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Role of the Transporter Regulator Protein (RS1) in the Modulation of Concentrative Nucleoside Transporters (CNTs) in Epithelia[S]

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ABSTRACT

SLC28 genes encode three plasma membrane transporter proteins, human concentrative nucleoside transporter (CNT)1, CNT2, and CNT3, all of which are implicated in the uptake of natural nucleosides and a variety of nucleoside analogs used in the chemotherapy of cancer and viral and inflammatory diseases. Mechanisms determining their trafficking toward the plasma membrane are not well known, although this might eventually become a target for therapeutic intervention. The transporter regulator RS1, which was initially identified as a short-term, post-transcriptional regulator of the high-affinity, Na^+-coupled, glucose transporter sodium-dependent glucose cotransporter 1, was evaluated in this study as a candidate for coordinate regulation of membrane insertion of human CNT-type proteins. With a combination of studies with mammalian cells, *Xenopus laevis* oocytes, and RS1-null mice, evidence that RS1 down-regulates the localization and activity at the plasma membrane of the three members of this protein family (CNT1, CNT2, and CNT3) is provided, which indicates the biochemical basis for coordinate regulation of nucleoside uptake ability in epithelia and probably in other RS1-expressing cell types.

Introduction

SLC28 genes encode CNT1, CNT2, and CNT3 proteins. CNTs mediate the translocation of nucleosides and nucleoside-derived drugs across the plasma membrane of cells (Huber-Ruano and Pastor-Anglada, 2009; Errasti-Murugarren and Pastor-Anglada, 2010). Mammalian orthologs exhibit similar kinetic properties, with CNT1 and CNT2 being pyrimidine- and purine-preferring nucleoside transporters, respectively, but CNT3 showing broad substrate selectivity. They all are Na^+-coupled but their stoichiometry differs, being one Na^+ ion per one nucleoside for CNT1 and CNT2 and two Na^+ ions per one nucleoside for CNT3 (Pastor-Anglada et al., 2008). The latter tolerates the substitution of one of the two Na^+ ions required for nucleoside translocation by a proton (Slugoski et al., 2008).

In (re)absorptive epithelia, CNT proteins are localized at the apical side of enterocytes and tubular epithelial cells. Apical expression of CNT proteins seems to confer vectoriality to nucleoside flux across these epithelial barriers, with the proteins thus becoming major factors in the absorption/reabsorption of nucleosides and their derivatives and contributing to whole-body nucleoside homeostasis and nucleoside-derived drug pharmacokinetics (Lai et al., 2002; Errasti-Murugarren et al., 2007). Their anatomical localization along the gastrointestinal tract and the nephron is consistent with this view. In the human gastrointestinal tract, CNT-related activities were reported to follow a proximal-to-distal gradient, with maximal transport rates in the jejunum (Ngo et al., 2001). Human CNT1 and hCNT2 proteins were shown in immunohistochemical analyses to be predominantly on the apical side of duodenal enterocytes, rather than in crypt cells (Govindarajan et al., 2007). Human CNT1 and hCNT2 mRNA and proteins were detected predominantly in proxi-

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ABBREVIATIONS: CNT, concentrative nucleoside transporter; hCNT, human concentrative nucleoside transporter; mCNT, mouse concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; hRS1, human RS1; ER, endoplasmic reticulum; BBMV, brush border membrane vesicle; siRNA, short interfering RNA; GST, glutathione transferase; IRIP, ischemia/reperfusion-inducible protein; OCT, organic cation transporter; PCR, polymerase chain reaction; SGLT1, sodium-dependent glucose cotransporter 1.

mal tubular cells (Govindarajan et al., 2007), which supports data obtained with microdissected rat nephron segments that showed high levels of expression of CNT1, CNT2, and CNT3 mRNAs in proximal convoluted tubules (Rodríguez-Mulero et al., 2005).

CNT trafficking mechanisms that allow export of transporter proteins from the ER to the plasma membrane have not been studied in detail, but some structural determinants in the transporters themselves seem to be relevant for this process. A putative hCNT3 variant lacking 69 amino acid residues of the N-terminal domain of the protein is retained in the ER (Errasti-Murugarren et al., 2009). Membrane-sorting determinants of hCNT3 seem to reside within residues 50 to 62, the tripeptide Val57-Thr58-Val59 being the core of the export signal and acidic residues upstream and downstream of the core modulating the time course of this process (Errasti-Murugarren et al., 2010). Regulated hCNT3 trafficking in leukemia cells suggests a probable chemotherapeutic role for agents able to up-regulate hCNT3-related drug uptake activity by promoting hCNT3 insertion into the plasma membrane (Fernández-Calotti and Pastor-Anglada, 2010). Understanding CNT ER export and plasma membrane insertion mechanisms also seems to be of pharmacological relevance.

The transporter regulator RS1, which was initially identified as a short-term post-transcriptional regulator of the high-affinity Na^+-coupled glucose transporter SGLT1 (Korn et al., 2001), has been shown to modulate (among other physiological roles) the localization of this and a few other transporter proteins in the plasma membrane, acting as an inhibitor of their exocytosis (Veyhl et al., 2006; Vernaleken et al., 2007). In this study, we have addressed whether CNT-type function might also be under RS1 regulation. With a combination of studies with mammalian cells, *Xenopus laevis* oocytes, and RS1-null mice, evidence is provided that RS1 coordinately down-regulates the localization and activity at the plasma membrane of three members of this protein family (i.e., CNT1, CNT2, and CNT3).

Materials and Methods

Reagents. Uridine, cytidine, guanosine, and brefeldin A were obtained from Sigma-Aldrich (St. Louis, MO). [5,6-^{3}H]Uridine (35–50 Ci/mmol) was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). [5-^{3}H(N)]Cytidine (21.5 Ci/mmol) and [8-^{3}H(N)]guanosine (7 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA).

Animals. Mice were handled in compliance with institutional guidelines and German and Spanish laws. Knockout mice used in this study were previously generated and phenotypically characterized (Osswald et al., 2005). The mice were back-crossed on a C57BL/6 background (14 generations of back-crossing); the strain was designated C57BL/6/Rsc1A1del. Genotyping was performed with PCR assays, as described previously (Osswald et al., 2005). Three-month-old male mice were used. Animals were kept in a temperature-controlled environment with a 12-h light/dark cycle and received standard chow (Altromin GmbH, Large-Lippe, Germany) and water ad libitum.

Cell Culture. HeLa cells were maintained at 37°C in a humidified 5% CO_2 environment in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. The hepatocyte-derived cell line HHL5 was a generous gift from Dr. Arvin H. Patel (University of Glasgow, Glasgow, Scotland) (Clayton et al., 2005). HHL5 cells were maintained as described for HeLa cells, with the same culture medium but supplemented with 1% nonessential amino acids.

Preparation of Mouse Intestinal and Renal Brush Border Membranes. Renal and intestinal BBMVs were prepared from wild-type and knockout mice as described previously (Biber et al., 2007). The final pellets were resuspended in preloading medium consisting of 250 mM sucrose, 0.2 mM $CaCl_2$, 20 μM $MgCl_2$, 10 mM HEPES, pH 7.5, and 150 mM KSCN (Castaño et al., 1997) and were used for nucleoside transport assays or stored in liquid nitrogen for Western blot analyses.

Nucleoside Uptake Measurements in Cell Lines. Nucleoside uptake measurements were performed with HeLa and HHL5 cell cultures, 4 days and 1 day after seeding, respectively (2×10^4 and 6×10^4 cells/cm^2, respectively). Uptake rates were measured as described previously (del Santo et al., 1998). Replicate cultures were exposed at room temperature to ^{3}H-labeled uridine (1 μM, 1 μCi/ml) in a sodium-replete (137 mM NaCl) or sodium-free (137 mM choline chloride) transport buffer that also contained 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM HEPES, pH 7.4. Initial rates of transport were determined by using incubation periods of 1 min, and transport was terminated through washing with an excess volume of chilled buffer.

Nucleoside Transport Assays in Isolated BBMVs. Uptake studies with renal and intestinal BBMVs were performed by using the rapid filtration technique, as described previously (Ruiz-Montasell et al., 1992). Uptake was initiated by mixing 10 μl of vesicle suspension with 40 μl of uptake medium (250 mM sucrose, 0.2 mM $CaCl_2$, 20 μM $MgCl_2$, 10 mM HEPES, pH 7.5) containing either 150 mM NaSCN or KSCN and 1 μM ^{3}H-labeled substrate. Reactions were terminated at the indicated times with the addition of 1 ml of ice-cold stop solution (250 mM sucrose, 150 mM NaCl, 0.2 mM $CaCl_2$, 10 mM HEPES, pH 7.5), and mixtures were filtered through 0.45-μm nitrocellulose filters. Filters were washed with 4 ml of ice-cold stop solution, and radioactivity on the filters was determined through liquid scintillation counting. All experimental values were corrected by subtracting nonmediated uptake values, which were obtained by adding the stop solution to the transport buffer before the vesicles.

RNA Extraction and Real-Time PCR. Total RNA was isolated from kidney, intestine, heart, and liver lysates from wild-type and knockout mice by using the SV total RNA isolation system (Promega, Madison, WI). One microgram of total DNase-treated RNA was used to generate cDNA, with Moloney murine leukemia virus reverse transcriptase and random hexamers for reverse transcription (Invitrogen, Carlsbad, CA).

Real-time PCR analysis of mCNT1, mCNT2, mCNT3, mouse ENT1, and mouse ENT2 mRNAs was performed. The primers and probes used to amplify mCNT1 (Mm01315368_m1), mCNT2 (Mm00445488_m1), mCNT3 (Mm00491586_m1), mouse ENT1 (Mm00452176_m1), mouse ENT2 (Mm00432817_m1), and mouse glyceraldehyde 3-phosphate dehydrogenase (4352339E) with real-time PCR were purchased from Applied Biosystems (Foster City, CA). Real-time monitoring of PCR amplification of cDNAs was performed by using the TaqMan Universal Master Mix (Applied Biosystems), 700 nM probe, and 150 nM concentrations of each primer, with an ABI Prism 7700 sequence detection system (Applied Biosystems). Relative quantification of gene expression and validation of the method were performed as described in the TaqMan user's manual, with 18S rRNA as an internal control. The threshold cycle is defined as the cycle number at which fluorescence corresponding to the amplified PCR product is detected. The threshold cycle difference for each transporter, expressed in arbitrary units, is defined as the threshold cycle of these genes normalized to the 18S rRNA level and referred to the expression level in wild-type mice, which is considered 1.

Western Blot Analysis of mCNT1, mCNT2, and mCNT3 Expression. Twenty micrograms of renal and intestinal BBMV preparations were separated on 10% polyacrylamide gels and transferred

to nitrocellulose membranes (Bio-Rad 162-0115; Bio-Rad Laboratories, Hercules, CA). Membranes were incubated with primary anti-mCNT1, anti-mCNT2, or anti-mCNT3 antibodies diluted 1:1000. These were polyclonal antibodies raised in rabbits on demand (Antibody Barcelona, Barcelona, Spain), by using N-terminal peptides as antigens. Chosen sequences for that purpose were as follows: amino acids 50 to 69, SRSKAVWKPFSRWRSLQPT-C (mCNT1); amino acids 54 to 71, SLWSRRIFQPFTKARSF-C (mCNT2); amino acids 26 to 44, PSDLGRSNEAFQDEDLERQ-C (mCNT3). After incubation with primary antibodies, proteins were detected by using a horseradish peroxidase-conjugated secondary antibody preparation and an enhanced chemiluminescence detection kit (GE Healthcare). Expression levels for concentrative nucleoside transporters were normalized to those for β-actin (ab8229; Abcam plc, Cambridge, UK), which was used as a loading control. In this regard, we tried stripping of the anti-mCNT antibody-treated membranes and use of duplicates of the samples, one treated with anti-mCNT antibody and the other treated with anti-β-actin antibody; results were identical.

Cloning and Purification of GST-Fusion Protein with N-Terminal Fragment of hRS1. GST was fused to a fragment of hRS1 containing amino acids 16 to 98 (GST-hRS1-F). The corresponding coding DNA fragment was amplified from full-length hRS1 cDNA (Lambotte et al., 1996) through PCR with the following oligonucleotides: 5′-AATGGATCCGTTCTTCAGGACAGAGTCC-3′ (containing a BamHI restriction site) and 5′-ATTGTCGACTACTGCATAGGCATAGCTGG-3′ (containing a SalI restriction site). The PCR product was digested with BamHI and SalI restriction enzymes and ligated to the BamHI/SalI restriction sites of the pGEX-5X-3 vector (GE Healthcare). In-frame cloning was confirmed with DNA sequencing. Expression of the GST fusion protein in *Escherichia coli* BL21-Star cells and purification with glutathione-Sepharose beads were performed according to protocols provided by GE Healthcare.

cRNA Synthesis. Human CNT1 was cloned from human fetal liver (Lostao et al., 2000), and hCNT3 (Errasti-Murugarren et al., 2007) and hCNT2 (this study) were cloned from human kidney. All three were inserted into the oocyte expression vector pBSII(KS), which contained a poly(A) tail of 54 bp (Lostao et al., 2000; Gorraitz et al., 2010). To prepare sense cRNA from hRS1 (Lambotte et al., 1996), hCNT1, hCNT2, or hCNT3, the respective purified plasmids were linearized with XbaI (hCNT1), SalI (hCNT2), SacII (hCNT3), or MluI (hRS1). cRNA was synthesized by using T3 polymerase (hCNTs) or SP6 polymerase (hRS1). cRNAs were prepared by using a mMESSAGE mMACHINE kit (Ambion, Austin, TX), with sodium acetate precipitation. cRNA concentrations were estimated from ethidium bromide-stained agarose gels by using polynucleotide markers as standards.

Expression of CNTs and hRS1 in *X. laevis* Oocytes. Mature female *X. laevis* frogs were anesthetized through immersion in fresh water containing 0.1% tricaine. Oocytes at stages V and VI were obtained through partial ovariectomy and were treated overnight with 1 mg/ml collagenase I in Ori buffer (5 mM HEPES-Tris, pH 7.6, 110 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$). The oocytes were washed twice with Ca^{2+}-free Ori buffer and were kept at 16°C in modified Barth's solution [15 mM HEPES, pH 7.6, 88 mM NaCl, 1 mM KCl, 0.3 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$] containing 12.5 μg/ml gentamicin. Selected oocytes were injected with 25 nl of water containing cRNAs (hCNT1, 0.5 ng; hCNT2, 5 ng; hCNT3, 10 ng; hRS1, 10 ng). For expression, injected oocytes were kept for 3 days at 16°C in modified Barth's solution with gentamicin. Noninjected oocytes served as controls.

Injection of Fusion Protein GST-hRS1-F and/or Brefeldin into Oocytes. Three days after cRNA injections into oocytes and before initiation of flux experiments, 25 nl of K-Ori buffer (5 mM HEPES, pH 7.6, 100 mM KCl, 3 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$) containing 16 μM GST-hRS1-F and/or 0.5 pmol of brefeldin A (inhibitor of vesicle budding from the trans-Golgi network) were injected. Oocytes were incubated for 1 h at room temperature in Ori buffer, and uptake of 5 μM [^{3}H]uridine was measured after incubation for 15 min at room temperature. Uridine uptake in noninjected oocytes was subtracted. For each experimental condition, three independent experiments were performed; in each experiment, 8 to 10 individual oocytes were analyzed. The significance of differences was determined with Student's *t* test or analysis of variance with post hoc Tukey's comparisons.

RS1 Suppression by RNA Interference in HeLa and HHL5 cells. Two siRNAs directed against hRS1 (Applied Biosystems) were tested at different concentrations through reverse transcription-PCR, to determine their efficiency in suppressing RS1 expression. HeLa cells were grown on 60-mm plates. Cells were transfected with hCNT1-pcDNA3.1, hCNT2-pcDNA3.1, or hCNT3-pcDNA3.1 plasmids by using calcium phosphate precipitation, and 14 h later the same cells were transfected with siRNAs by using Lipofectamine 2000 (Invitrogen). As a control, cells were transfected with hCNT1-pcDNA3.1, hCNT2-pcDNA3.1, or hCNT3-pcDNA3.1 and in parallel with a silencer siRNA (AM4613; Applied Biosystems). RNA was extracted and transport assays were performed 24 h later. The human hepatocyte-derived cell line HHL5 was grown in 24-well plates for 24 h and then transfected with siRNA by using Lipofectamine 2000 (Invitrogen). Uptake assays were performed 24 h after transfection, as described above.

Results

Nucleoside Transport in Intestinal and Renal BBMVs from RS1-Null Mice. Nucleoside uptake was characterized by using uridine, cytidine, and guanosine as substrates. Figure 1 shows the time courses of uridine (Fig. 1, A and D), cytidine (Fig. 1, B and E), and guanosine (Fig. 1, C and F) uptake (1 μM) into BBMVs derived from intestines from wild-type and RS1-knockout mice (four or five independent preparations). Sodium-dependent transport was observed for all three nucleosides when uptake was measured in the presence of an outside-to-inside sodium gradient (150 mM, outside greater than inside) (Fig. 1, A–C). RS1-knockout mice showed ~2-fold higher transport activity for all three assayed nucleosides (Fig. 1), whereas sodium-independent activity reached similar uptake values in BBMV preparations from wild-type and knockout mice (Fig. 1, D–F). Identical results were obtained when renal BBMVs from wild-type and RS1-knockcout mice were analyzed (data not shown). To analyze which transport agencies were responsible for this sodium-dependent component of nucleoside transport, the profiles of ^{3}H-labeled natural nucleoside uptake into intestinal and renal BBMVs were analyzed through cross-inhibition of [^{3}H]cytidine uptake by unlabeled guanosine and vice versa (Fig. 2). Sodium-dependent uptake of ^{3}H-labeled cytidine and guanosine (1 μM) was not significantly blocked by 100 μM guanosine or cytidine, respectively, in intestinal BBMVs from wild-type or knockout mice (Fig. 2A), which suggests the expression of both mCNT1 and mCNT2 proteins but not mCNT3. In contrast, sodium-dependent uptake of ^{3}H-labeled cytidine and guanosine (1 μM) was significantly blocked by 100 μM guanosine and cytidine, respectively, in renal BBMVs from both wild-type and knockout mice (Fig. 2B), which suggests the presence of all three concentrative nucleoside transporter proteins (mCNT1, mCNT2, and mCNT3) at the plasma membrane.

Nucleoside Transporter Protein Expression in BBMVs from RS1-Null Mice. The amounts of concentrative nucleoside transporter proteins in BBMVs isolated from wild-type and RS1-knockout mice were analyzed through Western blotting. To this end, polyclonal antibodies against mouse CNTs were gener-

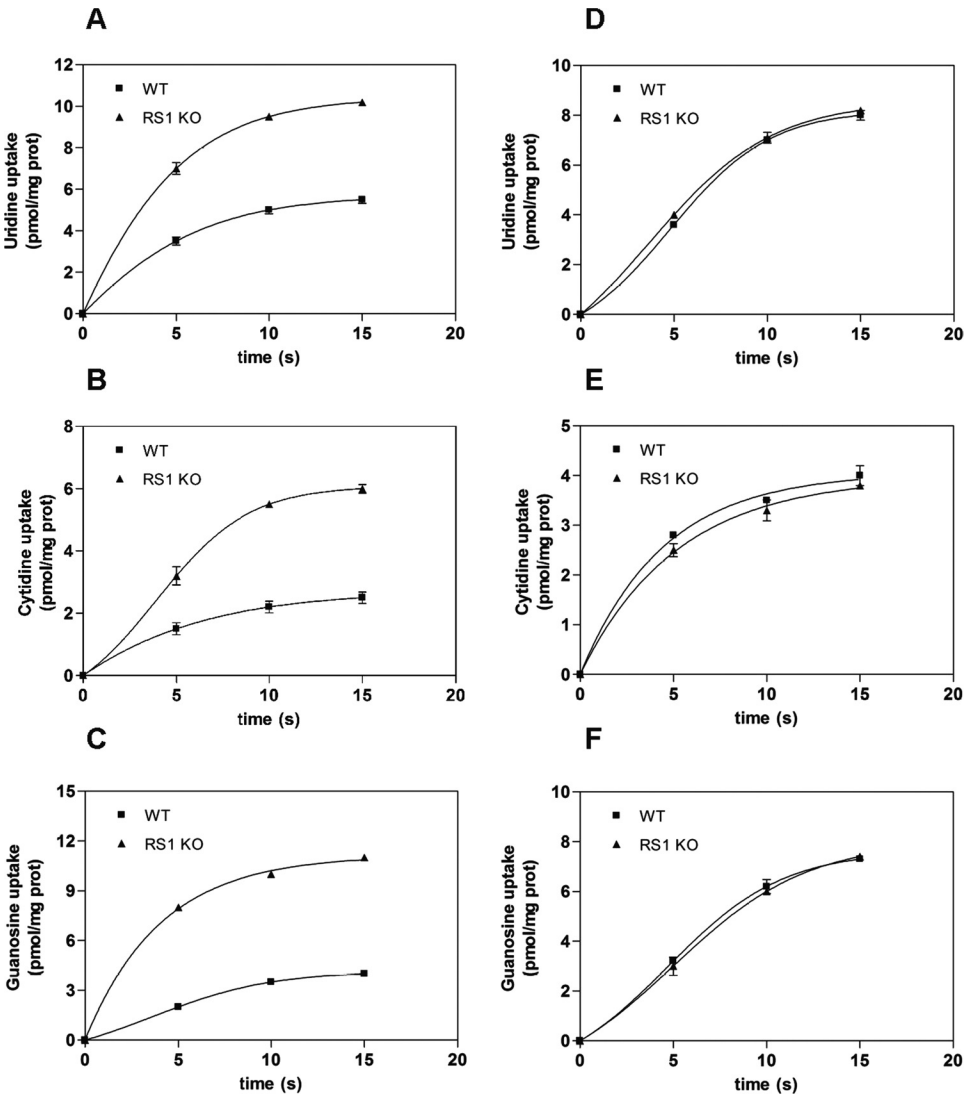

Fig. 1. Functional characterization of nucleoside uptake in intestinal brush border membrane vesicles derived from wild-type and RS1-null mice. Time courses of sodium-dependent (CNT activity) (A–C) and sodium-independent (ENT activity) (D–F) [^{3}H]uridine (A and D), [^{3}H]cytidine (B and E), and [^{3}H]guanosine (C and F) uptake in brush border vesicles derived from wild-type (WT) and RS1-knockout (KO) mouse intestines were determined. Uptake (1 μM) was measured in transport medium containing 150 mM NaSCN or 150 mM KSCN. Sodium-dependent transport was calculated as uptake in sodium medium minus uptake in potassium medium. Data are expressed as the mean ± S.E.M. of four experiments performed on different days with different batches of vesicles.

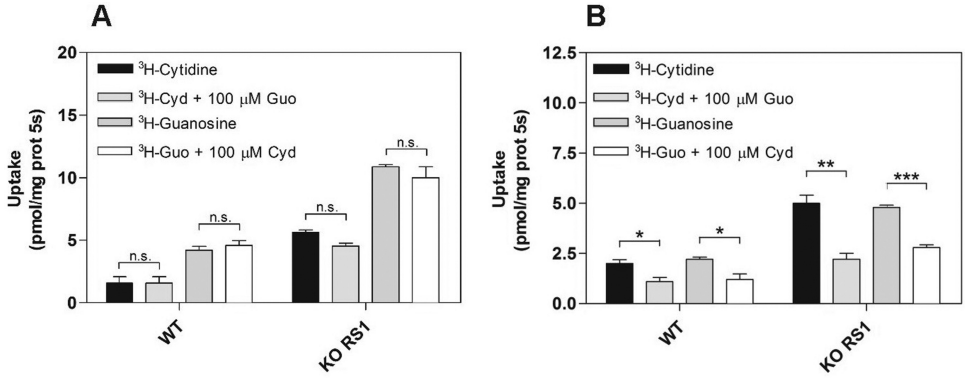

Fig. 2. Characterization of sodium-dependent transport activity present in plasma membrane vesicles. CNT activities in brush border vesicles derived from wild-type (WT) and RS1-knockout (KO) mouse intestines (A) and kidneys (B) were analyzed. Sodium-dependent uptake of 1 μM tritiated cytidine (Cyd) inhibited with 100 μM unlabeled guanosine (Guo) and of 1 μM guanosine inhibited with 100 μM unlabeled cytidine (5 s) was measured in transport medium containing 150 mM NaSCN or 150 mM KSCN. Sodium-dependent transport was calculated as uptake in sodium medium minus uptake in potassium medium. Data are expressed as the mean ± S.E.M. of triplicate measurements made with three independent pools of three samples from individual animals. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant.

ated. As positive controls for mCNT expression and antibody characterization, mouse spleen (mCNT1 and mCNT2) (Valdés et al., 2006; Paproski et al., 2010) and the PCT cell line (mCNT3) (Errasti-Murugarren et al., 2007) were used (Fig. 3A). Western blot analysis showed that each antibody recognized endogenously expressed mCNT1 (~60 kDa), mCNT2 (~60 kDa), or mCNT3 (~75 kDa), whereas the negative (prebleeding) serum did not recognize them (Fig. 3A). Protein expres-

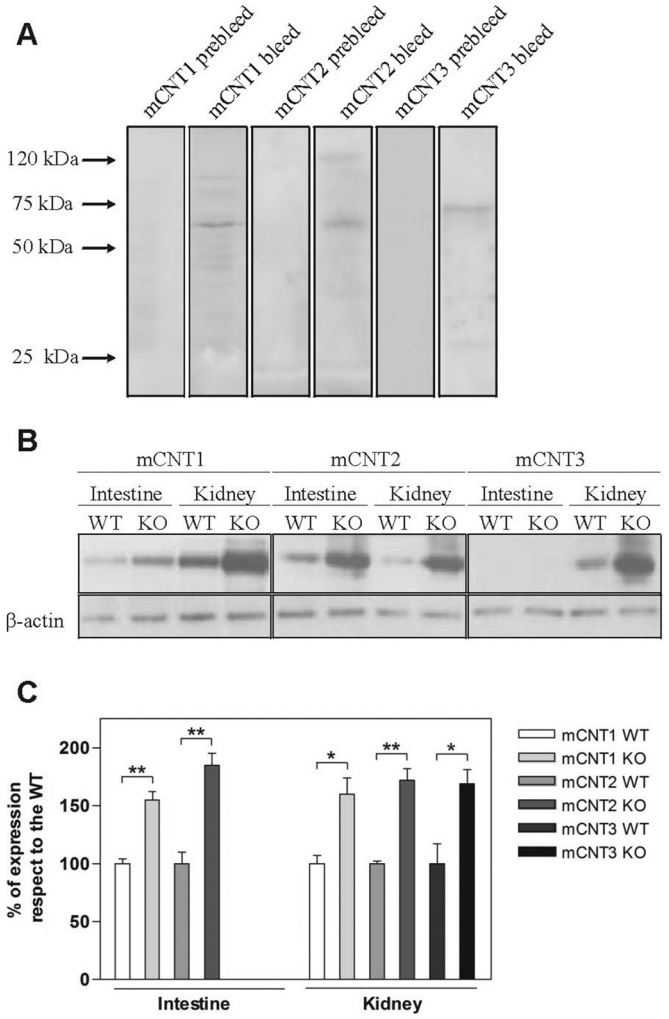

Fig. 3. CNT protein expression in plasma membrane-derived vesicles. A, characterization of polyclonal anti-mCNT1, anti-mCNT2, and anti-mCNT3 antibodies generated in rabbits. Mouse spleen (mCNT1 and mCNT2) and the PCT cell line (mCNT3) were used as positive controls. B, Western blot analysis of mCNTs in brush border vesicles derived from wild-type (WT) and RS1-knockout (KO) mouse intestines and kidneys. C, densitometric analysis of Western blots. Data were normalized to control values. Data are expressed as the mean ± S.E.M. of triplicate estimations made with three preparations from different groups of animals. *, $p < 0.05$; **, $p < 0.01$.

sion results for mCNTs in both intestinal and renal BBMVs from wild-type and RS1-knockout mice were consistent with apical plasma membrane localization of mCNT1 and mCNT2 in intestinal membranes and the three mCNT proteins in renal epithelial cells (Fig. 3B). Moreover, the expression levels observed for all three transporters in intestinal and renal brush border vesicles from RS1-knockout mice were 1.5- to 2-fold higher, compared with those observed for wild-type mice (Fig. 3, B and C). These results were consistent with the reported changes (see above) in nucleoside transport activity in BBMVs from RS1-null mice, which suggested that the up-regulation of mCNT-related biological activity was mainly attributable to greater plasma membrane abundance of concentrative nucleoside transporter proteins in both the intestine and the kidney.

Post-Transcriptional hRS1 Blockade of hCNT Expression. To determine whether RS1 blocked the expression of CNTs post-transcriptionally, as described previously for SGLT1 (Veyhl et al., 1993, 2006; Lambotte et al., 1996; Vernaleken et al., 2007), we separately coexpressed hCNT1, hCNT2, and hCNT3 with hRS1 in *X. laevis* oocytes (Fig. 4A). Blast searches of the available databases showed that amphibians do not express RS1, and RS1 immunoreactivity could not be detected in *X. laevis* oocytes (Valentin et al., 2000). Because oocytes do not express RS1, in contrast to most mammalian cells (Veyhl et al., 1993), they are an ideal system for investigation of the function of RS1. Figure 4A shows that coexpression decreased uridine uptake associated with hCNT1, hCNT2, or hCNT3 by more than 80%. This effect was post-transcriptional, because proteins were translated from the corresponding injected cRNAs. In Fig. 4B, we investigated whether hRS1 protein promoted short-term, high-affinity inhibition of CNTs, as described for SGLT1 (Veyhl et al., 2006; Vernaleken et al., 2007). We injected cRNAs encoding hCNT1, hCNT2, or hCNT3 into oocytes and incubated them for 3 days before functional analysis. At that point, 0.4 pmol of a GST fusion protein with an N-terminal fragment of hRS1 comprising amino acids 16 to 98 (GST-hRS1-F) were injected. This fragment of hRS1 is known to decrease the abundance of human SGLT1 in the trans-Golgi network in a post-transcriptional manner (Korn et al., 2001). With the assumption that the aqueous volume of oocytes is 0.4 μl (Taylor and Smith, 1987), the concentration of GST-hRS1-F is estimated to have been ~1 μM. Figure 4B indicates that the expression of the three hCNTs was inhibited by ~50%. To prove that the post-transcriptional inhibition of hCNTs occurred at the trans-Golgi network, we measured the effect of GST-hRS1-F on the expression of hCNT1, hCNT2, and hCNT3 in the presence of the trans-Golgi network inhibitor brefeldin. Figure 4, C, D, and E, shows that GST-hRS1-F was not effective if trans-Golgi network functions were blocked. The data indicated that hRS1 is a short-term inhibitor of human CNTs at the trans-Golgi network.

High hCNT-Related Activities in Plasma Membrane with Endogenous hRS1 Inhibition. To demonstrate that the observed effect of hRS1 on mCNTs could be reproduced in human cell lines endogenously expressing this regulator and the human transporters, hRS1 was silenced in either HeLa or HHL5 cells and the effect on either heterologously expressed or endogenously present hCNT-related transport activities was monitored. The greatest silencing efficiency of hRS1 (60%) was achieved by using the combination of the two siRNAs (see *Materials and Methods*) at 100 nM (Fig. 5A), 24 and 48 h after transfection. Under these conditions, hRS1 was silenced in HeLa cells separately transfected and transiently expressing each of the three isoforms of hCNTs. Partial hRS1 silencing was sufficient to impair significantly the hRS1-mediated down-regulation of hCNTs, thereby increasing their corresponding transport activities (Fig. 5B). Data were normalized to the basal transport activity found in hCNT-transfected cells with intact hRS1 endogenous function. Basal uptake rates were 84.8 ± 13.5, 94.9 ± 11.4, and 113.5 ± 19.2 pmol · mg of protein^{-1} · min^{-1} for hCNT1-, hCNT2-, and hCNT3-expressing HeLa cells, respectively. Control siRNA did not induce any significant change in hCNT-related activities (data not shown).

The use of transient transfection to monitor the effects of hRS1 on hCNT-related transport activities was undertaken because hCNT expression is associated with cell differentiation, a status that is often lost in immortalized cell lines. We

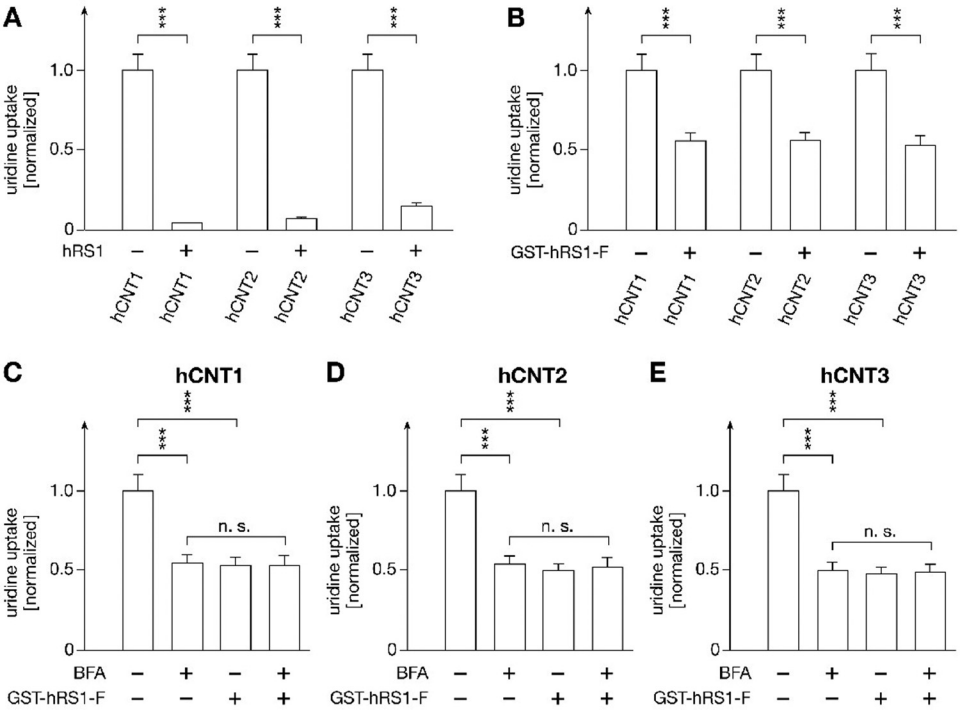

Fig. 4. Post-transcriptional inhibition of hCNTs by hRS1 and an N-terminal fragment of hRS1. A, hCNT1, hCNT2, or hCNT3 was coexpressed with hRS1 through coinjection of the respective cRNAs. Three days after the injections of cDNAs, uridine uptake was measured after 15-min incubations with 5 μM [^{3}H]uridine. The uptake rates were corrected for uridine uptake measured in noninjected oocytes. B, hCNT1, hCNT2, or hCNT3 cRNA was injected and the oocytes were incubated for 3 days. Purified GST-hRS1-F protein was then injected and uridine uptake was measured as in A. C–E, hCNT1, hCNT2, or hCNT3 was expressed in oocytes through cRNA injection and incubation for 3 days. Brefeldin A (BFA), GST-hRS1-F, or GST-hRS1-F plus brefeldin was injected and uridine uptake was measured 1 h later. Data are the mean ± S.E.M. of uptake measurements in three (C) or two (D and E) independent experiments with nine oocytes each. ***, $p < 0.001$; n.s., not significant.

also used a human hepatocyte-derived cell line, HHL5, because it retained significant endogenous hCNT2-related transport activity. Silencing of endogenous hRS1 expression in HHL5 cells similarly modified basal endogenous hCNT2 activity, inducing a dramatic increase in Na^+-coupled guanosine uptake (Fig. 5C). As in the experiments with hCNT-expressing HeLa cells, the control siRNA did not modify basal hCNT2-related uptake rates in HHL5 cells (Fig. 5C).

Discussion

Evidence for coordinate regulation of CNT1, CNT2, and CNT3 proteins by RS1 in (re)absorptive epithelia is provided here. This is based on data generated with a combination of approaches, involving RS1 knockdown in both in vitro (RS1 silencing in cell lines) and in vivo (RS1-null mice) models. A more-direct analysis of the inhibitory role RS1 exerts on the plasma membrane expression of these three transporter proteins is provided by experiments using *X. laevis* oocytes injected either with a RS1-encoding cRNA or with a GST-fused chimera including an N-terminal fragment of hRS1 comprising amino acids 16 to 98.

The intronless *RSC1A1* gene encodes RS1, a 67- to 68-kDa ubiquitous protein apparently found only in mammals (Valentin et al., 2000). RS1, which was initially described as a post-transcriptional regulator of the Na^+/D-glucose cotransporter SGLT1, was shown to inhibit its exocytosis, thus compromising its biological activity at the plasma membrane (Veyhl et al., 2006). This effect is not restricted to SGLT1 and might affect unrelated transporter proteins such as human OCT2 (Veyhl et al., 2006). RS1 actions are not promiscuous, however, because no changes were found when the effects of RS1 coexpression with transporters such as glucose transporter 1 and peptide transporter 1 were analyzed (Veyhl et al., 2003). Here the list of putative RS1 targets is being expanded to include the whole family of proteins encoded by *SLC28* genes. However, the inhibitory action of RS1 on CNT expression and activity at the plasma membrane should be considered somehow restricted to this family of nucleoside transporters, because ENT-related activities in renal and intestinal BBMVs from RS1-null mice were not modified and neither was the Na^+-independent component (ENT-related) of transport in cell lines after RS1 gene silencing (data not shown). Although ENT expression might be more abundant at the basolateral side of these epithelia, apical occurrence of ENT-related transport activity has been consistently demonstrated (Errasti-Murugarren et al., 2007).

RS1 is localized in several subcellular compartments. RS1 is found on the inner side of the plasma membrane (Valentin et al., 2000; Korn et al., 2001), it coats the trans-Golgi network (Kroiss et al., 2006), and it can be present in the nucleus, where it is involved in transcriptional down-regulation of the Na^+/D-glucose cotransporter SGLT1 in the renal epithelial cell line LLC-PK1 (Korn et al., 2001). Nuclear localization of RS1 seems to depend on cell density, progressively decreasing when cells are reaching confluence (Kroiss et al., 2006). This might explain why endogenous SGLT1 activity increases with cell density, and this suggests that transcriptional inhibition of SGLT1 expression by RS1 is regulated through nuclear migration. A putative transcriptional inhibitory effect of RS1 on *SLC28* genes could be ruled out with the RS1-null mice, because real-time PCR analysis of the mRNA levels for the three genes revealed no significant differences between wild-type and knockout animals (Supplemental Fig. 1). In the same way, mRNA levels for the *SLC29* gene family members *SLC29A1* (ENT1) and *SLC29A2* (ENT2) were not affected in these mice (Supplemental Fig. 1). Changes in CNT-related expression associated with cell density might be cell type-dependent and not necessarily consistent with the reported changes in RS1 nuclear localization and inhibitory function. Although CNT1 protein amounts were shown to increase as cultures became confluent for rat hepatocyte-derived cell lines (Dragan et al., 2000), CNT-related activities

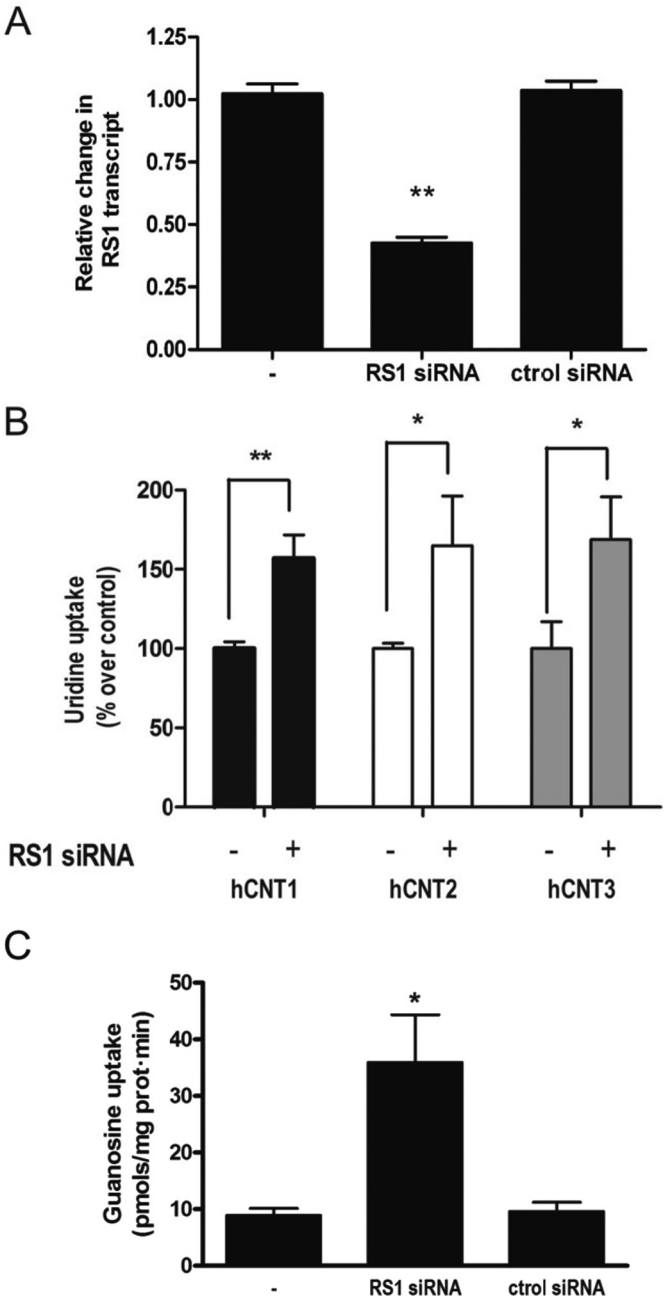

Fig. 5. Effects of hRS1 on hCNT-related activities in human cell lines. A, HeLa cells were transfected with a mixture of two RS1 siRNAs (118930 and 118931; 100 nM each) or control (ctrol) siRNA (AM4613; 200 nM). Human RS1 real-time PCR was performed 24 h later. B, HeLa cells were transiently transfected with hCNT1, hCNT2, or hCNT3 and 14 h later with the mixture of RS1 siRNAs or control siRNA, as described under *Materials and Methods*. After an additional 24 h, sodium-dependent [^{3}H]uridine uptake was measured. Results are based on quadruplicate measurements from three independent experiments. All values were normalized to the basal activity found in hCNT-transfected cells with preserved endogenous hRS1. Data (means ± S.E.M.) are shown as the percentage increase in uridine uptake over control (nontreated cells) values. C, HHL5 cells were transiently transfected with the mixture of RS1 siRNAs or control siRNA, as described under *Materials and Methods*. Twenty-four hours after transfection, sodium-dependent [^{3}H]guanosine uptake was monitored. Results (mean ± S.E.M.) are based on quadruplicate measurements from three independent experiments. Significance of differences, relative to control cells, was determined with Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

were reported to be density-independent in other epithelial models (García-Manteiga et al., 2003) or even to decrease progressively as cell density increased for lymphoid cells (Soler et al., 1998). Moreover, CNT1 and CNT2 were shown to be cell cycle-regulated in hepatoma cells (del Santo et al., 1998; Valdés et al., 2002), and RS1 nuclear import and export also seem to be dependent on cell cycle progression (Filatova et al., 2009). RS1 has been localized in the nucleus in synchronized subconfluent cells and was shown to be exported during the G_2/M transition, through a mechanism that seems to be dependent on the protein kinase C-mediated phosphorylation of a RS1 nuclear shuttling domain that promotes RS1 export (Filatova et al., 2009). The sequence of events regarding nuclear import/export of RS1 is not consistent with reported changes in the expression of CNT1 and CNT2, which are up-regulated before the peak of thymidine incorporation into DNA, which suggests G_1/S-related activation (del Santo et al., 1998; Valdés et al., 2002). Information on the transcriptional regulation of CNT1 and CNT2 is still scarce. Hepatocyte nuclear factor 4α is known to modulate *SLC28A1* gene expression in hepatocytes (Fernández-Veledo et al., 2007; Klein et al., 2009), whereas the CNT2-encoding gene is transcriptionally activated by transforming growth factor-β (Valdés et al., 2006), CCAAT enhancer-binding protein α, hepatocyte nuclear factor 3γ (Fernández-Veledo et al., 2007), and hepatocyte nuclear factor 1α (Yee et al., 2009). Data from this study support a major role for RS1 in the trafficking of CNT proteins from the trans-Golgi network to the plasma membrane, without consistent proof of transcriptional modulation of the *SLC28* genes.

The complexity of the biological features of RS1 is supported by the variety of structural determinants relevant for function that have been described to date. In addition to the RS1 nuclear shuttling domain (Filatova et al., 2009), evidence for selected domains promoting the inhibition of transporter export from the trans-Golgi network comes from peptide-mapping dissection of this biological effect in *X. laevis* oocytes injected with peptides mimicking particular sequences within RS1 (Vernaleken et al., 2007). As shown in this study, a fragment of RS1 including residues 16 to 98 of the N-terminal tail of the protein similarly inhibited CNT activity. This was mimicked by the fungal metabolite brefeldin, which is known to promote the release of various coat proteins from the Golgi apparatus. RS1 has been identified as a protein partner of the protein IRIP (Jiang et al., 2005). In fact, IRIP (which apparently is also ubiquitous in mammals) similarly down-regulates, when overexpressed, OCT2 activity. This inhibitory effect was also shown for related proteins belonging to the *SLC22* gene family, such as OCT3 and organic anion transporter 1, and for unrelated transporters such as the dopamine, norepinephrine, and serotonin transporters (Jiang et al., 2005). IRIP was shown to modulate expression levels at the plasma membrane for the multidrug resistance transporter protein P-glycoprotein, thus modulating doxorubicin-induced cytotoxicity in HeLa cells (Prokopenko and Mirochnitchenko, 2009). Whether RS1 similarly modulates drug efflux remains to be established. However, the effects of RS1 and IRIP on OCT2 function were not additive, and the inhibition of OCT2 by RS1 was prevented by coexpression of a dominant-negative mutant of IRIP (Jiang et al., 2005), which suggests a common regulatory pathway for the two proteins. It is particularly interesting that

IRIP is up-regulated during ischemia, a process in which extracellular adenosine is known to accumulate (Eltzschig et al., 2009). Luminal adenosine exerts a variety of physiological effects in both renal and intestinal epithelia. Adenosine is a modulator of tubuloglomerular feedback (Thomson et al., 2000) and a protective agent in intestinal inflammatory disease (Ye and Rajendran, 2009). CNT proteins, particularly CNT2 and CNT3, are major candidates to remove adenosine from the extracellular space, because they are high-affinity concentrative adenosine transporters (Pastor-Anglada et al., 2008). Both are present not only in proximal convoluted tubules but also in distal segments of the nephron (collecting duct), where they are likely to play a major role in adenosine reabsorption (Rodríguez-Mulero et al., 2005). CNT2 is also expressed in the colon, where it can contribute to adenosine removal from the lumen (Wojtal et al., 2009). The possibility that a hypoxia/ischemia-sensitive mechanism, implicating the IRIP-RS1 partnership, might contribute to down-regulating the adenosine reuptake capacity by coordinately inhibiting CNT protein export from the trans-Golgi network to the plasma membrane is an interesting hypothesis that should be further explored.

In summary, we have identified RS1 as a novel regulator of CNT-type protein abundance and activity at the plasma membrane, which provides the biochemical basis for coordinate regulation of nucleoside uptake ability in epithelia and probably in other RS1-expressing cell types. This regulatory function seems to involve exclusively trafficking phenomena, being independent of the transcriptional regulation of the CNT protein-encoding *SLC28* genes.

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Authorship Contributions

Participated in research design: Errasti-Murugarren, Fernández-Calotti, Pinilla-Macua, Koepsell, and Pastor-Anglada.

Conducted experiments: Errasti-Murugarren, Fernández-Calotti, Veyhl-Wichmann, Diepold, Pinilla-Macua, Pérez-Torras, and Kipp.

Contributed new reagents or analytic tools: Errasti-Murugarren, Pérez-Torras, Koepsell, and Pastor-Anglada.

Performed data analysis: Errasti-Murugarren, Fernández-Calotti, Veyhl-Wichmann, and Pinilla-Macua.

Wrote or contributed to the writing of the manuscript: Errasti-Murugarren, Fernández-Calotti, Pinilla-Macua, Koepsell, and Pastor-Anglada.

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