Zip14 is a complex broad-scope metal-ion transporter whose functional properties support roles in the cellular uptake of zinc and nontransferrin-bound iron

Jorge J. Pinilla-Tenas,1 Brian K. Sparkman,1 Ali Shawki,1 Anthony C. Illing,1 Colin J. Mitchell,1 Ningning Zhao,2 Juan P. Liuzzi,2 Robert J. Cousins,2 Mitchell D. Knutson,2 and Bryan Mackenzie1

1Department of Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, Ohio; and 2Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida

Submitted 19 November 2010; accepted in final form 6 June 2011

Materials and Methods

Reagents. Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Research Products International (Prospect, IL) unless otherwise indicated.

Expression of mouse Zip14 and human DMT1 in Xenopus oocytes. We performed laparotomy and ovarioectomy on adult female Xenopus laevis frogs (Nasco, Fort Atkinson, WI) under 3-aminoethylbenzoxoate methanesulfonate anesthesia (0.1% in 1:1 water/ice, by immersion) following a protocol approved by the University of Cincinnati Institutional Animal Care and Use Committee. Ovarian tissue was isolated and treated with collagenase A (Roche Diagnostics, Indianapolis, IN), and oocytes were isolated and stored at 17°C in modified Barths’ medium as previously described (28). We expressed in oocytes the Zip14 short isoform transcript, the product of the mouse SLC39A14 gene, GenBank sequence accession number BC021530, in pCMVSPORT6 as previously described (25, 35, 42), the major sites of organ damage in iron overload. Our previous data identified Zip14 as a candidate route for NTBI uptake since overexpression of Zip14 in human embryonic kidney (HEK)293, SF9, or HeLa cell lines stimulated NTBI uptake (14, 25), whereas small interfering RNA (siRNA) suppression of endogenous Zip14 in AML12 mouse hepatocytes decreased NTBI uptake (25).

We have expressed mouse Zip14 in RNA-injected Xenopus oocytes, an efficient heterologous expression system ideal for direct assays of membrane transport and tolerant of broad manipulation of experimental conditions. We used radiotracer assays to test the hypothesis that Zip14 transports free iron and to examine the functional properties and metal-ion substrate profile of Zip14.

Iron-Overload Conditions (e.g., thalassemia, hereditary hemochromatosis) are characterized by the appearance in plasma of nontransferrin-bound iron (NTBI) and result in cardiomyopathy, diabetes, hepatic cancer, and cirrhosis. Identification of the routes of cellular NTBI uptake will therefore provide novel targets for therapeutics.

Zrt- and Irt-like protein-14 (Zip14) is a member of a large family of mammalian metal-ion transporters, the SLC39 gene family (6, 11, 12, 22, 26). Zip14 (synonyms SLC39A14, KIAA0062) is strongly expressed in the intestine (25, 35) but its subcellular localization there is not yet clear. Notably, Zip14 is abundantly expressed in the liver, heart, and pancreas (25, 35, 42), the major sites of organ damage in iron overload. Our previous data identified Zip14 as a candidate route for NTBI uptake since overexpression of Zip14 in human embryonic kidney (HEK)293, SF9, or HeLa cell lines stimulated NTBI uptake (14, 25), whereas small interfering RNA (siRNA) suppression of endogenous Zip14 in AML12 mouse hepatocytes decreased NTBI uptake (25).

We have expressed mouse Zip14 in RNA-injected Xenopus oocytes, an efficient heterologous expression system ideal for direct assays of membrane transport and tolerant of broad manipulation of experimental conditions. We used radiotracer assays to test the hypothesis that Zip14 transports free iron and to examine the functional properties and metal-ion substrate profile of Zip14.

Iron-Overload Conditions (e.g., thalassemia, hereditary hemochromatosis) are characterized by the appearance in plasma of nontransferrin-bound iron (NTBI) and result in cardiomyopathy, diabetes, hepatic cancer, and cirrhosis. Identification of the routes of cellular NTBI uptake will therefore provide novel targets for therapeutics.

Zrt- and Irt-like protein-14 (Zip14) is a member of a large family of mammalian metal-ion transporters, the SLC39 gene family (6, 11, 12, 22, 26). Zip14 (synonyms SLC39A14, KIAA0062) is strongly expressed in the intestine (25, 35) but its subcellular localization there is not yet clear. Notably, Zip14 is abundantly expressed in the liver, heart, and pancreas (25, 35, 42), the major sites of organ damage in iron overload. Our previous data identified Zip14 as a candidate route for NTBI uptake since overexpression of Zip14 in human embryonic kidney (HEK)293, SF9, or HeLa cell lines stimulated NTBI uptake (14, 25), whereas small interfering RNA (siRNA) suppression of endogenous Zip14 in AML12 mouse hepatocytes decreased NTBI uptake (25).

We have expressed mouse Zip14 in RNA-injected Xenopus oocytes, an efficient heterologous expression system ideal for direct assays of membrane transport and tolerant of broad manipulation of experimental conditions. We used radiotracer assays to test the hypothesis that Zip14 transports free iron and to examine the functional properties and metal-ion substrate profile of Zip14.

MATERIALS AND METHODS

Reagents. Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Research Products International (Prospect, IL) unless otherwise indicated.

Expression of mouse Zip14 and human DMT1 in Xenopus oocytes. We performed laparotomy and ovarioectomy on adult female Xenopus laevis frogs (Nasco, Fort Atkinson, WI) under 3-aminoethylbenzoxoate methanesulfonate anesthesia (0.1% in 1:1 water/ice, by immersion) following a protocol approved by the University of Cincinnati Institutional Animal Care and Use Committee. Ovarian tissue was isolated and treated with collagenase A (Roche Diagnostics, Indianapolis, IN), and oocytes were isolated and stored at 17°C in modified Barths’ medium as previously described (28). We expressed in oocytes the Zip14 short isoform transcript, the product of the mouse SLC39A14 gene, GenBank sequence accession number BC021530, in pCMVSPORT6 as previously described (25, 27, 47) under the SP6 promoter. We linearized the pCMVSPORT6-Zip14 construct using HpaII and synthesized RNA in vitro using the mMESSAGE mMACHINE/SP6 RNA polymerase transcription kit (Applied Biosystems/Ambion, Austin, TX) according to the manufacturers’ protocols. Human divalent metal-ion transporter-1 (DMT1) isoform 1A/IRE(+) RNA was prepared as described (30, 41). Defolliculate stage V-VI oocytes were injected with 50 ng of RNA and incubated for 3–5 days (Zip14) or 6 days (DMT1) before being used in functional assays.

We also expressed in oocytes enhanced green fluorescence protein (EGFP) fusion proteins of Zip14 and DMT1. To construct N-terminal-EGFP-Zip14 (EGFP-Zip14) in pCMVSPORT6, we used forward (5’-CTGGCGCCGCTCTACTACTACGCGCCACCC-3’) and reverse (5’-GGAGGTTGGACACTGAGGCGGGCGACG-3’) primers to amplify the EGFP sequence from pEGFP-N1 (Clontech, Mountain View, CA) flanked by Spiol restriction sites and ligated the EGFP sequence into the N-terminal region of pCMVSPORT6-Zip14 at an Spiol restriction site we created by site-directed mutagenesis. The
NH2-terminal-EGFP-Zip14 sequence was then cut out and ligated into the pOX(+) oocyte expression vector (30) using KpnI and NotI. COOH-terminal-EGFP-tagged DMT1 (DMT1-EGFP), a gift of Dr. Elizabetta Nemeth and Dr. Bo Qiao (David Geffen-UCLA School of Medicine), was generated by subcloning human DMT1 isoform 1A/H11001 and replacing of CaCl2 with additional MgCl2.

**Media for functional assays in oocytes.** We prepared ion-substituted media as follows:

- For functional assays, we prepared ion-substituted media as follows:
  - For functional assays, we prepared ion-substituted media as follows:
    - 2 mM CaCl2, and 1 mM MgCl2, and buffered using 0–5 mM piperazine (GFS Chemicals, Columbus, OH) to obtain pH 7.5 or as otherwise indicated. Media were supplemented with 1 mM t-ascorbic acid. Oocytes were then solubilized using 3% Nonidet P-40 and RNA synthesized as before. Oocytes were injected with 50 ng of RNA and incubated 4 (EGFP-Zip14) or 6 days (DMT1-EGFP) before being used for confocal microscopy or immunoblotting of oocyte membrane fractions.

**Analysis of EGFP-Zip14 and DMT1-EGFP fusion-protein expression in oocytes.** We imaged EGFP-Zip14 and DMT1-EGFP protein expression in the oocyte by using the Zeiss LSM 7 DUO confocal laser-scanning microscope (excitation at 488 nm) fitted with the EC Plan-Neofluar ×10/0.3 and LD C-Apochromat ×40/1.1 W Korr objectives to measure emission in the band 500–531 nm at a pinhole setting of 9.9 μm.

**Western blot analysis of membrane fractions from oocytes expressing EGFP-Zip14 or DMT1-EGFP.** The total membrane fraction from homogenates of oocytes (≈20 of each) expressing EGFP-Zip14 or DMT1-EGFP by sucrose-density fractionation as described (5), except that we added protease inhibitor cocktail set I (EMD Biosciences, Gibbstown, NJ) to all solutions. We used the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) to estimate protein concentration. Oocyte membrane fractions containing ~3 μg of total protein were mixed with Laemmli buffer (1× final concentration), heated for 30 min at 37°C, and electrophoretically separated on a sodium dodecylsulfate (SDS)-polyacrylamide gel (7.5% acrylamide). Proteins from the gel were transferred to an OptiTRAN BA-85 nitrocellulose blotting membrane (Whatman, Piscataway, NJ). The blot was incubated for 1 h in blocking solution (5% nonfat dry milk in Tris-buffered saline (TBS), pH 7.4, containing 0.01% Tween 20 (TBST), followed by a 1-h incubation with a 1:5,000 dilution of anti-EGFP Mab-2 mouse antibody (Thermo Scientific, Rockford, IL). The blot was washed in TBST and then incubated for 40 min with a 1:5,000 dilution of Zymax goat anti-mouse IgG horseradish peroxidase conjugate (Invitrogen, Carlsbad, CA). After washing in TBST and TBS was completed, immunoreactivity was visualized by using SuperSignal West Pico enhanced chemiluminescent substrate (Thermo Scientific) and X-ray film. We performed reversible Ponceau staining (40) of the blot to obtain an index of protein loading in each lane. Signal intensities of the immunoreactive bands and of Ponceau staining were quantified by densitometry by using GENETOOLS software (SynGene, Frederick, MD).

**RESULTS**

**EGFP-Zip14 expression in Xenopus oocytes.** We used confocal laser-scanning microscopy to image the expression of an NH2-terminal EGFP-fusion protein of murine Zip14 (EGFP-Zip14) in RNA-injected oocytes (Fig. 1). We observed strong fluorescence throughout most of the plasma membrane but no detectable intracellular fluorescence. We also examined the expression of a COOH-terminal EGFP-fusion protein of hu-
man DMT1 isoform 1A/IRE(+) (DMT1-EGFP), DMT1-EGFP was expressed throughout the plasma membrane, consistent with the pattern of anti-DMT1 immunofluorescence (same isoform) described previously (30). We detected no fluorescence in control oocytes. Whereas the expression pattern of EGFP-Zip14 was similar to that of DMT1-EGFP, fluorescence was more intense in oocytes expressing DMT1-EGFP; however, since EGFP fluorescence varies depending on the microenvironment and steric mobility, fluorescence signals for the two fusion proteins are not directly comparable.

For a semiquantitative comparison of expression levels, we used Western blot analysis (anti-GFP) of membrane fractions isolated from oocytes expressing EGFP-Zip14 or DMT1-EGFP (Fig. 2). We observed for DMT1-EGFP two strong bands at ≈80 and ≈110 kDa. The band at ≈110 kDa likely represents glycosylated DMT1 (3, 30). For EGFP-Zip14, we observed three strong bands, at ≈40, ≈80, and ≈170 kDa. The band at ≈40 kDa may represent a Zip14 monomer, precursor, or degradation product, whereas the band at ≈170 kDa likely represents an oligomer. We performed densitometric analysis of immunoreactive bands and used as an index of gel loading the densities of reversible Ponceau staining (40) of the blot (not shown). After normalizing by the amount of protein loaded (which was ~25% more for DMT1-EGFP than for Zip14-EGFP), we found that the ratio of protein expression between DMT1-EGFP and Zip14-EGFP was 1.7. We considered the possibility that the 40-kDa Zip14-EGFP band represents a nonfunctional peptide, in which case the ratio of protein expression is 2.9; however, the 40-kDa band is immunoreactive with anti-Zip14 antibody, and we do not expect any synthesis of free EGFP in the oocyte system, so we suspect the 40-kDa band represents a degradation product. In either event, the two proteins are expressed in oocytes on the same order, DMT1-EGFP modestly higher in this preparation (see Discussion for a comparison of their functional activities).

Zip14 mediates cellular uptake of free iron. We expressed murine Zip14 in RNA-injected oocytes and used radiotracer assays to characterize its functional properties. In the presence of L-ascorbic acid, expression of Zip14 stimulated up to 150-fold the uptake of 2 μM 55Fe2+ compared with that in control oocytes (Fig. 3). 55Fe2+ accumulation was linear from 2 min up to at least 2 h (Fig. 3A). Subsequent transport experiments were conducted over 10 min, within the linear phase of 55Fe2+ uptake (except see Fig. 6A). Zip14-mediated 55Fe2+ uptake was saturable (Fig. 3B); the Fe2+ concentration at which 55Fe2+ uptake was half-maximal (K_0.5 [Fe]) was 2.3 ± 0.5 μM. The Hill coefficient (n_st) for Fe2+ was ≈1, indicating a lack of cooperativity. Whereas Zip14 readily transported Fe2+ in the presence of ascorbate, the uptake of 55Fe added as FeCl3 in the presence of the Fe(III) chelator nitritiotriacetic acid (NTA) and in the absence of an exogenous reducing agent did not differ between control oocytes and oocytes expressing Zip14 (Fig. 3C). Therefore, Zip14 transports ferrous ion (Fe^{2+}) and not ferric ion (Fe^{3+}).

Properties of Zip14-mediated Fe2+ transport. Zip14-mediated 55Fe2+ transport was inhibited by 10-fold excess concentrations of Cd2+, Co2+, Mn2+, Ni2+, Pb2+, or Zn2+ (Fig. 4A). Whereas a 10-fold excess of unlabeled Fe2+ inhibited 55Fe2+ uptake less potently (by 57 ± 7% SE) than we expected (~90%) for a simple homogeneous system (44), Cd2+, Co2+, and Zn2+ afforded complete inhibition of 55Fe2+ transport.

We found that Zip14-mediated 55Fe2+ transport was temperature dependent in the tested range 16–28°C (Fig. 4B).
Arrhenius plot was linear over this temperature range and the apparent activation energy (E_a) was 15 ± 2 kcal/mol. An alternative index of temperature dependence, the factor by which activity increased with every 10-degree increase in T (Q10), was 2.4 ± 0.3 (obtained from the fit parameters of a 3-parameter single-exponential growth function; adjusted r² = 0.91, P = 0.003). Zip14-mediated 55Fe²⁺ transport was pH sensitive (Fig. 4C). Zip14 was active over a narrow pH range, the optimal pH was 7.5, and Zip14-mediated 55Fe²⁺ transport was abolished at pH 6.0 and below.

Comparison of iron-transport activities mediated by Zip14 and DMT1. We compared 55Fe²⁺ transport in oocytes expressing Zip14 and DMT1. DMT1 is known to be maximally stimulated at low pH (30). The 55Fe²⁺ transport activity at pH 5.5 in oocytes expressing DMT1 was 1.6-fold (±0.5-fold SE) the 55Fe²⁺ transport activity at pH 7.5 in oocytes expressing Zip14 (Fig. 5). We verified that the 55Fe²⁺ transport activities of the EGFP-fusion proteins of Zip14 and DMT1 were similar to those of their nontagged counterparts (data not shown). Given the slightly higher protein levels for DMT1 compared with Zip14 (Fig. 2), these data indicate that Zip14 and DMT1 mediate similar 55Fe²⁺ fluxes per functional unit, i.e., the turnover rates of the transport cycle are similar.

Ion dependence of Zip14-mediated Fe²⁺ transport. When we applied voltage-clamp protocols as described previously (30, 31, 41) in studies of DMT1, we did not observe any metal-ion-evoked currents or presteady-state currents in oocytes expressing Zip14 (data not shown). We provisionally conclude that Zip14 is not rheogenic (i.e., net charge movement is zero) and considered that Zip14-mediated Fe²⁺ uptake may be associated with an anion influx or countertransport of (i.e., exchange with) (a) cation(s). Since HCO₃⁻ stimulated zinc uptake via human Zip2 expressed in K562 erythroleukemia cells (13), we tested the effects of HCO₃⁻ on Zip14 activity. We found that addition of 30 mM HCO₃⁻ to the medium stimulated by 130% ± 6% (SE) the uptake of 2 μM 55Fe²⁺ in oocytes expressing Zip14 (Fig. 6A). In a second preparation, we found that excess Zn²⁺ completely inhibited Zip14-mediated 55Fe²⁺ transport both in the absence and presence of HCO₃⁻ (data not shown, P < 0.001); in a third preparation, excess Mn²⁺ inhibited Zip14-mediated 55Fe²⁺ transport in the absence of HCO₃⁻ (by 40% ± 11%, SE) to a similar degree to that in the presence of HCO₃⁻ (by 65% ± 5%, SE) (data not shown; P = 0.06 for absence cf. presence of HCO₃⁻). Cl⁻ replacement with the organic anion isethionate had no effect on Zip14-mediated 55Fe²⁺ uptake (Fig. 6B). These data may be interpreted in one of two ways, such that Zip14-mediated iron-transport activity is stimulated by extracellular HCO₃⁻ but is not associated with an anion influx; or 2) is associated with a nonspecific anion influx and that HCO₃⁻ is a preferred anion. In any event, Zip14 does not appear to be an obligatory HCO₃⁻ cotransporter (although nominally HCO₃⁻-free media will contain micromolar amounts of HCO₃⁻ arising from atmospheric CO₂ alone).

Zip14-mediated 55Fe²⁺ transport was dependent on the extracellular Ca²⁺ concentration (Fig. 6C). 55Fe²⁺ transport was half-maximal at Ca²⁺ concentration of 0.4 ± 0.1 mM (i.e., apparent Kₘ(Ca²⁺)). In separate experiments (not shown), we found that the effect of raising the Ca²⁺ concentration from 0.3 to 2.0 mM was to increase nearly fivefold the flux in control oocytes (P = 0.018, by Student’s t-test) without effect on Kₘ(Ca²⁺) (P = 0.96) (data were fit by Eq. 1); i.e., increasing Ca²⁺ accelerated iron transport without altering the affinity of Zip14 for Fe²⁺.

Metal-ion substrate profile of Zip14. The ZIP (SLC39) family of transporters is known primarily for its role in zinc homeostasis (22), but some ZIP transporters are capable of also transporting other metal ions. We therefore examined the substrate profile of Zip14 by direct measurement of radiotracer uptake. As well as stimulating the uptake of 55Fe²⁺, expression of Zip14 in oocytes increased the uptake of 2 μM 110Cd²⁺, 54Mn²⁺, and 65Zn²⁺ (Fig. 7A); of these, the flux of 55Fe²⁺ was the greatest and 54Mn²⁺ the lowest. Zip14 expression did not alter the ratio of 64Cu²⁺ uptake either in the presence or absence of l-aspartic acid compared with control oocytes (Fig. 7B). Therefore, Zip14 is capable of transporting Fe²⁺, Cd²⁺, Mn²⁺, and Zn²⁺ but not Cu⁺ or Cu²⁺.

Properties of Zip14-mediated Zn²⁺ transport. The uptake of 65Zn²⁺ in oocytes expressing Zip14 was saturable (Fig. 8A);
the Zn\textsuperscript{2+} concentration at which 65Zn\textsuperscript{2+} uptake was half-maximal (K_{0.5}^\text{65Zn\textsuperscript{2+}}) was 1.9 ± 0.6 \mu M and was not significantly different from the K_{0.5}^\text{55Fe\textsuperscript{2+}} we obtained for 55Fe\textsuperscript{2+} transport (Fig. 3B) (P = 0.66, by Student’s t-test). Zip14-mediated uptake of 2 \mu M 65Zn\textsuperscript{2+} was strongly inhibited by 10-fold excess unlabelled Zn\textsuperscript{2+} (by 88% ± 3% SE) and only weakly by Cd\textsuperscript{2+} (56% ± 3%) but was only marginally inhibited by Fe\textsuperscript{2+} (14% ± 5%) and not by any other metal ion tested (Fig. 8B). Therefore, the metal-ion inhibition profile for 65Zn\textsuperscript{2+} transport differed from that for 55Fe\textsuperscript{2+} transport.

We found that Zip14-mediated 65Zn\textsuperscript{2+} transport was temperature dependent in the tested range 15–30°C (Fig. 8C). The Arrhenius plot was linear over this temperature range; the apparent E_a was 14 ± 2 kcal/mol and was not significantly different from that obtained for 55Fe\textsuperscript{2+} transport (Fig. 4B) (P = 0.65, by Student’s t-test). HCO_3\textsuperscript{-} stimulated 65Zn\textsuperscript{2+} transport in oocytes expressing Zip14 (data not shown, P < 0.001) in the same manner as it did for 55Fe\textsuperscript{2+} transport (Fig. 6A). Zip14-mediated 65Zn\textsuperscript{2+} transport was pH sensitive (Fig. 8D). The optimal pH for 65Zn\textsuperscript{2+} transport was 7.5 (as for 55Fe\textsuperscript{2+} transport, Fig. 4C); however, 65Zn\textsuperscript{2+} transport activity was observed over a broader pH range than was 55Fe\textsuperscript{2+} transport, and 65Zn\textsuperscript{2+} transport activity persisted at pH 6.0 or lower. Zip14-mediated transport of 2 \mu M 65Zn\textsuperscript{2+} was not affected by replacement of extracellular Ca\textsuperscript{2+} by Mg\textsuperscript{2+} (Fig. 8E). Therefore, whereas Zip14 transported Fe\textsuperscript{2+} and Zn\textsuperscript{2+} with identical K_{0.5} and temperature-dependence parameters, the properties of Zip14-mediated 65Zn\textsuperscript{2+} transport differed from those of 55Fe\textsuperscript{2+} transport with respect to the metal-ion inhibition profile, pH dependence, and Ca\textsuperscript{2+} dependence.

Metal-ion inhibition profiles for Zip14-mediated Cd\textsuperscript{2+} and Mn\textsuperscript{2+} transport. Uptake of 2 \mu M 109Cd\textsuperscript{2+} was inhibited by 10-fold excess unlabeled Cd\textsuperscript{2+} or Zn\textsuperscript{2+} but not by any other metal ion tested (Fig. 9A). In contrast, the uptake of 2 \mu M 54Mn\textsuperscript{2+} was inhibited by a broad range of divalent metal ions at 10-fold excess, including Cd\textsuperscript{2+}, Co\textsuperscript{2+}, Fe\textsuperscript{2+}, Ni\textsuperscript{2+}, Pb\textsuperscript{2+}, and Zn\textsuperscript{2+} (Fig. 9B). Therefore the metal-ion inhibition profile for Zip14-mediated Cd\textsuperscript{2+} transport resembles that for Zn\textsuperscript{2+} transport (Fig. 8B), whereas the metal-ion inhibition profile for Zip14-mediated Mn\textsuperscript{2+} transport resembles that for Fe\textsuperscript{2+} transport (Fig. 4A). Likewise, the pH ranges of Zip14-mediated Cd\textsuperscript{2+} and Mn\textsuperscript{2+} transport activities resembled those of Zn\textsuperscript{2+} and Fe\textsuperscript{2+} transport activities, respectively (data not shown). HCO_3\textsuperscript{-} stimulated Zip14-mediated 109Cd\textsuperscript{2+} transport (interaction P = 0.040) and 54Mn\textsuperscript{2+} transport (interaction P < 0.001) (data not shown) in the same manner as it did for 55Fe\textsuperscript{2+} transport (Fig. 6A); however, whereas HCO_3\textsuperscript{-} markedly stimulated transport of Fe\textsuperscript{2+} and Mn\textsuperscript{2+}, HCO_3\textsuperscript{-} only modestly stimulated transport of Zn\textsuperscript{2+} and Cd\textsuperscript{2+}.

Zip14-mediated Ca\textsuperscript{2+} transport and the effects of divergent metal ions. Since we found Zip14-mediated 55Fe\textsuperscript{2+} transport to be Ca\textsuperscript{2+} dependent, we examined whether Zip14 could mediate the influx of 45Ca\textsuperscript{2+} (at physiological concentrations) either in the presence or absence of divalent metal ions. To reduce background activity, we included in the media 100 \mu M niflumic acid since we found (in experiments not shown) that doing so reduced by 86% ± 20% (SE) the endogenous uptake of 150 \mu M 45Ca\textsuperscript{2+} in control oocytes. Expression of Zip14 stimulated the uptake of 45Ca\textsuperscript{2+} at 150 or 300 \mu M in the absence of divalent transition metal ions (Fig. 10, A and B). Notably, the 45Ca\textsuperscript{2+} fluxes at these concentrations were lower than the fluxes we had obtained for 55Fe\textsuperscript{2+} or 65Zn\textsuperscript{2+} transport at ~1/100th the concentration (Figs. 3 and 8). 45Ca\textsuperscript{2+} transport was inhibited by

Fig. 4. Properties of Zip14-mediated Fe\textsuperscript{2+} transport. A: metal-ion inhibition profile of Zip14-mediated 55Fe\textsuperscript{2+} transport. Uptake of 2 \mu M 55Fe\textsuperscript{2+} in the absence (None) or presence of a range of candidate inhibitor metal ions each at 20 \mu M, in the presence of 1 mM L-ascorbic acid, in control oocytes (gray bars) and oocytes expressing Zip14 (black bars) (n = 10–14). Within Zip14, all metals inhibited 55Fe\textsuperscript{2+} uptake (P < 0.001). B: temperature (T) dependence of Zip14-mediated uptake of 2 \mu M 55Fe\textsuperscript{2+} (n = 9–15). Data were fit by Eq. 2 to obtain activation energy (E_a) = 15.2 ± 2.0 kcal/mol, ln(A) = 25.0 ± 3.5 (adjusted r^2 = 0.90, P < 0.001). For clarity, control data are not displayed. C: uptake of 2 \mu M 55Fe\textsuperscript{2+} as a function of extracellular pH in oocytes expressing Zip14 (black symbols and line, n = 27–30). Within Zip14, uptakes at each pH differed from one another (unadjusted P < 0.001) except pH 6.5 cf. pH 8.5 (unadjusted P = 0.20), pH 7.5 cf. pH 8.0 (unadjusted P = 0.44), and pH 6.0 cf. pH 5.5 (unadjusted P = 0.54). For this experiment, uptakes in control oocytes (gray symbols and line) were tested only at pH 5.5 and 8.5 (n = 31–32). Zip14 did not differ from control at pH 5.5 (unadjusted P = 0.77) but did at pH 8.5 (unadjusted P = 0.004).

Fig. 5. Comparison of the iron-transport activities of mouse Zip14 and human DMT1. Uptake of 2 \mu M 55Fe\textsuperscript{2+} was measured at pH 5.5 and 7.5 in control oocytes (gray bars) and oocytes expressing mouse Zip14 (black bars) or human DMT1 isoform 1A/IRE(+) (hatched bars) (n = 9–12). ANOVA, P < 0.001; *Unadjusted P = 0.90 cf. control at pH 5.5; *unadjusted P = 0.52 cf. control at pH 7.5; DMT1 at pH 5.5 differed from Zip14 at pH 7.5 (unadjusted P < 0.001).
lower concentrations of unlabeled Cd\(^{2+}\) (30 \(\mu\text{M}\)) or Zn\(^{2+}\) (15 \(\mu\text{M}\)) but not by Fe\(^{2+}\) (30 \(\mu\text{M}\)) (Fig. 10, A and B).

**DISCUSSION**

Functional properties of Zip14 support a role in the cellular uptake of nontransferrin-bound iron. We have examined the functional properties of mouse Zip14 expressed in RNA-injected Xenopus oocytes and found that Zip14 is capable of transporting free iron. Zip14 is specific for ferrous ion (Fe\(^{2+}\)), whereas ferric ion (Fe\(^{3+}\)) is not transported. It is generally considered that most NTBI is present in plasma as Fe(III)citrate; however, a significant portion of the iron at the plasma membrane is expected to be reduced since 1) plasma l-ascorbic acid concentrations are typically \(\approx\)70 \(\mu\text{M}\), 2) mammalian hepatocytes (34) and other cell types (19, 20) express surface ferrireductases, and 3) citrate is capable of forming chelates of both Fe(II) and Fe(III).

Zip14-mediated \(^{55}\text{Fe}^{2+}\) uptake was saturable (\(K_{d}^{\text{Fe}^{2+}}\approx 2 \mu\text{M}\)), temperature-dependent (apparent activation energy, \(E_a\) = 15 kcal/mol), Ca\(^{2+}\) dependent (\(K_{d}^{\text{Ca}^{2+}}\) = 0.4 \(\mu\text{M}\)), and inhibited by Co\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\). These data agree well with those obtained from measurements of NTBI uptake in other preparations. For example, NTBI uptake in the perfused rat liver was temperature dependent (\(E_a\) = 14 kcal/mol), Ca\(^{2+}\) dependent (\(K_{d}^{\text{Ca}^{2+}}\) = 0.6 \(\mu\text{M}\)), and inhibited by Co\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\). The \(K_{d}^{\text{Fe}^{2+}}\) obtained in the study just cited was 16 \(\mu\text{M}\), higher than the \(K_{d}^{\text{Fe}^{2+}}\) we obtained in oocytes expressing mouse Zip14; however, higher \(K_{d}^{\text{Fe}^{2+}}\) estimates are expected in perfusion studies since binding by other membrane proteins cannot easily be controlled. In a second study, iron uptake in isolated rat hepatocytes was mediated by a high-affinity transport system (\(K_{d}^{\text{Fe}^{2+}}\) = 1.3 \(\mu\text{M}\)) that was Ca\(^{2+}\) dependent (\(K_{d}^{\text{Ca}^{2+}}\) of 0.6–0.75 \(\mu\text{M}\)); however, inhibition by other divalent metal ions was not observed in that preparation (2).

Substantial increases in plasma NTBI are characteristic of iron-overload disorders (e.g., thalassemia, hereditary hemochromatosis) (1, 8, 16, 18, 37). Consistent with the functional properties of Zip14, a role for Zip14 in NTBI uptake in vivo is supported by the tissue distribution of Zip14 and its subcellular localization. Zip14 is a plasma-membrane protein (25) that is abundantly expressed in the liver, heart, and pancreas (35, 42), the three organs that preferentially accumulate iron during iron overload.

Zip14 is a complex, broad-scope metal-ion transporter. Zip14 also mediated the uptake of \(^{109}\text{Cd}^{2+}\), \(^{54}\text{Mn}^{2+}\), and \(^{65}\text{Zn}^{2+}\) but not \(^{64}\text{Cu}^{(I \text{ or II})}\). Zip14-mediated \(^{65}\text{Zn}^{2+}\) uptake in oocytes also was saturable (\(K_{d}^{\text{Zn}^{2+}}\approx 2 \mu\text{M}\)) and displayed kinetic properties reminiscent of zinc uptake in cultured rat hepatocytes (38). Notably, the properties of Zn\(^{2+}\) transport in oocytes expressing Zip14 differed from those of Fe\(^{2+}\) transport. \(^{109}\text{Cd}^{2+}\) and \(^{65}\text{Zn}^{2+}\) transport escaped inhibition by all metals except those two (Fe\(^{2+}\) afforded only weak inhibition of \(^{65}\text{Zn}^{2+}\) transport), whereas \(^{55}\text{Fe}^{2+}\) and \(^{54}\text{Mn}^{2+}\) transport was inhibited by every divalent metal ion tested. This observation reminds us of the importance of determining the substrate profile of transporters by directly measuring transport of each candidate substrate instead of relying on inferences from the inhibition profile for just one radiolabeled test substrate. At
least one group has relied on the latter approach and interpreted the lack of inhibition of Zip14-mediated $^{65}$Zn$^{2+}$ uptake by Fe$^{2+}$ in viral-infected mouse fetal fibroblasts (15) to contradict our earlier observation that Zip14 could transport iron (25). Given the likelihood that Zip14 shares basic mechanistic properties in common with other members of the mammalian SLC39 family of Zip transporters, the substrate profiles of metals share the first translocation pathway. Zn$^{2+}$ ing for the first translocation pathway and by inhibiting the 65Zn$^{2+}$ transport was Ca$^{2+}$ dependent, whereas 65Zn$^{2+}$ transport was not, and that Cd$^{2+}$ and Zn$^{2+}$, but not Fe$^{2+}$, inhibited the modest $^{45}$Ca$^{2+}$ fluxes observed in oocytes expressing Zip14. A model that can explain these observations is one in which there exist two metal-ion translocation pathways within Zip14. Fe$^{2+}$ transport via the first of these translocation pathways is dependent on (i.e., functionally coupled with) Ca$^{2+}$ transport via the second. Mutual inhibition of Fe$^{2+}$ and Mn$^{2+}$ transport activities indicates that these metals share the first translocation pathway, Zn$^{2+}$ and Cd$^{2+}$ however are capable of coordinating with both pathways, thereby completely inhibiting Fe$^{2+}$ transport both by competing for the first translocation pathway and by inhibiting the Ca$^{2+}$ transport otherwise supporting Fe$^{2+}$ transport. The additional ionic species that account(s) for the apparent net neutral transport activity of Zip14 are (is) not presently understood.

We provisionally conclude that Zip14 is not rheogenic (i.e., net charge movement is zero) based on the lack of any metal-ion-evoked currents in oocytes expressing Zip14. While the ratio of protein expression of DMT1-EGFP relative to human ZIP4 and ZIP5 are zinc-specific transporters based on the lack of inhibition by other metals (9, 10, 45) may need to be reexamined.

The pattern of incomplete mutual inhibition observed here between the divergent metal ions tested is not consistent with a single saturable transport process (44). Whereas Zip14-mediated $^{55}$Fe$^{2+}$ transport was completely inhibited by excess Zn$^{2+}$, we found that Fe$^{2+}$ only marginally inhibited $^{65}$Zn$^{2+}$ transport. We found that $^{55}$Fe$^{2+}$ transport was Ca$^{2+}$ dependent, whereas $^{65}$Zn$^{2+}$ transport was not, and that Cd$^{2+}$ and Zn$^{2+}$, but not Fe$^{2+}$, inhibited the modest $^{45}$Ca$^{2+}$ fluxes observed in oocytes expressing Zip14. A model that can explain these
that each operates as a monomer). Whereas DMT1 exhibits large Fe^{2+}-evoked currents (30), Zip14 does not. Fluxes of up to 2.5 pmol/min observed in nonclamped Zip14-expressing oocytes in this study should correspond to a pure divalent metal-ion current of ~8 nA (converted using the Faraday) over an order of magnitude greater than the noise in the voltage clamp (~0.5 nA root mean square), and much larger currents would be expected under voltage clamp at ~70 mV. In a recent study (24), investigators reconstituted a bacterial homolog ZIPB into liposomes and observed a nonsaturable, voltage-dependent channel-like activity for ZIPB. However, bacterial ZIPB shares with mouse Zip14 only 15% identity at the amino acid level (pairwise alignment using VectorNTI software, Invitrogen, Carlsbad, CA), and ZIPB differs from Zip14 with respect to nearly every property tested, including its pH dependence, temperature dependence, saturation kinetics, substrate profile, and anion dependence (24), providing no basis for anticipating that ZIPB and Zip14 share a common mechanism. Moreover, currents observed for ZIPB were specific for zinc and were obtained with millimolar zinc concentrations and we expect Zip14 to be functional, albeit suboptimally, at pH 7.5. We therefore expect that wherever Zip14 is expressed on plasma membranes, such as is observed in hepatocytes (25), this transporter should be capable of mediating cellular uptake of NTBI characteristic of iron-overload conditions. The observation that zinc potently inhibits iron uptake suggests that Zip14 expressed at the plasma membrane should primarily serve zinc transport under normal conditions.

Zip14 may also participate in transferrin-associated iron uptake in hepatocytes (47) by mobilizing iron from early endosomes to cytoplasm. Suppression of Zip14 expression by siRNA resulted in the inhibition of iron assimilation in HepG2 cells by ~50% (47). Iron is liberated from the transferrin-transferrin receptor complex after only relatively modest endosomal acidification (50% dissociation at pH ~6.5; Ref. 36), and we expect Zip14 to be functional, albeit suboptimally, at pH 6.5. In contrast, DMT1, which is H^{+} coupled and maximally stimulated at low pH (Fig. 5) (30, 31), may serve as the predominant route of iron mobilization from late endosomes and lysosomes, and DMT1 is essential for erythroid iron assimilation (17).

Our previous study indicated that, in mice, Zip14 is most highly expressed in the small intestine among the tissues tested (25). Although its plasma-membrane localization and apical/basolateral distribution in intestine is yet to be established, Zip14 could play a role in zinc and iron acquisition in the expression in hepatocytes and lead to Zip14-dependent zinc accumulation (23, 27). ChIP assays have shown AP-1 association with the Zip14 promoter and Zip14 mRNA, indicative of transcription, is increased by nitric oxide (23). Under such conditions there is an increase in Zip14 associated with the plasma membrane. These responses have been interpreted as physiological homeostatic responses to stresses and infections. How such responses are factored into pathophysiological outcomes, such as iron-overload disorders, warrants investigation.

**pH dependence of Zip14-mediated Fe^{2+} transport and its implications for cell-specific iron and zinc transport.** Our data indicated that Zip14 transports iron within a narrow pH range (pH ~6.5) and optimally at pH ~7.5. We therefore expect that wherever Zip14 is expressed on plasma membranes, such as is observed in hepatocytes (25), this transporter should be capable of mediating cellular uptake of NTBI characteristic of iron-overload conditions. The observation that zinc potently inhibits iron uptake suggests that Zip14 expressed at the plasma membrane should primarily serve zinc transport under normal conditions.
By 10.220.32.247 on June 27, 2017 http://ajpcell.physiology.org/ Downloaded from