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ZIP8, Member of the Solute-Carrier-39 (SLC39) Metal-Transporter Family: Characterization of Transporter Properties

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ABSTRACT

Cadmium is a dangerous metal distributed widely in the environment. Members of our laboratory recently identified the ZIP8 transporter protein, encoded by the mouse *Slc39a8* gene, to be responsible for genetic differences in response to cadmium damage of the testis. Stable retroviral infection of the ZIP8 cDNA in mouse fetal fibroblast cultures (rvZIP8 cells) leads to as much as a 10-fold increase in the rate of intracellular cadmium influx and accumulation. In the present study, we showed that cadmium uptake operated maximally at pH 7.5 and a temperature of 37°C and was inhibited by cyanide. Of more than a dozen cations tested, manganese(II) was the best competitive cation for cadmium uptake. The K_m for Cd^{2+} uptake was 0.62 μM, and the K_m for Mn^{2+} uptake was 2.2 μM; thus, manganese is probably the physiological substrate for ZIP8. Cadmium uptake was independent of sodium, potassium or chloride ions, but strongly dependent on the presence of bicarbonate. By Western blot analysis of rvZIP8 cells, we showed that ZIP8 protein was glycosylated. Using Z-stack confocal microscopy in Madin-Darby canine kidney polarized epithelial cells, we found that ZIP8 was localized on the apical side—implying an important role for manganese or cadmium uptake and disposition. It is likely that ZIP8 is a Mn^{2+}/HCO_3^- symporter, that a HCO_3^- gradient across the plasma membrane acts as the driving force for manganese uptake, and that cadmium is a rogue hitchhiker displacing manganese to cause cadmium-associated disease.

Cadmium is a highly toxic metal—widely distributed in contaminated soil, cigarette smoke, toxic waste dump sites, and polluted seafood. Short-term exposure to large cadmium doses can result in damage to the central nervous system, gastrointestinal tract, lung, liver, bone, ovary, placenta, and testes (Waisberg et al., 2003; Zalups and Ahmad, 2003).

Long-term exposure to low doses of cadmium results predominantly in nephropathy and osteomalacia; cadmium is eliminated slowly and thus accumulates with age. The level of cadmium in the environment has risen with the rise of industrialization, and cadmium-induced human disease is of growing concern. The increasing levels of environmental cadmium, in combination with longer life expectancy, work together to enhance the body's cadmium burden: the average accumulation of cadmium in the kidneys of a person who smokes at least two packs a day for 50 years, for example, is beyond the threshold sufficient for causing overt cadmium nephrotoxicity (http://www.trace-elements.org.uk /cadmium.htm).

Cadmium is classified as an IARC category I human carcinogen. People who are at highest risk for cadmium-associated lung cancer include cigarette smokers, women with low body-iron stores, people with a habitual diet rich in high-fiber foods and contaminated shellfish, and malnourished populations (for review, see Jarup et al., 1998; Waalkes, 2003).

Because cadmium is not essential to living organisms, cadmium-transporting proteins are expected to be transporters of one or more essential metal ions. There have been numerous studies about cadmium transport in bacteria, yeast and

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ABBREVIATIONS: ZIP, Zrt-like, Irt-like protein; MFF, mouse fetal fibroblast; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ha, hemagglutinin tag on C terminus; rv, retrovirus; LUC, luciferase; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyl tetrasodium bromide; PNGase F, *N*-glycosidase F; PAGE, polyacrylamide gel electrophoresis; MDCK, Madin-Darby canine kidney; GFP, green fluorescent protein; NBC1, sodium-bicarbonate-1 cotransporter; PNA, peanut agglutinin; pH_{out}, pH of the transporting medium; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; PCT, proximal convoluted tubule; ACDP4, ancient conserved domain protein-4.

plants; until recently, however, very little was known about the molecular mechanisms of cadmium transport in vertebrates. Focusing on testicular necrosis as a sensitive endpoint for genetic differences in response to cadmium, researchers at this laboratory set out to identify the *Cdm* locus—defined more than 3 decades ago (Taylor et al., 1973). Using mouse genetics, recombinant inbred lines and advances in knowledge about the mouse genome, this laboratory demonstrated that the *Slc39a8* gene is the *Cdm* locus responsible for genetic differences in damage to the vasculature endothelium specific to the testis (Dalton et al., 2000, 2005).

Slc39a8 is one of 14 members of the mouse *Slc39* gene family. The human *SLC39* gene family also has 14 members, all of which are orthologous and highly conserved with the mouse genes. The *Slc39* genes encode Zrt-like, Irt-like proteins (ZIP), first characterized in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*; the gene product of the mouse *Slc39a8* gene is thus called ZIP8 (Eide, 2004). This laboratory has produced a stable retrovirally infected ZIP8 cDNA into mouse fetal fibroblast (MFF) cultures, to generate ZIP8-expressing rvZIP8 cells, which exhibit as much as a 10-fold increase in the rate of intracellular cadmium uptake and accumulation and a ~30-fold increase in sensitivity to cadmium-induced cell death (Dalton et al., 2005).

In the present study, we have characterized further the membrane-bound ZIP8 transporter protein. These data include: dependence of cadmium uptake by ZIP8 on temperature, cellular ATP-mediated energy, pH, and HCO_3^-; cation competition studies; effects of cation or anion substitution or depletion; glycosylation; and membrane localization of the transporter.

Materials and Methods

Chemicals. $CdCl_2$, $MnCl_2$, and $ZnCl_2$ were bought from Fisher Scientific (Pittsburgh, PA). Bovine serum albumin and the remainder of the chemicals—including Chelex 100—were purchased from Sigma (St. Louis, MO). Cadmium uptake studies were performed with $^{109}CdCl_2$ [710 mCi/mg (1 mCi = 37 mBq) in 0.1 M HCl], purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Manganese uptake studies were carried out with $^{54}MnCl_2$ (7734 mCi/mg in 0.5 M HCl), purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Zinc uptake studies were done with $^{65}ZnCl_2$ (140 mCi/mg in 0.1 M HCl), purchased from the National Laboratory of Oak Ridge (Oak Ridge, TN). Transporting medium for these divalent metal ions was a modified version of Hanks' balanced salt solution (HBSS), shown in Table 1.

Cell Culture and Transfection Methods. MFF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen; Carlsbad, CA) plus 10% fetal bovine serum (FBS) from Hyclone (Logan, UT) at 37°C in 5% CO_2. All culture medium contained 100 units/ml of penicillin and 100 μg/ml streptomycin. The ZIP8-infected cells were maintained in selection medium (Dalton et al., 2005) supplemented with hygromycin (400 μg/ml) and puromycin (3 μg/ml). Transfection was carried out according to the manufacturer's protocol for Lipofectamine 2000 (Invitrogen).

Cloning of the ZIP8 cDNA and Delivery into MFF Tet-Off Cells. Oligo-dT-primed reverse transcription was carried out with C57BL/6J mouse testis total RNA. Primers for amplification began at the start codon and ended at the stop codon; a consensus Kozak sequence at the start-site was included for efficient expression. For ZIP8, restriction sites were added at the 5′ (BamHI) and 3′ (ClaI) ends of the coding sequence for cloning into the pRevTRE vector (Invitrogen); for ZIP4, the sites are 5′ (BamHI) and 3′ (SalI). In each 3′ primer, the coding sequence of a hemagglutinin (ha) tag was also inserted in-frame, before the termination codon of that protein coding sequence. An MFF cell line was generated by continuous passage of primary MFF cultures. These cells were infected with a retrovirus encoding the Tet-off receptor (Bergwitz et al., 2000), and a clone was selected using puromycin (3 μg/ml) resistance. These cells were infected with retrovirus (rv)-encoding control luciferase (LUC), ZIP8ha, or ZIP4ha cDNAs, to generate, respectively, the stable rv-LUC, rvZIP8, and rvZIP4 cell pools that were selected for hygromycin (400 μg/ml) resistance (Dalton et al., 2005). These cell pools constitutively express the transporters via retroviruses expressed from pRevTRE.

Determination of Cadmium, Zinc, and Manganese Uptake. The cells were seeded at a density of ~80% confluence in 24-well plates. The next day, the DMEM was replaced with modified HBSS as the transport medium, unless otherwise specified; $^{109}CdCl_2$,

TABLE 1

Composition of Hanks' balanced salt solution (HBSS)

Where indicated, transport studies were conducted in HBSS (Invitrogen), which lacks Ca^{2+} and Mg^{2+}. The standard HBSS that we made was modified to contain 1.26 mM $CaCl_2$, 0.493 mM $MgCl_2$, 0.407 mM $MgSO_4$, and 4.17 mM $NaHCO_3$ (second column). Adjustments were made to maintain these ion concentrations, using stock solutions of the respective salt. To determine anion and cation requirements for ZIP8 transport, the following modifications were made to the standard HBSS. For Na^+-free K^+-HBSS (third column), Na^+ salts of PO_4^{3-}, HCO_3^-, and Cl^- were replaced with K^+ salts. For Na^+-free NMDG$^+$-HBSS (fourth column), NaCl was replaced with NMDG chloride, and the Na^+ salts of HCO_3^- and PO_4^{3-} were replaced with K^+ salts. For salt solutions in which K^+ was varied (fifth column), KCl was replaced with NaCl. For salt solutions in which HCO_3^- was varied (sixth column), $NaHCO_3$ was replaced with NaCl. For Cl^--free HBSS, Cl^- in all salts was replaced by gluconate salts (last column).

Components	Normal	Na^+ Replaced with K^+	Na^+ Replaced with NMDG$^+$	K^+ Adjusted	HCO_3^- Adjusted	Glu-HBSS
			mM			
D-Glucose (dextrose)	5.56	5.56	5.56	5.56	5.56	5.56
$CaCl_2$	1.26	1.26	1.26	1.26	1.26	
$MgCl_2$	0.493	0.493	0.493	0.493	0.493	
$MgSO_4$	0.407	0.407	0.407	0.407	0.407	0.407
KCl	5.33	143.26	5.33	Varied	5.33	
NaCl	137.93			Varied	Varied	
(NMDG$^+$)Cl			137.93			
$NaHCO_3$	4.17			4.17	Varied	4.17
$KHCO_3$		4.17	4.17			
Na_2HPO_4	0.338			0.338	0.338	0.338
K_2HPO_4		0.338	0.338			
NaH_2PO_4	0.441			0.441	0.441	0.441
KH_2PO_4		0.441	0.441			
$Ca(Glu)_2$						1.26
$Mg(Glu)_2$						0.493
KGlu						5.33
NaGlu						137.93

NMDG$^+$, *N*-methyl-D-glucamine ion; Glu^-, gluconate ion $(C_6O_7H_{11})^-$.

$^{54}MnCl_2$, or $^{65}ZnCl_2$ was added to the transport medium to make the final concentrations 0.25, 0.25, or 10 μM, respectively, so that each well contained 0.5 ml of transport medium and 0.1 μCi/ml radioactivity. The cells were then incubated for 20 min at 37°C. For each sampling point, the uptake was stopped by placing the plate on ice and quickly removing the transport medium. The cells were washed three times with 0.5 ml of ice-cold phosphate-buffered saline (PBS) (containing 1 mM EDTA). After the final wash, 0.5 ml of 0.5 N NaOH was added to each well, and the plates were then incubated at 37°C overnight to digest the cells. The next day, 200 μl of cell lysate from each well was used for liquid scintillation counting, whereas the remaining portion of each sample was used for determination of protein concentration, measured by the BCA protein assay.

Measurement of Metal-Induced Cell Death. The viability of cells was assessed, after exposure to metal ions including Cd^{2+}, Co^{2+}, Cs^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}. All cations were in the chloride form. Pb^{2+} and Ag^{1+} could not be precisely tested because of precipitation with chloride ions present. Cell viability was determined as the cleavage of 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT), as described by the manufacturer's protocol (Promega; Madison, WI). The Hoechst assay replaced the MTT assay in studying Cu^{2+} toxicity, because Cu^{2+} interferes with the MTT assay. In brief, after incubating the cells with Cu^{2+} for 32 h, the cells were washed once with PBS; the cells were then fixed with 75% ethanol at −20°C for 1 h. Next, the ethanol was removed, and the wells were air-dried in the hood. Then 250 μl of Hoechst 33258 solution (5 μg/ml) was added, and the cells were incubated at room temperature in the dark for 20 min. Fluorescence was determined in a Wallac Victor2 1420 multilabel counter (PerkinElmer Life and Analytical Sciences) at 355 nm excitation and 460 nm emission.

Western Immunoblot Analysis of ZIP8 Protein Glycosylation. Cell pellets were dissolved in denaturing buffer (0.5% SDS and 1% β-mercaptoethanol), and protein concentrations of each cell lysate sample were determined. Cell lysate (5 μg of protein) was denatured at 37°C for 30 min. Nonidet P-40 and sodium phosphate (pH 7.5, 25°C) were added to make final concentrations of 1% and 50 mM, respectively. PNGase F (500 U; New England Biolabs, Beverly, MA) was added, and the mixture was incubated at 37°C for 2 h. Samples were then denatured with SDS-PAGE loading buffer (37°C for 30 min), and then run on a 10% SDS-PAGE gel before immunoblotting. Next, these protein samples, separated by SDS-PAGE gels, were transferred to nitrocellulose and blotted (Dalton et al., 2005). For detection, a rabbit affinity-purified polyvalent anti-ha antibody (α-ha; Bethyl Laboratories; Montgomery, TX) was used at 1/5000 dilution.

Z-Stack Confocal Microscopy for Detecting Cell Surface-Bound ZIP Proteins. Zinc depletion of FBS was carried out using Chelex 100. In brief, a solution of 5% (w/v) Chelex 100 resin in FBS was incubated overnight, with constant stirring, followed by filtration through a 0.2-μm filter. This FBS was then added into DMEM to make Chelex 100 medium. Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA) were seeded onto (noncoated) cover slips in a 24-well plate. The next day, each well of cells was transfected with the combination of the following plasmids—pRevTet-Off and pRevTRE-ZIP8—according to the manufacturer's transfection protocol for Lipofectamine 2000 (Invitrogen). Some wells of these cells were transfected with the plasmid encoding green fluorescent protein fused with Na^+-HCO_3^- cotransporter-1 protein GFP-NBC1 (Li et al., 2004). Two days after transfection, the cells were incubated in Chelex 100 medium for 1 h and then fixed with 3% formaldehyde for 20 min. Next, the cells were permeabilized with 0.1% Triton X-100 for 4 min and blocked with 10% FBS-containing PBS medium for 1 h. Fixation, permeabilization, and blocking were all done at room temperature. The α-ha (Bethyl Laboratories), at 1:500 dilution, was incubated at 4°C overnight with cells in 1% bovine serum albumin containing PBS. The next day, the primary antibody solution was removed, and the cells were washed three times with PBS for 5 min each time. The secondary antibody Alex488-α-rabbit or Alex568-α-rabbit (Invitrogen) was incubated with the cells at room temperature for 1 h. Next, cells transfected with GFP-NBC1 were mounted for confocal analysis; cells that were not transfected with GFP-NBC1 were costained with an apical membrane marker peanut agglutinin (PNA)-lectin (Invitrogen) at 1/200 dilution for 1 h at room temperature and then mounted for confocal analysis. Images were taken on a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany). Both Z-line and Z-stack images were obtained, using the LSM5 Image software to analyze specific cellular membrane targeting.

Statistical Analysis. Statistical significance between groups was determined by analysis of variance between each group and Student's t test. All assays were performed in duplicate or triplicate and repeated at least twice. Statistical analyses were performed with the use of SAS statistical software (SAS Institute Inc., Cary, NC). The determinations of K_m and V_{max} values for ZIP8, and TD_{50} values for divalent cation-induced cell death, were determined using Sigma Plot (SPSS Inc., Chicago, IL).

Results

We have shown previously that ZIP8 is a robust rogue cadmium transporter and is not expressed in MFF cells. Addition of a C-terminal ha tag does not alter cadmium transport by ZIP8, and rvZIP8 cells possess cadmium uptake properties that are clearly separable from wild-type Tet-off cells or the control rvLUC cells (Dalton et al., 2005). Therefore, rvZIP8 cells or rvZIP8ha cells may be used interchangeably to investigate the transport properties of ZIP8.

Temperature-Dependent Transport. Temperature sensitivity is a salient feature of many carrier-mediated, energy-dependent transport processes. ZIP8-mediated cadmium uptake was more than four times greater at 37°C than at 25°C (Fig. 1A). At 4°C, ZIP8-mediated cadmium transport was indistinguishable from that seen in rvLUC cells, and very close to background. Between 25°C and 37°C, we found that the temperature coefficient Q_{10} (for review, see Bennett, 1984; http://www.csupomona.edu/~seskandari/physiology/physiological_calculators/Q10.html) was 3.25 for rvZIP8 cells. A Q_{10} of ~1.0 represents the physical diffusion of ions or molecules, whereas a Q_{10} of >2.1 denotes an energy-dependent activity. These data confirm that the ZIP8 transporter-mediated process requires cellular energy. Cadmium uptake by rvLUC cells was only slightly influenced by temperature changes.

ATP-Dependent Transport. An active transporter can move its substrate "uphill," or against, a concentration gradient of the substrate. The driving force can be the energy from ATP hydrolysis (in this case, the transporter being called a "pump"); the driving force can also be the energy stored in the electrochemical gradient of a coupled substrate. In this case, a "symporter" carries two substrates in the same direction, whereas an "antiporter" carries two substrates in opposite directions. Symporters and antiporters are both examples of cotransporters.

KCN is a strong inhibitor of the mitochondrial respiratory chain and significantly decreases ATP production—thus further disrupting the electrochemical gradient across the cell membrane. KCN (0.5 mM) was added to rvZIP8 cells at 5, 15, or 30 min before the addition of $^{109}CdCl_2$ (Fig. 1B); at 5 min of pretreatment, KCN completely inhibited cadmium uptake. KCN had no effect on cadmium uptake in the control rvLUC cells, even during the longest incubation period. These data,

combined with the Q_{10} temperature coefficient data, confirm that cadmium uptake is an active process, requiring energy—either directly from ATP, or indirectly from energy stored in the electrochemical gradient across the plasma membrane.

Dependence of Transport on pH_{out}. Some cotransporters use protons as the coupled ion. The concentration gradient of protons across the cell membrane provides energy for the transport of the substrate. The SLC11A2 (DMT1) protein, for example, cotransports divalent metal ion (such as Fe^{2+} or Cd^{2+}) with protons into the cells; as would be expected, SLC11A2 functions optimally at pH_{out} 5.5 (Mackenzie and Hediger, 2004). We adjusted the pH of the uptake medium at intervals between 5.5 and 8.0 and examined cadmium uptake (Fig. 1C). After incubation, the pH of uptake solutions was again measured and, under these conditions, did not vary by more than 0.1 pH units. Control rvLUC cells, as well as rvZIP4 cells, had only a slight change in cadmium uptake. On the contrary, rvZIP8 cells were very sensitive to pH changes, having a maximal activity at pH 7.5—which is the physiological pH of most tissue fluids. This result indicates that, unlike SLC11A2, the ZIP8 protein is probably not a proton-coupled transporter.

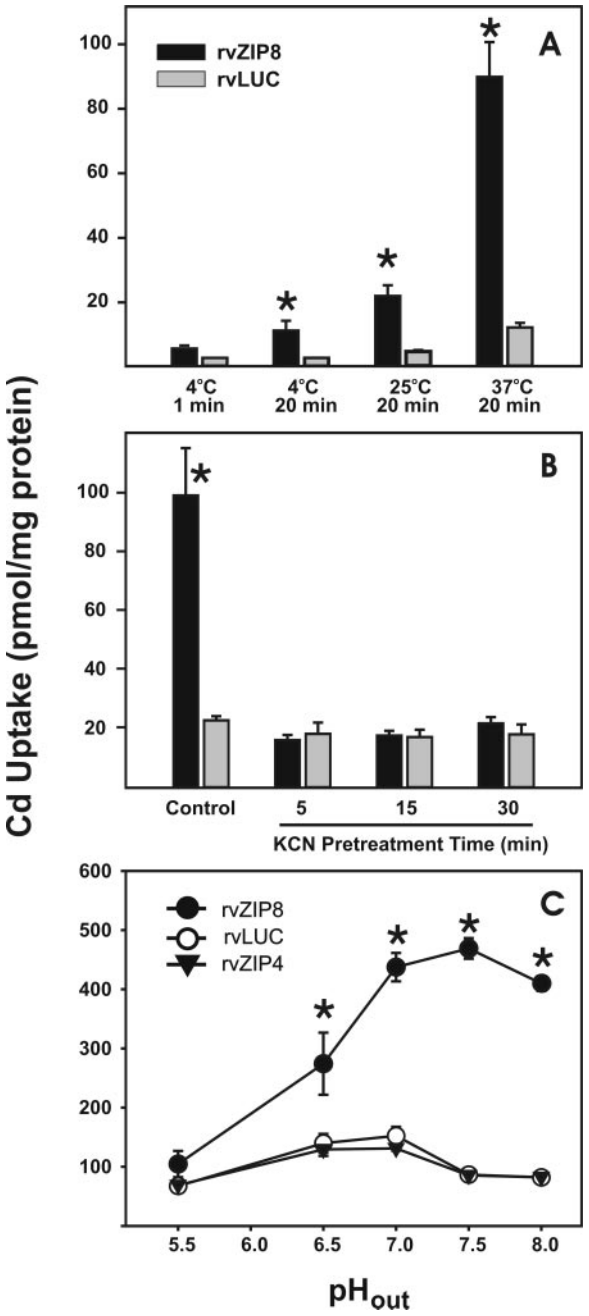

Fig. 1. Effects of temperature (A), KCN pretreatment (B), and pH_{out} (C) on cadmium uptake in cultured rvZIP8 and rvLUC cells. In addition, the rvZIP4 cell line is shown in the pH study. The temperature and KCN dependence studies were carried out in DMEM with 10% FBS as uptake medium to more closely approximate physiological conditions. On the other hand, the pH_{out} study (extracellular pH of the medium) was performed in HBSS to allow adjustment of the pH more easily. Asterisks denote significant ($P < 0.05$) differences between rvZIP8 and rvLUC in A and B, and between rvZIP8 and both rvLUC and rvZIP4 in C (Student's t test).

Metal Cation Competition Experiments. Figure 2 shows the degree to which each of 10 metal ions competes with cadmium uptake. We found that Mn^{2+} and Hg^{2+} were the two best inhibitors, with the order of the inhibitory effect as $Mn^{2+} > Hg^{2+} \gg Pb^{2+} = Cu^{2+} = Zn^{2+} = Cs^{2+}$. This result suggests that ZIP8 may have a very high affinity for Mn^{2+}.

Cadmium and Manganese Uptake Kinetics. Cadmium was shown previously to be taken up by rvZIP8 cells (Dalton et al., 2005). When the rvZIP8 cells were studied in HBSS, the kinetics of cadmium transport (Fig. 3A) confirmed that the process fits the Michaelis-Menten model very well, with a Hill coefficient of 0.92, a V_{max} of 92.1 pmol/mg/min, and a K_m value of 0.62 μM.

Because manganese was found to be the most potent inhibitor of cadmium uptake (Fig. 2), we carried out direct transport studies with radiolabeled manganese (Fig. 3B). Under our experimental conditions, manganese uptake in rvZIP8 cells was 5-fold greater than that by rvLUC cells over a broad concentration range. After subtraction of rvLUC uptake from rvZIP8 uptake, the ZIP8 transporter was found to have a high affinity for Mn^{2+}: a Hill coefficient of 0.71, a

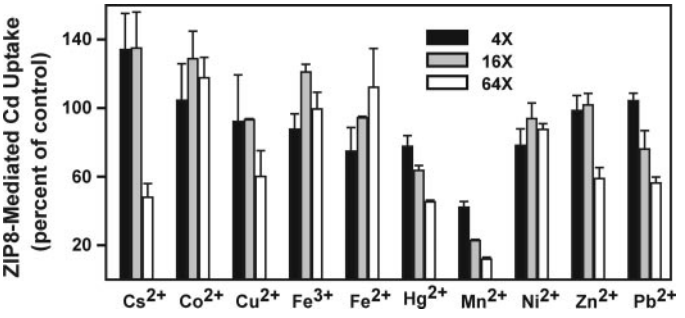

Fig. 2. Metal cation competition for cadmium uptake in rvZIP8 cells. $^{109}CdCl_2$ was added to make a final cadmium concentration of 0.25 μM; the competing metal cations (in chloride or nitrate anion) at concentrations of 0, 1, 4. or 16 μM were added at the same time as cadmium, and the cells were incubated at 37°C for 20 min, after which cadmium accumulation was determined. Because Pb^{2+} precipitates in chloride-rich medium, we tested Pb^{2+} as the acetate salt, and its uptake medium was gluconate-HBSS. The Ag^{1+} ion (no effect on cadmium uptake; data not shown) likewise precipitates with chloride and was also studied in gluconate-HBSS. All the other metals were chloride salts, and these studies were carried out in regular HBSS containing Cl^-. Mn^{2+} was significantly ($P = 0.0007$) inhibitory at 1 μM compared with 0 μM Mn^{2+} added, and significantly ($P = 0.0007$) inhibitory at 16 μM compared with 4 μM Mn^{2+} added. Hg^{2+} was significantly ($P = 0.0305$) inhibitory at 1 μM compared with 0.25 μM cadmium added; inhibitory trends seen with Cs^{2+}, Cu^{2+}, Zn^{2+}, and Pb^{2+} were not statistically significant ($P > 0.05$). These two-tailed P values were calculated from Student's t test with 4 degrees of freedom.

V_{max} of 73.8 pmol/min/mg of protein, and a K_m of 2.2 μM. These parameters are very similar to that of cadmium kinetics, indicating that the ZIP8 protein behaves similarly in transporting either manganese or cadmium.

Heavy-Metal-Induced Cell Death. ZIP8, which transports cadmium with high affinity, greatly sensitizes cells to cadmium-induced cell death (Dalton et al., 2005). We therefore studied rvZIP8 cell viability in the presence of the 10 cations that had been tested above for competitive inhibition of cadmium uptake. Figure 4, A, B, and C, shows that rvZIP8 cells were most sensitive to cadmium, and much more sensitive than rvLUC cells to manganese or Hg^{2+} toxicity, with TD_{50} values of 4 versus 11 μM for Mn^{2+} and 40 versus 65 μM for Hg^{2+}, respectively. No differential toxicity was observed for any of the other metals (data not shown). We found no difference in zinc toxicity between rvZIP8 cells and rvLUC cells (Fig. 4D), even though zinc uptake (at 10 μM) in rvZIP8 cells is at least twice as rapid as that in rvLUC cells (not shown). One possible explanation is that zinc might be complexed or compartmentalized within the cell (Outten and O'Halloran, 2001); thus, an elevation in total cellular zinc might not mean a proportionate increase in free zinc and therefore might not reflect a proportionate increase in zinc toxicity. The Fig. 4 data strongly suggest that—in addition to cadmium—manganese and mercury are also specific substrates for ZIP8. Furthermore, these results are consistent with the metal ion competition experiments of Fig. 2.

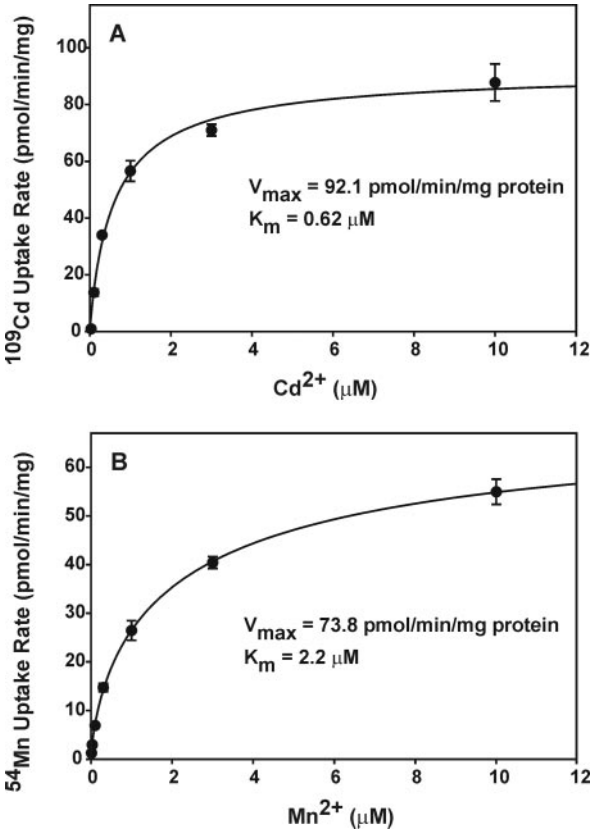

Fig. 3. Comparison of the kinetics of cadmium (A) and manganese (B) uptake in rvZIP8 cells. The cells were incubated (with the indicated concentration of cation) for 20 min at 37°C. The closed circles and brackets denote the means ± S.E. for five experiments done each time in triplicate.

Effects of Sodium, Potassium, or Chloride Substitution on Cadmium Uptake. ZIP8 has no ATP-binding domains, and no ATP hydrolysis activity has been reported. Therefore, we reasoned that ZIP8 is probably a cotransporter that uses an electrochemical gradient of one or more ions as the driving force for the active transport of cadmium (and perhaps manganese and other endogenous metals). Sodium (Na^+) is the most commonly coupled ion for animal cells. When Na^+ moves into the cell and down its electrochemical gradient, the coupled substrate is "dragged" into the cell, or pumped out of the cells, along with Na^+. The greater the electrochemical gradient for Na^+, the greater will be the transport rate of the substrate. Therefore, we tested the dependence of cadmium uptake on a Na^+ gradient by substituting the Na^+-containing uptake medium with *N*-methyl-D-glucamine—an inert organic cation generally believed not to be carried by any transporter (Fig. 5A). We found no change in cadmium uptake, whatsoever, indicating that ZIP8 does not cotransport Na^+ with cadmium.

Animal cells maintain a slight negative charge on the intracellular side of the plasma membrane; this membrane potential is generated by a "leaky" outward potassium (K^+) current, driven by a K^+ gradient across the membrane (high K^+ inside the cell; low K^+ outside). During electrogenic transport processes in which the substrate may be either a charged or a neutral molecule, the membrane potential is an important driving force. For example, if the net charge that ZIP8 brings into the cell is positive, an increase of the membrane potential in the negative direction can accelerate the velocity of cadmium uptake by ZIP8. Membrane potential can be altered by modifying extracellular K^+ concentration. We found that ZIP8-mediated cadmium uptake was not influenced by altering the K^+ concentration (Fig. 5B); we therefore conclude that ZIP8-mediated transport is independent of K^+ and membrane potential alteration.

Chloride (Cl^-) ion is another candidate for coupled ion(s). After the same rationale, we substituted Cl^- in the uptake medium with the inert organic anion, gluconate. No influence upon cadmium uptake was found (Fig. 5C); thus, we conclude that ZIP8 does not cotransport Cl^- with cadmium.

Dependence of Cadmium Uptake on Bicarbonate. To test the effects of extracellular HCO_3^- on cadmium uptake, we grew the cells in HBSS with varying concentrations of HCO_3^- but with the same osmolar pressure and same pH, 7.5. Figure 6A shows that cadmium uptake was dependent on the presence of HCO_3^-; with increasing concentrations of HCO_3^-, ZIP8-mediated uptake of cadmium increased as well. At 4 mM HCO_3^-, cadmium uptake had already reached its maximum. This finding justifies all the earlier transporter experiments in this article, in which 4.17 mM HCO_3^- in HBSS had been used.

The "zero" mM HCO_3^- in rvZIP8 cells in HBSS (Fig. 6A) is actually not free of HCO_3^-, because there is exogenous HCO_3^- from dissolved CO_2 in the air, as well as the CO_2/HCO_3^- derived from metabolism in the cells. From the Henderson-Hasselbach equation of this buffer system, $pH = pK + \log([HCO_3^-]/0.03 \times pCO_2)$, one can calculate that putatively "HCO_3^--free" medium actually contains 171 μM HCO_3^- at 37°C and pH 7.5; this is probably the reason why we found substantial amounts of ZIP8-mediated cadmium uptake in "~0 mM HCO_3^-" HBSS.

To further confirm the role of HCO_3^-, we added 4,4′-diiso-

thiocyanostilbene-2,2′-disulfonic acid (DIDS), a well known competitive HCO_3^- transporter inhibitor (Cabantchik and Greger, 1992), to the 4.17 mM HCO_3^--containing HBSS medium before adding cadmium (Fig. 6B). At 1 mM DIDS, a concentration that is regularly used to inhibit 4 mM HCO_3^- uptake, DIDS was found to impede ZIP8-mediated cadmium uptake by more than half. When HCO_3^- concentrations were decreased to negligible amounts (171 μM), low doses of DIDS almost completely abolished ZIP8-mediated cadmium uptake: ZIP8-mediated cadmium uptake was lowered by 0.25 μM DIDS from 110 to 20 pmol/mg (Fig. 6B). Hence, DIDS is more effective at low HCO_3^- concentrations than at high HCO_3^- concentrations. These data strongly suggest that ZIP8 mediates HCO_3^--dependent cadmium uptake, consistent with ZIP8 being a Cd^{2+}/HCO_3^- symporter.

Glycosylation of the ZIP8 Protein. Many plasma membrane and secretory proteins contain one or more carbohydrate chains linked via asparagine (*N*-linked) or via threonine or serine (*O*-linked). Glycosylation is a common post-translational modification of proteins in eukaryotic cells. We found two potential *N*-linked glycosylation sites in the ZIP8 protein sequence, Asn-40 and Asn-88; no predicted *O*-linked glycosylation sites were found. PNGase F treatment, followed by Western immunoblot analysis, was employed to confirm this prediction. *N*-glycosidase F, also known as PNGase F, is an amidase that cleaves between the innermost *N*-acetylglucosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoprotein (Maley et al., 1989). PNGase F will not cleave *N*-linked glycans containing core α1,3-fucose. After PNGase F treatment (Fig. 7), there is a significant shift in apparent molecular mass of the ZIP8ha protein band—from ~80 kDa to ~50 kDa—the latter of which is close to the calculated molecular mass of the core ZIP8ha protein. The ZIP8ha protein is 51.3 kDa with the signal peptide, and 49.5 kDa without the signal peptide. The heterogeneity of ZIP8ha bands in the 80-kDa region is probably due to the varying degrees of saturation of available glycosylation sites. It is interesting to note that, after PNGase F treatment of ZIP4ha, on the other hand, any cleavage resulting in the lower-molecular-weight band was negligible.

Localization of ZIP8 to the Apical Surface of Polarized Cells. Western immunoblot analysis had shown only that the ZIP8 protein is detected in the membrane fraction of MFF cells, and confocal images showed clearly that ZIP8 protein is localized, in large part, on the plasma membrane of MFF cells (Dalton et al., 2005). It should be noted that ZIP8 expression on intracellular membranes was also detected in the cell confocal pictures; this probably represents ZIP8 protein procession and transportation in the endoplasmic reticulum, Golgi apparatus, and "transporting" vesicles.

Uptake of cadmium into living cells is a vectorial process that happens in the epithelial cells of the small intestine, the lung, and the kidney reabsorption tubules. Epithelial cells are polarized cells. Their apical sides face the lumen and basolateral sides face the basal membrane and tissues; these two sides of epithelial cells have very different expression patterns of transporters and represent the structural basis for their distinct substrate-handling capacity. In this regard, MFF cells are not the best system to study ZIP8 protein, because they are not polarized cells. We therefore chose MDCK cells, a distal convoluted tubule polarized epithelial cell line (Simmons et al., 1984), to study further the subcellular localization of the ZIP8 protein.

We confirmed that the ZIP8ha protein is localized predominantly on the MDCK cell surface membrane (Fig. 8). GFP fused to the NBC1 is known to be localized on the basolateral membrane of MDCK cells (Li et al., 2004). Z-stack analysis (Fig. 8, left) clearly shows that the ZIP8 protein localization is distinct from the basolaterally localized GFP-NBC1 pro-

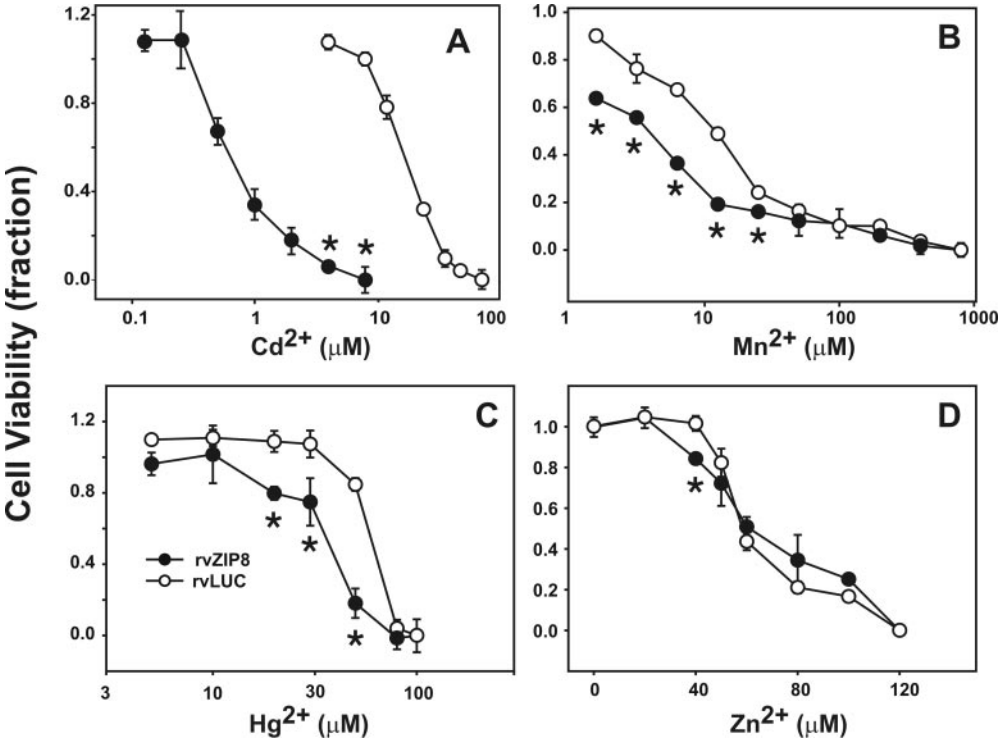

Fig. 4. Cell killing by 32-h exposure to cadmium (A), Mn^{2+} (B), Hg^{2+} (C) or Zn^{2+} (D) in rvZIP8 versus rvLUC cells. A, B, and C are semi-log plots, whereas D is a linear plot. Asterisks denote significant ($P < 0.05$) differences between rvZIP8 and rvLUC—at the indicated concentration of the indicated cation (Student's *t* test). Pb^{2+} toxicity could not be done, because chloride-rich medium precipitates Pb^{2+}, and the gluconate solution is perishable in less than 32 h at 37°C.

tein. Moreover, ZIP8ha was localized to the surface of MDCK cells that is in contact with the culture medium. Taken together, these data suggest that ZIP8 is localized on the apical surface of this polarized epithelial cell line. This conclusion is further supported by the observation that ZIP8ha is localized to the same cell surface as the apical marker PNA-lectin (Fig. 8, right). PNA-lectin is known to bind exclusively to the apical membrane (Cooper, 1984; Li et al., 2004). As recent studies have shown (Li et al., 2004), PNA-lectin binds to only a subset of MDCK cells and, because transient transfection is only effective in allowing expression in a subset of cells, ZIP8 and PNA-lectin were not easily colocalized. These markers, however, could be visualized in adjacent cells in many microscopic fields, and their localization consistently showed a similar membrane localization pattern.

In general, the apical-basolateral orientation of a transporter in a polarized epithelial cell of one organ is almost always consistent with the localization pattern in epithelial cells of other organs (Li et al., 2004). Thus, our result implies that the ZIP8 protein takes up endogenous or exogenous metals—from food (in small intestine), blood (in endothelial

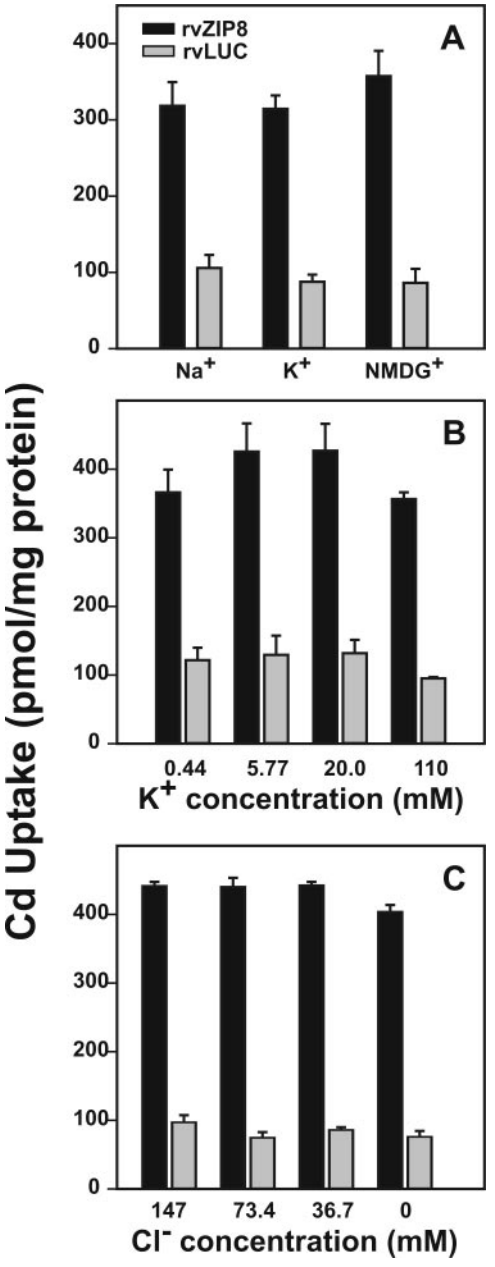

Fig. 5. Effects of Na^+ (A), K^+ (B), or Cl^- (C) substitution on cadmium uptake in rvZIP8 and rvLUC cells. NMDG+, *N*-methyl-D-glucamine. In all cases, cells were incubated with 0.25 μM cadmium, containing radiolabeled $^{109}CdCl_2$, for 20 min at 37°C. The closed bars are not significantly ($P > 0.05$) different from one another in any of the panels (analysis of variance), and the stippled bars are not significantly ($P > 0.05$) different from one another in any of the panels (analysis of variance).

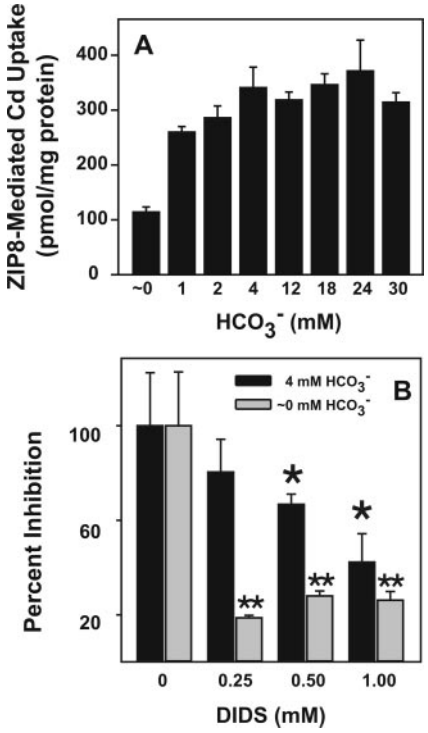

Fig. 6. Dependence of cadmium uptake on bicarbonate in rvZIP8 cells. A, cadmium uptake as a function of HCO_3^- concentration in HBSS. B, percentage inhibition of cadmium uptake as a function of increasing amounts of DIDS, a well known competitive inhibitor of HCO_3^- transporters. The DIDS solution was prepared fresh in DMSO and added to the uptake medium 30 min before addition of the $^{109}CdCl_2$. For HBSS (~4 mM), the medium contained 4.17 mM HCO_3^-. For HCO_3^--depleted HBSS (~0 mM), the medium contained nominal amounts (171 μM) of HCO_3^-. In all cases, cells were incubated with 0.25 μM cadmium, containing radiolabeled $^{109}CdCl_2$, for 20 min at 37°C. *, mean is significantly ($P < 0.05$) different from the mean when no DIDS was present at 4 mM HCO_3^- (Student's *t* test). **, mean is significantly ($P < 0.005$) different from the mean when no DIDS was present at 171 μM HCO_3^- (Student's *t* test).

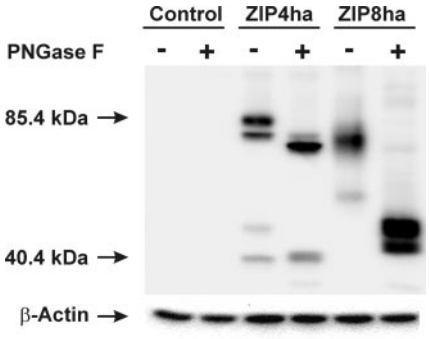

Fig. 7. Western immunoblot of control, ZIP4ha, and ZIP8ha proteins, with or without treatment with PNGase F, which cleaves glycoproteins. Lane-loading was confirmed by the protein β-actin.

cells), or glomerular filtrate (in renal proximal or distal tubules)—into tissues or organs where they are distributed.

Discussion

There are currently a total of 360 putatively functional genes, divided into 46 families, within the solute carrier gene (*SLC*) superfamily (http://www.bioparadigms.org/slc/menu.asp). The ZIP transporter proteins comprise the SLC39 family, having 14 members—highly conserved orthologs—between mouse and human. A protein is assigned to a specific family if it shows at least 20 to 25% amino acid sequence identity to other members of that family. In plants, several ZIP proteins have been implicated in zinc, Fe^{2+}, and manganese transport (Eide, 2004); some plant ZIP proteins, such as Irt1 and TcZNT1, are capable of transporting cadmium (Hall and Williams, 2003). In mammals, all ZIP proteins cloned to date are able to transport zinc at micromolar concentrations; until our recent report (Dalton et al., 2005), no transport study of cadmium by a ZIP protein in vertebrates had previously been reported. The mouse *Slc39a8* gene encodes ZIP8.

Our study shows that mouse ZIP8 can transport cadmium very efficiently. This uptake process is: energy-dependent; optimal at pH 7.5 [and, thus, ZIP8 is unlikely to be a proton pump (Fig. 1)]; independent of any K^+, Na^+, or Cl^- gradient; and dependent on HCO_3^- in the transport medium (Fig. 6). Physiological concentrations of HCO_3^- range from ~20 mM in glomerular filtrate, and ~29 mM in extracellular fluids such as blood, to ~12 mM inside most types of animal cells (http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid = mcb. table.4057). Thus, it is reasonable to speculate that ZIP8 is a Cd^{2+}/HCO_3^- symporter, with the HCO_3^- gradient as the driving force for cadmium uptake. Zinc uptake in yeast by Zrt1 and Zrt2 has been shown to be energy-dependent (Zhao and Eide, 1996a,b). Zinc uptake by human ZIP1 and ZIP2 has been shown to be independent of K^+ or Na^+ gradients (Gaither and Eide, 2000, 2001). Furthermore, HCO_3^- dependence has been noted for ZIP2-mediated zinc uptake (Gaither and Eide, 2000).

In various types of cultured cells, a stimulatory effect of HCO_3^- on cadmium uptake has been reported. These cells include human erythrocytes (Lou et al., 1991), rat and rabbit erythroid cells (Savigni and Morgan, 1998), and LLC-PK1 cells (epithelial cell line originally derived from porcine kidneys) (Endo et al., 1998); these stimulatory effects can be blocked by DIDS, a specific inhibitor of HCO_3^--dependent transporters. No specific transporters, however, had been identified in these cell types. Experiments presented herein may shed light into the underlying molecular mechanism of these studies on HCO_3^- stimulation of cadmium uptake.

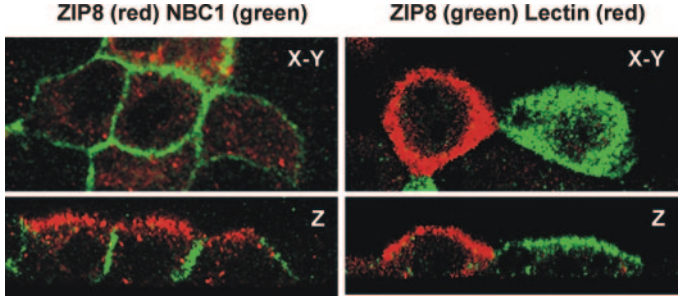

Fig. 8. Z-stack confocal microscopy of MDCK cells, showing that NBC1-GFP transporter is basolateral (left), whereas ZIP8ha (left and right) and PNA-lectin (right) are apical. Expression vectors carrying the indicated transporter proteins were transiently transfected into cells. After 2 days, cells were fixed, blocked and incubated with primary anti-ha antibody, then the secondary fluorescence antibody. Stained cells were examined under a Zeiss 510 laser-scanning confocal microscope. X-Y and Z denote the three axes examined. NBC1, sodium bicarbonate-1 transporter; GFP, green fluorescent protein.

The toxicological implications of HCO_3^- dependence in the intact animal are intriguing. The HCO_3^- concentration is 24 mM in the glomerular filtrate but is efficiently reabsorbed as it passes through the proximal convoluted tubule (PCT). The concentration drops to ~4 to 6 mM when it reaches the S3 segment, the straight portion of the PCT, and is negligible by the time the filtrate reaches the collecting duct. One major complication of cadmium toxicity is kidney damage, which is manifested as proximal renal tubular acidosis. We speculate that the apical localization of ZIP8, along with its HCO_3^- dependence, may explain why cadmium damage to the kidney is almost exclusively localized to the PCT; thus, ZIP8 may be the main cadmium transporter in the kidney, and it functions well only in the PCT because of the presence of high HCO_3^- concentrations there.

For the first time among mammalian ZIP proteins, our study has demonstrated that ZIP8 has an extremely high affinity for cadmium (Fig. 3A). The K_m of ZIP8 is only 0.62 μM in our established mouse cell culture system, which is half as much as the K_m of SLC11A2 (DMT1) for cadmium when the DMT1 transporter mRNA was expressed in *Xenopus laevis* oocytes (Okubo et al., 2003). SLC11A2 is believed to be important in cadmium uptake from the small intestine. Given the fact that SLC11A2 is located apically, it will be important to determine the cell-type-specific expression of ZIP8, because ZIP8 is a likely candidate for cadmium uptake from the intestine, lung, glomerular filtrate, and plasma. Indeed, uptake from the plasma into the testis is predominantly mediated by ZIP8 (Dalton et al., 2005). It will also be important to determine the patterns of expression and transport characteristics of the other ZIP transporters.

Cadmium is not an essential metal. Cadmium levels in the environment have risen markedly because of industrialization; therefore, cadmium has begun to pose a risk to human populations only during the past 150 years. Hence, human and other animals are not expected to be "genetically prepared" to handle this noxious metal, and cadmium must be a "hitchhiker" using one or more existing transporters. What, then, are the physiological substrates of mammalian ZIP8?

Our radiolabeled manganese uptake study (Fig. 3B) confirms that ZIP8 has a very high affinity for manganese. Because manganese is an essential metal, ZIP8 may act principally as a Mn^{2+} transporter under physiological conditions. The metal concentration and the transporter affinity will determine, in general, the metal(s) transported. The K_m of 2.2 μM for Mn^{2+} is close to physiological concentrations: manganese in mammalian tissues ranges between 0.3 and 2.9 μg of Mn^{2+}/g wet tissue weight (Rehnberg et al., 1982; Keen and Zidenberg-Cherr, 1994). The K_m of 2.2 μM for Mn^{2+} is also within the same range determined in many cell lines or tissues: 2 μM for HepG2 cells (Finley, 1998); 18 μM for glial cells of the chick cerebral cortex (Wedler et al., 1989); 0.3 μM for rat astrocytes (Aschner et al., 1992); and 32.2 μM for Caco-2 cells (Leblondel and Allain, 1999). Very little is known about manganese transport pathways at the molecular level; manganese competition experiments have sug-

gested that some mammalian ZIP proteins might be capable of transporting manganese, but no direct transport study has been reported until the present study. That cadmium and manganese might share the same transport mechanism was speculated in studies with a cadmium-resistant cell line (Yanagiya et al., 2000)—although their apparent K_m values were much lower (40 nM for Cd^{2+}; 36 nM for Mn^{2+}) than those in the present study (Fig. 3). Rat spinal cord dorsal horn neurons express ancient conserved domain protein-4 (ACDP4); recently, ACDP4 was found by a yeast two-hybrid system to interact specifically with an intracellular metal ion chaperone in human embryo renal cortical HEK293 cells and enhance cell killing by Cu^{2+}, Co^{2+}, and Mn^{2+}(Guo et al., 2005).

Zinc uptake was reported to be increased when human ZIP8, termed BIGM103 in that study, was expressed in Chinese hamster ovary cells (Begum et al., 2002). It is possible, therefore, that ZIP8 is also an important zinc transporter in certain cell types devoid of other efficient zinc transporters.

All of the above studies cited are from cell culture experiments. One must be cautious in extrapolating, into the intact animal, the conclusion that ZIP8 is a specific manganese or zinc transporter, because the ability of this protein to transport a particular cation does not necessarily mean that this protein is physiologically well used. Maintaining metal homeostasis is essential for the function of many important enzymes, transcription factors, and other subcellular proteins. Disruption of metal homeostasis can sometimes lead to disease, and even death. Thus, *Slc39* gene deletion, or overexpression—at the whole-animal level—would be definitive in assessing the physiological role of a transporter. For example, human ZIP4 is a well established essential zinc transporter, because the loss of ZIP4 function causes zinc-deficient acrodermatitis enteropathica, a disease marked by skin lesions on the extremities, and immune and reproductive dysfunction, as a result of decreased uptake of dietary zinc by enterocytes in the small intestine (Kury et al., 2002).

Western blot and confocal analysis of MFF cells have shown that ZIP8 is localized mainly in the plasma membrane (Dalton et al., 2005). In the present study, using MDCK cell cultures as a model system, we have determined that the expressed recombinant ZIP8 protein is localized on the apical side of these polarized epithelial cells (Fig. 8). This observation with ZIP8 is similar to many reports showing that ZIP1, ZIP2, ZIP3, and ZIP4 are located in the apical side of mouse enterocytes and visceral yolk sac epithelial cells. However, ZIP5 is located on the basolateral membrane in enterocytes, acinar cells, visceral endoderm cells, and cultured MDCK cells (Dufner-Beattie et al., 2004; Wang et al., 2004). Thus, ZIP5 is the only ZIP protein so far found to be localized on the basolateral side of polarized cells. It is noteworthy that—in addition to the apical localization—ZIP8 also shows cytoplasmic distribution, raising the possibility of trafficking between the intracellular compartments and the membrane during physiological and pathophysiological states.

The vectorial transport of a substrate, and the apical-basolateral expression pattern of a specific transporter, generally are quite similar in many ways—across various types of epithelial cells. This is one of the reasons why MDCK cells have become the standard model system for studying polarized epithelial cells. The present study supports the notion that most ZIP protein members, including ZIP8, are internalizing metal ions from the environment, from the blood or glomerular filtrate, into tissues such as intestine, lung, pancreas, kidney tubules or endothelial cells of the testicular vasculature. The only exception is ZIP5, which seems to move metal ions in the reverse direction, from these tissues into the blood stream. Further studies in MDCK cells, *X. laevis* oocytes, *Slc39a8*-containing BAC-transgenic mice, and *Slc39a8*(−/−) knockout mice are underway to elucidate the physiological importance of this newly characterized ZIP8 transporter protein.

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