3B). Due to the presence of cysteine residues, the hRS1-Reg peptides dimerize in the absence of reducing agents (Fig. 3B); therefore, the SPR measurements were performed in the presence of DTT. For SPR analysis, purified hODC1 was immobilized on a sensor chip. The chip was superfused with running buffer (25°C, pH 7.4) containing 150 mM NaCl, 3.5 mM EDTA, 0.005% (v/v) Tween 20, 1 mM DTT, and different concentrations of analyzed peptides. For measurements in the presence of glucose or DFMO, either the running buffer was supplemented with 1 mM α-glucose or the immobilized hODC1 was pretreated with 1 mM DFMO.

Both peptide variants, hRS1-Reg(S20A) and hRS1-Reg(S20E), bind with high affinity to hODC1 (Fig. 4; Table 1). For both variants, similar association rate constant (kₐ) values, similar dissociation rate constant (kₐ) values, and similar equilibrium binding dissociation constant (K_D) values were observed. The constants determined for hRS1-Reg(S20E) were not significantly altered by α-glucose, and the constants obtained for hRS1-Reg(S20A) were also not significantly changed after modification of hODC1 with the covalently binding inhibitor DFMO (Table 1). The kₐ values ranged between 0.5 and 0.9 × 10⁶ M⁻¹ s⁻¹, the kₐ values ranged between 0.6 and 0.9 × 10⁻³ s⁻¹, and the deduced K_D values ranged between 68 and 161 nM (Table 1). The K_D value for the hRS1-Reg(S20A)–hODC1 interaction (68 ± 20 nM) was similar to the EC₅₀ determined for downregulation of hSGLT1 in oocytes (48 ± 8 nM) (Veyhl-Wichmann et al., 2016). In contrast, the K_D value determined for the hRS1-Reg(S20E)–hODC1 interaction (102 ± 25 nM) was 5000 times higher than the EC₅₀ value (19 ± 0.2 pM) measured for downregulation of hSGLT1 in oocytes (Veyhl-Wichmann et al., 2016). Furthermore, in contrast to the highly differing EC₅₀ values obtained for downregulation of hSGTL1 in the absence and presence of the glucose analog AMG (Veyhl-Wichmann et al., 2016), the binding affinities for the peptide-hODC1 interaction were not influenced significantly by glucose.

**Demonstration and Characterization of Inhibition of ODC Activity by hRS1-Reg.** Next, we investigated whether binding of the hRS1-Reg peptides to hODC1 influences the enzymatic activity. We measured the CO₂ generation when
10 ng of purified recombinant hODC1 was incubated with hRS1-Reg(S20A) or hRS1-Reg(S20E) in the presence of 8 μMornithine and 0.7 μMcosubstrate pyridoxal-5-phosphate (Fig. 5A). In the presence of 10 μMhRS1-Reg(S20A) or hRS1-Reg(S20E), the enzymatic activity was inhibited by 66 or 79%, respectively. For hRS1-Reg(S20A), an IC50 of 1.10 ± 0.19 μM was calculated, whereas for hRS1-Reg(S20E), an IC50 value of 0.27 ± 0.04 μM was determined (mean values ± S.D., n = 3 each, P < 0.001 for difference). The IC50 values obtained for inhibition of hODC1 by hRS1-Reg are 16-fold or 2.6-fold higher compared with the KD values determined for peptide binding to hODC1 measured by SPR in the absence of substrate and cosubstrate. The lower IC50 value of hRS1-Reg(S20E) for inhibition of ODC activity compared with hRS1-Reg(S20A) correlates with the lower EC50 value of hRS1-Reg(S20E) versus hRS1-Reg(S20A) observed for downregulation of hSGLT1 in oocytes (Veyhl-Wichmann et al., 2016). This suggests that inhibition of hODC1 by hRS1-Reg is critically involved in hRS1-Reg–mediated downregulation of hSGLT in the plasma membrane.

Previously, we observed that the EC50 of hRS1-Reg(S20E) for downregulation of SGLT1 in oocytes was 19,000-fold decreased in the presence of nonmetabolizable glucose analog AMG (Veyhl-Wichmann et al., 2016). Measuring the inhibition of the enzymatic activity of purified recombinant hODC1 by hRS1-Reg(S20E) in the presence of 1 mM D-glucose (Fig. 5A), we observed a 3-fold lower IC50 value compared with the absence of glucose (absence of glucose: 0.27 ± 0.04 μM, 1 mM D-glucose: 0.084 ± 0.014 μM; mean value ± S.D., n = 3 each, P < 0.01). Thus, glucose has a qualitatively similar but quantitatively much smaller effect on IC50 for inhibition of hODC1 activity compared with the EC50 for downregulation of hSGLT1.

ODC is endogenously expressed in X. laevis oocytes (Osborne et al., 1989). In lipid-depleted homogenates prepared from oocytes, we measured a specific ODC activity of 83 ± 17 pmol CO2 × mg protein −1 × h−1 (mean value ± S.D., n = 3). To determine whether the observed differences between IC50 values obtained with hODC1 and the EC50 values for downregulation of SGLT1 in Xenopus oocytes are due to different properties of recombinant hODC1 versus endogenous ODC of...
X. laevis, we measured inhibition of ODC activity by hRS1-Reg(S20E) in the lipid-depleted oocyte homogenates in the absence and presence of 1 mM D-glucose. The IC$_{50}$ values determined in oocyte homogenates were in the same range of magnitude as the values obtained with purified hODC1, indicating similar efficacy for interaction of hRS1-Reg(S20E) with hODC1 and ODC in oocytes (absence of glucose: 0.66 ± 0.13 μM; 1 mM AMG: 0.23 ± 0.05 μM; mean values ± S.D., n = 3 each, P < 0.01). In oocyte homogenates, a similar glucose-induced increase of efficacy for inhibition by hRS1-Reg(S20E) was observed as with hODC1.

**d-Glucose Decreases Efficacy of DFMO for Inhibition of Enzymatic Reaction.** We measured the substrate dependence of the enzymatic activity of purified hODC1 with and without 1 mM D-glucose. Under both conditions, Michaelis-Menten--type kinetics with similar $K_m$ and $V_{max}$ values were obtained (Fig. 5B). In the absence and presence of glucose, $K_m$ values of 0.27 ± 0.06 and 0.26 ± 0.06 mM and $V_{max}$ values of 4.0 ± 0.8 and 4.1 ± 0.3 μmol CO$_2$ × mg protein$^{-1}$ × h$^{-1}$ (mean values ± S.D., n = 3 each) were determined, respectively. We also measured the inhibition of hODC1 activity by DFMO in the absence or presence of 1 mM D-glucose using 0.1 μg of purified recombinant hODC1 × ml$^{-1}$ and a substrate concentration of 8 μM ornithine (Fig. 5C). In the absence of glucose, 0.1 mM DFMO inhibited 95% of the enzymatic activity of hODC1. For inhibition of hODC1 in the presence of 1 mM D-glucose, a 2.6-fold higher IC$_{50}$ value was obtained, as in the absence of glucose (12.6 ± 2.0 vs. 32.8 ± 6.8 μM; mean values ± S.D., n = 3 each, P < 0.01). For DFMO inhibition of endogenous ODC activity in lipid-depleted oocyte homogenates measured in the absence of glucose using 1 mg protein × ml$^{-1}$ for the enzymatic assay, 1 mM DFMO was required to obtain 90% inhibition. Similar to the glucose effect on DFMO inhibition of purified hODC1 (Fig. 5C), a significantly higher IC$_{50}$ value for inhibition by DFMO was obtained in the presence of 1 mM AMG compared with the absence of glucose (432 ± 79 vs. 118 ± 13 μM, mean values ± S.D., n = 3 each, P < 0.01). The IC$_{50}$ values for DFMO inhibition of ODC in lipid-depleted oocyte homogenates were 9-fold higher compared with the IC$_{50}$ values measured with purified hODC1. Because the apparent IC$_{50}$ values determined in lipid-depleted oocyte homogenate compared with the IC$_{50}$ of purified hODC1 is probably due to a reduced concentration of free DFMO.

**Fig. 5.** Effects of hRS1-Reg mutants and DFMO in the absence and presence of glucose on hODC1 activity. CO$_2$ liberation after addition of ornithine by purified recombinant hODC1 was measured at 37°C in the presence of 0.7 μM pyridoxal-5-phosphate, 0.34 mM DTT, hRS1-Reg(S20A), hRS1-Reg(S20E), DFMO, and/or D-glucose. (A) Effects of hRS1-Reg(S20A) and hRS1-Reg(S20E) in the absence of glucose and of hRS1-Reg(S20E) in the presence of 1 mM D-glucose on the enzymatic activity of ODC at 8 μM ornithine. (B) Substrate dependence of hODC1-mediated ODC activity in the absence and presence of 1 mM D-glucose. (C) Concentration dependence of the inhibition of hODC1-mediated CO$_2$ liberation in the presence of 8 μM ornithine by DFMO in the absence and presence of 1 mM D-glucose. (D) Glucose dependence for glucose protection of inhibition of hODC1 by DFMO. Inhibition of hODC1-mediated CO$_2$ liberation in the presence of 8 μM ornithine by 12.5 μM DFMO was measured in the presence of different concentrations of D-glucose, and the protective effect of D-glucose on inhibition was calculated. Mean values ± S.E. of nine measurements from three independent experiments are shown. The indicated curves were obtained by fitting the Hill equation (A and C), the Michaelis-Menten equation (B), or a one-site binding model (D) to the compiled data sets.

**TABLE 1**

Kinetic rate and equilibrium binding constant values which were determined by SPR for the interaction of hRS1-Reg(S20A) and hRS1-Reg(S20E) with immobilized hODC1.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>$k_{on}$ (×10$^4$ M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (×10$^{-4}$ s$^{-1}$)</th>
<th>$K_{D}$ (μM)</th>
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<tbody>
<tr>
<td>hRS1-Reg(S20A)</td>
<td>0.9 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>68 ± 20</td>
</tr>
<tr>
<td>hRS1-Reg(S20E)</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.4</td>
<td>102 ± 25</td>
</tr>
<tr>
<td>hRS1-Reg(S20E) with 1 mM D-glucose</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>161 ± 35</td>
</tr>
<tr>
<td>hRS1-Reg(S20A) with 1 mM DFMO</td>
<td>0.9 ± 0.7</td>
<td>0.6 ± 0.4</td>
<td>85 ± 12</td>
</tr>
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in oocyte homogenates due to nonspecific binding of DFMO to proteins and/or lipids. We observed that 3 mM DFMO was required to inhibit ODC activity up to 70% in lipid-depleted oocyte homogenates when the assay was performed in the presence of 10 mg protein × ml⁻¹. Also under this condition, ODC inhibition was reduced significantly by addition of 1 mM AMG (data not shown).

To characterize the interaction of glucose with hODC1, we measured the inhibition of purified hODC1 by 12.5 µM DFMO in the presence of different glucose concentrations (Fig. 5D). A saturable protective effect of glucose with an EC₅₀ of 0.28 ± 0.09 mM (mean value ± S.D., n = 3) was observed. The data indicate that ODC contains a glucose binding site.

**Effect of ODC Activity on the Expression of hSGLT1-Mediated Glucose Transport Expressed in Oocytes.** We investigated whether hSGLT1-mediated AMG uptake was increased after coexpression of hODC1 or decreased after inhibition of endogenous ODC activity by DFMO. After coexpression of hODC1 with hSGT1 in oocytes, the hSGLT1-mediated uptake of 25 µM AMG was increased by 40% (Fig. 6A). We then investigated whether the endogenous ODC activity in oocytes has an effect on hSGLT1-mediated AMG uptake. We expressed hSGLT1 by injection of hSGLT1-cRNA into oocytes and incubation for 2 days, inhibited ODC activity by injection of 3 mM DFMO, and measured AMG uptake 1 hour later. DFMO inhibited hSGLT1-mediated AMG uptake by 50% (Fig. 6, B and C). Importantly, the inhibition by DFMO could be counteracted when 1 µM putrescine, the product of ODC-mediated decarboxylation of ornithine, or 1 mM AMG was coinjected with DFMO (Fig. 6C). The data suggest that SGLT1 is upregulated in response to ODC-mediated generation of putrescine, and that the upregulation is blunted by glucose binding to ODC.

To elucidate whether ODC influences an exocytotic pathway of hSGLT1 at the TGN, similar to hRS1-Reg (Veyhl-Wichmann et al., 2016), we investigated whether the inhibition of hSGT1-mediated AMG uptake by DFMO is dependent on Golgi integrity (Fig. 6B). One hour after injection of 12.5 µM BFA into hSGLT1-expressing oocytes, hSGLT1-mediated AMG uptake was inhibited by 40–50%, as described earlier (Veyhl et al., 2006; Veyhl-Wichmann et al., 2016). No further inhibition was observed when 3 mM DFMO was injected together with BFA.

**hRS1-Reg Blocks the Exocytotic Pathway of SGLT1 by Inhibiting the Enzymatic Activity of ODC.** To determine whether the post-transcriptional short-term regulation of hSGLT1 by ODC and hRS1-Reg is mediated by the same regulatory pathway, we investigated whether down-regulation of hSGLT1 by inhibition of ODC and by hRS1-Reg is synergistic. Injection of hRS1-Reg or DFMO into hSGLT1-expressing oocytes inhibited AMG uptake to a similar degree of 40–50%, and the inhibition was not further increased upon coinjection of hRS1-Reg and DFMO (Fig. 7A). To determine whether hRS1-Reg downregulates hSGLT1 via ODC inhibition, we investigated whether hRS1-Reg–mediated downregulation of AMG uptake could be prevented when putrescine was supplemented. Inhibition of hSGLT1-mediated AMG uptake by hRS1-Reg was indeed blunted when 1 µM putrescine was injected (Fig. 7B). The data suggest that downregulation of hSGLT1 by hRS1-Reg is mediated via inhibition of ODC activity. Because hRS1-Reg inhibits the exocytotic pathway of the Na⁺-nucleoside cotransporter hCNT1 at the Golgi independently of glucose (Veyhl-Wichmann et al., 2016), we investigated whether ODC activity is also involved in short-term regulation of CNT1. In oocytes expressing hCNT1, sodium-dependent uptake of 5 µM [³H]uridine was not inhibited by DFMO, whereas it was downregulated by hRS1-Reg (Fig. 7C).

**DFMO and hRS1-Reg(S20E) Decrease Plasma Membrane Abundance of hSGLT1 Expressed in Oocytes.** Based on supporting experimental evidence derived from different experimental approaches, we concluded in a recent study that hRS1-Reg downregulates plasma membrane abundance of hSGLT1 (Veyhl-Wichmann et al., 2016). The data described in the present manuscript suggesting that hODC1 is part of the hRS1-Reg–modulated exocytotic pathway also indicate that DFMO decreases the amount of SGLT1 in the plasma...
membrane. To demonstrate downregulation of hSGLT1 in the plasma membrane by hRS1-Reg and DFMO directly, we expressed an hSGLT1-YFP fusion protein in oocytes and injected DFMO, hRS1-Reg(S20E), or DFMO plus hRS1-Reg(S20E). After 1 hour, we measured short-circuit currents at 250 mV that were induced by a saturating D-glucose concentration and determined the concentrations of YFP-hSGLT1 associated with the plasma membrane by measuring YFP fluorescence (Fig. 8). After injection of hRS1-Reg(S20E), DFMO, or hRS1-Reg(S20E) plus DFMO, the glucose-induced currents were decreased 40–45%, whereas the membrane-associated fluorescence measured was decreased 30–36%. The data indicate nonadditive downregulation of SGLT1 abundance in the plasma membrane by hRS1-Reg(S20E) and DFMO. The slightly smaller effects on fluorescence compared with glucose-induced currents may be due to some plasma membrane-associated transporter that is not functional.

**DFMO and hRS1-Reg(S20E) Decrease Phlorizin-Inhibited AMG Uptake in Caco-2 Cells.** To evaluate the physiologic relevance of RS1/ODC-regulated membrane trafficking of SGLT1 in the small intestine, we investigated the effects of DFMO and hRS1-Reg(S20E) on transport function of hSGLT1 in differentiated Caco-2 cells. Differentiated Caco-2 cells grown in the presence of 1 mM D-glucose were incubated for 30 minutes at 37°C with 0.25 mg/ml nanohydrogel, 0.25 ng/ml hRS1-Reg(S20E), or 5 mM DFMO or hRS1-Reg(S20E) linked to 5 mM DFMO. The used concentration of nanohydrogel coupled with hRS1-Reg(S20E) was optimized to induce a maximal decrease of phlorizin-inhibited AMG uptake after 30-minute incubation. After washing of the incubated cells, phlorizin-inhibited AMG uptake was measured (Fig. 9). DFMO, hRS1-Reg(S20E), and DFMO plus hRS1-Reg(S20E) decreased phlorizin-inhibited AMG uptake by similar degrees. The
downregulation of transport by DFMO and hRS1-Reg(S20E) was not additive. The data suggest in vivo relevance of RS1/ODC-mediated short-term regulation of SGLT1 in the small intestine.

Discussion

Previously, we reported that differentially phosphorylated forms of RS1-Reg induce blockage of the release of vesicles that contain either SGLT1 or CNT1 from the TGN, and that this regulation alters transporter activity in the plasma membrane within minutes (Veyhl-Wichmann et al., 2016). We showed that short-term downregulation of SGLT1 by RS1-Reg is glucose-dependent. On the basis of these data, the hypothesis was raised that differently phosphorylated forms of RS1-Reg bind to different receptor proteins at the TGN, which steer release of different vesicle populations.

In the present work, we provide evidence that hODC1 is the receptor protein for hRS1-Reg that controls the exocytotic pathway of SGLT1. We showed that an N-terminal hRS1 fragment containing hRS1-Reg that is associated with membranes interacts with membrane-bound hODC1. SPR analysis of purified recombinant hODC1 with hRS1-Reg variants, in which phosphorylation of a motif that is critical for the efficacy of RS1-Reg bind to different receptor proteins at the TGN, which steer release of different vesicle populations.

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![Fig. 9](image)

**Fig. 9.** Nonadditive downregulation of phlorizin-inhibited AMG uptake in Caco-2 cells by DFMO and hRS1-Reg. Caco-2 cells that had been cultivated for 18 days in medium containing 1 mM glucose were incubated for 30 minutes at 37°C with medium, medium containing unloaded nanohydrogel (gel), medium containing hRS1-Reg(S20E) linked to nanohydrogel [gel-hRS1-Reg(S20E)], medium with 5 mM DFMO or medium containing gel-hRS1-Reg(S20E) plus DFMO. After washing, the monolayers were incubated for 10 minutes (37°C) in the presence of sodium with 0.7 μM [14C]AMG in the absence of phlorizin, or in the presence of 1 mM phlorizin. Phlorizin-inhibited uptake was calculated. Mean values ± S.D from six measurements performed in two independent experiments are shown. ***p < 0.001 for difference to medium control, analysis of variance with post hoc Tukey comparison.
are regulated on transcriptional, translational, and post-translational levels (Pegg, 2006). ODC is a pyridoxal-5-phosphate–dependent amino acid decarboxylase which is functionally active as a homodimer. Reglations of proteasomal degradation of ODC and of the equilibrium between functionally active dimers and inactive monomers have been described. Proteins called antizymes (AZ1, AZ2, and AZ3) and antizyme inhibitors (AZIN1 and AZIN2) are involved (Mangold and Leberer, 2005; Pegg, 2006). Antizyme binds to ODC monomers and thereby decreases the number of functionally active ODC dimers. Antizyme–ODC monomer complexes are directed to the 26S proteasome, where ODC is degraded independent of ubiquitinylation, whereas antizyme is recycled (Coleman et al., 1994; Hayashi and Murakami, 1995; Mangold, 2005). Antizyme inhibitor is highly homologous to ODC but has no enzymatic activity (Murakami et al., 1996; Mangold and Leberer, 2005). It binds tightly to antizyme and thereby prevents its association with ODC monomers. This leads to an increase of functionally active ODC dimers.

Previous data have suggested mutual interrelations between ODC and SGLT1. For instance, it has been described that polyamines influence the expression and membrane abundance of SGLT1 during cell differentiation of LLC-PK1 cells (Peng and Lever, 1993; Wild et al., 2007). On the other hand, it has been reported that AMG stimulates ODC mRNA expression in LLC-PK1 cells (Benis and Lundgren, 1993). In the small intestine, post-translational regulation of SGLT1 by ODC and/or polyamines has been described. One study reported that glucose uptake into brush-border membrane vesicles isolated from rabbit small intestine was increased independently from protein synthesis when the animals had received polyamines with drinking water 24 hours earlier, whereas glucose uptake was decreased upon application of DFMO (Johnson et al., 1995). Rapid post-translational upregulation of SGLT1 in the brush-border membrane was observed 15 minutes after luminal application of polyamines (Uda et al., 2002). This effect might be due to RS1/ODC-mediated regulation of SGLT1 at the TGN as described in the present report.

On the basis of our data, we propose a mechanism for this regulation as depicted in Fig. 10. A population of ODC is located or activated at or close to TGN regions where budding of SGLT1-containing vesicles occurs. Dynamin and caveolin may be involved in budding, because RS1-dependent regulation of the exocytic pathway of SGLT1 is dynamin-dependent (Veyhl et al., 2003), and SGLT1 colocalizes with dynamin at the TGN (Kroiss et al., 2006). In addition, a caveolin-dependent exocytic pathway of SGLT1, which can be inhibited by BFA, has been described (Elvira et al., 2013). After activation of ODC, the local concentration of putrescine is increased. On the basis of our data, we hypothesize that the release of SGLT1-containing vesicles from the Golgi is activated by putrescine and/or other polyamines (Fig. 10). Because the intracellular concentration of free polyamines is very low in nondividing cells (Shin et al., 2006), the local generation of putrescine by ODC allows a local activation of polyamine-dependent processes. Due to the widespread intracellular distribution of ODC protein, it is difficult to distinguish specific subcellular ODC locations. This may be the reason why the location of ODC at the TGN has not yet been identified (Schipper et al., 2004). However, it has been shown that AZIN2 is located at the TGN, and data have been presented suggesting that AZIN2 acts as regulator of vesicle trafficking via activation of ODC (Parkkinen et al., 1997; Kanerva et al., 2010). In humans, a high mRNA expression of AZIN2 mRNA has been observed in brain and testis, whereas only minor expression was detected in kidney and the gastrointestinal tract (Pitkänen et al., 2001). Performing reverse-transcription PCR with mouse tissues, we showed that AZIN2 is also expressed in the duodenum and jejunum (C. Chintalapati and H. Koepsell, unpublished data). After selective activation of RS1-Reg by protein kinases, binding of RS1 to ODC blocks the enzymatic activity of ODC, which leads to decreased release of SGLT1-containing vesicles from the TGN (Fig. 10). At high intracellular concentrations of glucose, glucose binds to ODC. This possibly leads to a conformational change, which decreases the affinity of RS1 to ODC and blunts the inhibitory effect of RS1-Reg binding on ODC activity.

Our data show that ODC mediates release from the TGN; however, it remains a challenge to determine the specific functional properties of ODC at the TGN and to elucidate how a local increase of polyamines at the TGN induces vesicle release. The identification of a new functional role of ODC and a high-affinity binding site for hRS1-Reg in ODC, where inhibition of enzymatic activity can be induced, has provided a new pharmacological target for medical interventions. For example, RS1-Reg–derived ODC inhibitors that downregulate SGLT1-mediated glucose uptake in the small intestine may be useful for the treatment of diabetes (Powell et al., 2013; Song et al., 2016).
Short-Term Regulation of SGLT1 by RS1 via Inhibition of ODC


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