Divalent metal-ion transporter-1 (DMT1) is a widely expressed mammalian iron transporter that is energized by the H⁺ electrochemical potential gradient (1–3). DMT1 plays critical roles in iron absorption and erythroid iron utilization (4–7). DMT1 exhibits promiscuity toward a broad range of metals of nutritional or toxicological importance; however, questions remain about which of these DMT1 actually transports, most notably copper (5, 8), and it is not known which metals rely upon DMT1 for their absorption.

We have found that, like Fe²⁺, a broad range of metal ions can evoke inward currents in *Xenopus* oocytes expressing DMT1 (1, 3); however, evoked currents are evidence of reactivity (as is also the inhibition of radiotracer transport) but do not provide evidence of transport per se, so direct measurement of transport is required. Whereas radiotracer assay is a robust and direct approach, the limited commercial availability of radioisotopes for candidate DMT1 substrates and the possibility of kinetic isotope effects (i.e., that DMT1 reactivity with the radioisotope may differ from its reactivity with the commonly occurring isotope) warranted the development of a method for the continuous monitoring of transport by fluorescence (CMTF) (9).

Our aims were as follows: 1) to determine the comprehensive substrate profile of DMT1 by direct methods for assaying metal-ion transport, and 2) to establish the order of selectivity for transported substrates under fixed conditions. First, we tested the hypothesis that DMT1 is a ferrous-ion (Fe²⁺) transporter that is also capable of transporting a broad range of transition metal ions (including copper), group-12 metal ions (zinc, cadmium, and mercury), and gallium by expressing DMT1 in RNA-injected *Xenopus* oocytes and determining metal-ion transport using radiotracer assays and CMTF with the metal-sensitive fluorescent probe PGSK. Second, we tested for competition between DMT1 substrates by using radiotracer assays. Finally, for those metal ions that we had found to be substrates...
Metal-ion Substrate Profile and Selectivity of Human DMT1

of DMT1, we determined the order of metal-ion selectivity by measuring metal-ion-evoked currents and determining saturation kinetics, taking advantage of the controlled conditions and internal controls achieved by using the voltage clamp.

We have previously found that calcium is not a transported substrate of DMT1 but instead is a low affinity noncompetitive inhibitor of DMT1 (K_i = 1–20 mM) (10). Therefore, we did not revisit calcium in this study.

EXPERIMENTAL PROCEDURES

Expression of Human DMT1 in Xenopus Oocytes—We performed laparotomy and ovariectomy on adult female *Xenopus laevis* frogs (Nasco, Fort Atkinson, WI) under 3-aminoethylbenzoate methanesulfonate anesthesia (0.1% w/v 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), and oocytes were isolated and stored at 17°C in modified Barth’s medium as described (11).

We expressed in *Xenopus* oocytes the 1A/IRE (+) isoform of DMT1, the product of the human SLC11A2 gene. We chose the 1A/IRE (+) isoform (so named because it is coded from an mRNA processing variant that is initiated from exon 1A and contains in its 3’-UTR an iron-responsive element (IRE)) because of the following: (i) it appears to be the predominant isoform expressed in enterocytes (12), and (ii) we found that it was expressed in oocytes more efficiently than the other three isoforms tested (3). The oocyte expression vector pOX (isoform expressed in enterocytes (12), and (ii) we found that it was expressed in oocytes more efficiently than the other three isoforms tested (3). The oocyte expression vector pOX (+) containing the 1A/IRE (+) DMT1 cDNA under the SP6 RNA polymerase promoter as described (3) was linearized using SnaBI (New England Biolabs Inc., Ipswich, MA), and RNA was synthesized in vitro using the mMESSAGE mMACHINE/SP6 RNA polymerase kit (Applied Biosystems/Ambion, Austin, TX) according to the manufacturers’ protocols. Defolliculate stage V–VI oocytes were injected with ~50 ng of human 1A/IRE (+) DMT1 RNA and incubated 4–6 days before being used in functional assays.

Reagents and Media—Reagents were obtained from Sigma or Research Products International Corp. (Prospect, IL) unless otherwise indicated. For functional assays, oocytes were superfused or incubated at room temperature (22–25°C) in low calcium transport media containing 100 mM NaCl, 1 mM KCl, 0.6 mM CaCl_2_, 1 mM MgCl_2_, buffered using 0–5 mM MES plus either 0–5 mM N’N’-diethylpiperazine or piperazine-1,4-bis(2-propanesulfonic acid) (all three buffers from GFS Chemicals, Columbus, OH) to obtain pH 5.2–7.5 as indicated and supplemented, where indicated, with l-ascorbic acid, nitritotriacetic acid (NTA), or l-histidine.

Radiotracer Transport Assays—We used $^{55}$Fe (added as FeCl_3_) at a final specific activity 0.31–1.6 GBq/mg$^{-1}$ and $^{54}$Mn (added as MnCl_2_) at final specific activity 276 MBq/mg$^{-1}$ (both obtained from PerkinElmer Life Sciences), $^{109}$Cd (added as CdCl_2_) at a final specific activity 87 MBq/mg$^{-1}$ and $^{65}$Zn (added as ZnCl_2_) at a final specific activity at 385 MBq/mg$^{-1}$ (both obtained from the Oak Ridge National Laboratory (Oak Ridge, TN)), and $^{64}$Cu (added as CuCl_2_) at a final specific activity 1.38 GBq/mg$^{-1}$ (obtained from Washington University, St. Louis School of Medicine (St Louis, MO)).

Radiotracer metal-ion uptake was measured over 10 min (except Fig. 1A, 20 min), i.e. within the linear portion of the time course of $^{55}$Fe$^{2+}$ uptake (3). We terminated radiotracer uptake by rapidly washing the oocytes three times in ice-cold pH 5.5 transport medium containing 1 mM l-ascorbic acid and (1 mM l-histidine in experiments involving copper). Oocytes were then solubilized by using 5% (w/v) SDS, and radiotracer content was assayed by liquid scintillation counting using Scintiscan-30% liquid scintillation mixture (Fisher).

Previously, we found that Fe$^{2+}$ saturation kinetics were described by a Hill function with a Hill coefficient for Fe$^{2+}$ ($n_{H}$) of 1 (Ref. 3), the Hill function thus being reduced to the Michaelis-Menten function. Radiotracer uptake data for 0.5–100 μM $^{55}$Fe$^{2+}$ were therefore fit by a modified Michaelis-Menten function (Equation 1) for which $V_{app}$ is the velocity (uptake) of $^{55}$Fe$^{2+}$; $V_{app}^{max}$ is the derived maximum velocity; $S$ is the Fe$^{2+}$ concentration, and $K_{0.5}$ is the substrate concentration at which velocity was half-maximal.

$$V_{app} = \frac{V_{app}^{max} \cdot S}{K_{0.5} + S}$$

(Eq. 1)

When we measured $^{55}$Fe$^{2+}$ uptake in the absence or presence of nonradioactive candidate inhibitor metal ions, we obtained instead $K_{0.5}^{app}$ (the apparent $K_{0.5}$ in the presence of inhibitor) by substituting these terms in Equation 1. Where we concluded that inhibition was competitive in nature, we then determined $K_{i}$, the inhibitor concentration eliciting half-maximal inhibition, using Equation 2 for competitive inhibition (13) for which $[I]$ is the experimental inhibitor concentration used (20 μM).

$$K_{0.5}^{app} = K_{0.5}^{app} \left(1 + \frac{[I]}{K_{i}}\right)$$

(Eq. 2)

We also measured $^{65}$Zn$^{2+}$ uptake in the absence or presence of 10 μM nonradioactive Fe$^{2+}$, and we fit our data by Equations 1 and 2 in which $V_{app}^{max}$, $V_{app}^{max, Fe}$, $K_{0.5}^{app}$, and $K_{0.5}^{app, Fe}$ were substituted by $V_{app}^{Zn}$, $V_{app}^{Zn, Fe}$, $K_{0.5}^{Zn}$, and $K_{0.5}^{Zn, Fe}$, respectively. $K_{i}$ is the derived Fe$^{2+}$ concentration eliciting half-maximal inhibition of $^{65}$Zn$^{2+}$ uptake.

Metal-sensitive Fluorophore for the Continuous Monitoring of Transport by Fluorescence—We developed an assay for CMTF (9) to examine DMT1-mediated metal-ion transport in oocytes. We chose for use in our assays PGSK (Molecular Devices, Sunnyvale, CA), a phenanthroline-based fluorophore with peak excitation at wavelength = 507 nm and peak emission at wavelength = 532 nm. PGSK fluorescence is known to be quenched by a range of metal ions, including Cd$^{2+}$, Cu$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, and Fe$^{3+}$ (14–16), without a shift in peak emission wavelength (17), but it is not quenched by K$^{+}$, Na$^{+}$, or Mg$^{2+}$ or by Ca$^{2+}$ at physiological concentrations (15, 16, 18).

Metal-ion Reactivity of PGSK in a Cell-free System—We verified the reactivity of PGSK (dipotassium salt) with a broad range of metal ions in a cell-free system. To do so, we measured fluorescence intensity of solutions containing 10 μM PGSK in water, alone or with chelators (1 mM l-ascorbic acid or NTA), with and without any of several transition metal ions, group-12 metal ions, and Ga$^{3+}$ over the range 0.1–300 μM by using the
Molecular Devices SpectraMax M2 fluorescent plate reader with these following settings: excitation at 507 nm, detection at 532 nm, and high pass filter at 530 nm. We normalized the fluorescence intensity (F) in the presence of metal by fluorescence intensity (F_0) in the absence of metal, and we took (F - F_0)/F_0 as an index of PGSK quenching (−Q). Data were fit by a one-site saturation ligand-binding function (Equation 3) to estimate the apparent relative affinity (K_q^M) of PGSK for each metal (M). Q_{max} is the derived maximum quenching and [M] is the concentration of metal.

\[
Q = \frac{Q_{\text{max}} \cdot [M]}{K_q^M + [M]} \quad \text{(Eq. 3)}
\]

**Fluorescence Imaging of Metal-ion Transport**—We injected control oocytes and oocytes expressing human DMT1 with 23.0 nl of a 10 mM solution (in water) of the cell-impermeant PGSK dipotassium salt (to obtain nominal intracellular concentration of 250 μM) 30–60 min prior to conducting fluorescence assays. We used the confocal laser-scanning microscope LSM510 (excitation at 514 nm) and the META detector (both Carl Zeiss Microscopy LLC, Thornwood, NY) to measure emission in the band 530–600 nm. We continuously superfused oocytes 1 min with transport medium (pH 5.5) in the absence of metal and then for 10 min with media containing metal ion (except Fig. 3, C, G, and H) and acquired images every 10 s. By using the AIM software suite version 4.2 (Carl Zeiss Microscopy), we randomly selected four regions of interest (ROI) within which we measured fluorescence intensity (F) as a function of time (t) after the addition of metal ion (at t = 0). We obtained first-order rate constants (k) for fluorescence quenching by fitting our data to a three-parameter exponential decay function (Equation 4) for which F_0 and F are fluorescence F at t = 0 and t = ∞, respectively.

\[
F = F_0 + F_e \cdot \exp(-k \cdot t) \quad \text{(Eq. 4)}
\]

Rate constants of fluorescence quenching in the presence of 0.5–100 μM Fe^{2+} were fit by the Michaelis-Menten function (Equation 1) in which V_{max} was substituted by the rate constant k_{Fe} for Fe^{2+} and V_{max} by the derived maximal rate constant k_{Fe}^M.

**Voltage Clamp Experiments**—We used the two-microelectrode voltage clamp (Dagan CA-1B amplifier) to measure currents in control oocytes and oocytes expressing human DMT1 as described (3, 10). Microelectrodes with resistance 0.5–5 megohms were filled with 3 M KCl. Voltage clamp experiments included two protocols. (i) Continuous current recordings were made at a holding potential (V_h) = −70 mV, low pass filtered at 1 Hz, and digitized at 10 Hz. (ii) Oocytes were clamped at V_h = −50 mV, and step-changes in membrane potential (V_m) were applied from +50 to −150 mV (in 20-mV increments) each for a duration of 200 ms, before and after the addition of metal-ion substrate. Current was low pass filtered at 500 Hz and digitized at 5 kHz. Steady-state data were obtained by averaging the points over the final 16.7 ms at each V_m step. Steady-state data from protocol i were fit by a modified Hill function (Equation 5) for which I_{max} is the current evoked by metal-ion substrate (M); I_{max} is the derived maximum current; S is the concentration of metal ion; n_H is the Hill coefficient (for the metal ion), and K_{0.5}^M is the metal-ion concentration at which current was half-maximal.

\[
\rho = \frac{I_{\text{max}}^M \cdot S^m}{(K_{0.5}^M)^{n_H} + S^m} \quad \text{(Eq. 5)}
\]

**Statistical and Regression Analysis**—Statistical and regression analyses were performed using SigmaPlot version 11 (Systat Software) with critical significance level α = 0.05. We have presented our data as means ± S.D. (except as noted below) for n independent observations. Between group comparisons for radiotracer data (Figs. 1A, 1B, and 4C), CMTF data (Figs. 3F and 4D), and voltage clamp data (Figs. 1B, 6, and 7) were made by using one-way or two-way ANOVA (repeated measures tests where indicated) followed by pairwise multiple comparisons by the Holm-Šidak test, the results of which were always reported as the adjusted P. Data were fit by Equations 1–6 using the least squares method of regression analysis, the results of which are expressed as the estimates of fit parameters ± S.E.; adjusted r^2 is the adjusted regression coefficient, and P describes the significance of the fit. Fluorescence quenching rates (k) (Equation 4) for 10 or 100 μM metal ion (Table 1) were compared between control oocytes and oocytes expressing DMT1 by using multiple Student’s t test to determine the individual probability (P_i), and significance was determined by using the false discovery rate (f_d) procedure for each of the 22 comparisons of interest (DMT1 versus control). Fit parameters for Equation 1 (Fig. 4, A and B, Fig. 5B, Table 2) were compared by using Student’s t test and those for 50 μM Fe^{2+} data (Fig. 5A and Table 2) by using multiple Student’s t tests controlled by the false discovery rate (f_d) procedure for three comparisons of interest (pairwise versus none). The mean K_{0.5}^M values derived from voltage clamp experiments (n independent trials) were expressed along with 95% confidence intervals (CI) (Table 3); K_{0.5}^M values were compared between metal ions by using multiple Student’s t tests controlled by the false discovery rate procedure for 21 comparisons of interest (all pairwise). Metal-ion selectivity was expressed as mean I_{max}^M/K_{0.5}^M and S.E. (Fig. 7), and metal ions were compared by using one-way ANOVA followed by all-pairwise multiple comparisons by the Holm-Šidak test.

**RESULTS**

**DMT1 Transports Ferrous Ion (Fe^{2+}) but Not Ferric Ion (Fe^{3+})**—Expression of DMT1 in RNA-injected *Xenopus* oocytes stimulated the transport of 2 μM ^{55}Fe^{2+} in the presence of 1 mM L-ascorbic acid at pH 5.5 by over 700-fold compared with control oocytes but did not stimulate the uptake of 2 μM ^{55}Fe^{3+} in the presence of 1 mM nitritotriacetic acid (NTA) (Fig. 1A). Although this NTA concentration was no higher than that of L-ascorbic acid, we considered whether the lack of transport observed in NTA-containing solution may have resulted from chelation of the iron. We therefore also tested iron uptake from a solution containing NTA at the lower concentration of 10 μM. We added ferrocenium hexafluorophosphate as an electron donor, because *Xenopus* oocytes exhibit measurable surface

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**Metal-ion Substrate Profile and Selectivity of Human DMT1**
ferrireductase activity (21). Expression of DMT1 again stimulated 50Fe2+ transport in the presence of L-ascorbic acid, whereas the uptake of 2 μM 55Fe3+ in the presence of 10 μM NTA, 200 μM ferrocenium hexafluorophosphate in oocytes expressing DMT1 did not differ from control oocytes (supplemental Fig. 1).

Addition of 20 μM Fe2+, but not Fe3+, evoked large inward currents in oocytes expressing DMT1 voltage clamped at −70 mV (at pH 5.5), whereas no currents were observed in control oocytes (Fig. 1B). Therefore, DMT1-mediated iron transport is specific to ferrous ion (Fe2+) over ferric ion (Fe3+).

**Metal-ion Substrate Profile of DMT1 Determined by Radiotracer Assay**—In addition to stimulating the transport of 50Fe2+, expression of DMT1 in oocytes strongly stimulated the uptake of 100Cd2+ and 54Mn2+ and, more weakly (only 4-fold), the uptake of 56Zn2+ (Fig. 2A); however, the transport of 64Cu2+ and 64Cu+ did not differ between control oocytes and oocytes expressing DMT1 (Fig. 2B). Therefore DMT1 is capable of transporting Cd2+, Fe2+, Mn2+, and Zn2+ but not copper.

**Validation of a Fluorescence-based Metal-ion Transport Assay Using PGSK**—We established the reactivity of Phen-Green SK (PGSK) with a range of metal ions in a cell-free system (supplemental Table 1). Apparent affinity constants (Kd) for a range of transition metal ions, group-12 metal ions, and Ga3+ were estimated in the range 10−7–10−5 M at a PGSK concentration of 10 μM. We observed no PGSK reactivity with the alkaline-earth metal ion Sr2+. We found that Co2+, Cu+, Ni2+, and Zn2+ exhibited biphasic quenching behavior, so data for these metal ions were fit over the narrower range of 0.1–30 μM. Based on measurements of maximal transport of 50Fe2+ in DMT1-expressing oocytes (3), we should not expect a change in intracellular metal-ion concentration of greater than 10 μM over the time course of the fluorescence assay in oocytes. Therefore, PGSK is reactive with a broad range of metal ions within concentration ranges appropriate to metal-ion transport assays in oocytes expressing DMT1.

We examined metal-ion transport in control oocytes and DMT1-expressing oocytes injected with PGSK by CMTF using laser-scanning confocal microscopy (Fig. 3). Strong quenching of PGSK fluorescence was apparent in an oocyte expressing DMT1 superfused at pH 5.5 following the addition of 100 μM Fe2+ for 10 min (Fig. 3A), and we observed no photobleaching of PGSK fluorescence within 20 min of continuous excitation (data not shown). Fluorescence quenching induced by the superfusion of the metal ion could be fit by a three-parameter exponential decay function from which we determined k, the first-order rate constant of quenching (Fig. 3B). Fluorescence quenching resulting from superfusion of 100 μM Fe3+ for 30-s bursts was promptly halted upon the removal of the metal ion (and the proton-motive driving force for transport) and recommenced upon the reintroduction of the metal ion at pH 5.5 (Fig. 3C). Using the same settings as in Fig. 3A, we observed no fluorescence signal (i.e., no autofluorescence) in a DMT1-expressing oocyte that had not been injected with PGSK (Fig. 3D).

We examined the effect of oocyte pigmentation on the assessment of fluorescence quenching by comparing rate constants (k) determined from sampling discrete regions of the oocyte (regions of interest, ROI), namely the whole oocyte (ROI1), the densely pigmented animal pole (ROI2), the lightly pigmented vegetal pole (ROI3), and the equator (ROI4), illustrated in Fig. 3E. Although initial absolute fluorescence levels differed between regions of interest (data not shown), the computed rate constants did not vary across the regions chosen (Fig. 3F).

To further test the specificity of the Fe2+-induced changes in PGSK fluorescence, we adopted a protocol described elsewhere (22) involving the use of nonpermeant and permeant metal-ion chelators. Rapid fluorescence quenching induced by superfusion of 20 μM Fe2+ at pH 5.5 in oocytes expressing DMT1 was promptly halted upon removal of the extracellular Fe2+ with the aid of the nonpermeant chelator diethylenetriaminepentaacetic acid (DTPA) (typical fluorescence records are illustrated in Fig. 3G and rates of change are illustrated in Fig. 3H). Continued superfusion of an oocyte at pH 7.5 resulted in a slow, partial recovery of fluorescence (<10% over 10 min), whereas
PGSK fluorescence recovered more rapidly (>40% over 10 min) as a result of superfusion with the cell-permeant chelator salicylaldehyde isonicotinoylhydrazone (SIH). These observations indicate that application of extracellular \( \text{Fe}^{2+} \) to oocytes expressing DMT1 induces the rapid, specific, and reversible iron quenching of intracellular PGSK fluorescence.

**Validation of PGSK as a Reporter of DMT1-mediated \( \text{Fe}^{2+} \) Transport in Oocytes**—To test whether \( k \), the first-order rate constant of fluorescence quenching, could be used as an index of DMT1-mediated \( \text{Fe}^{2+} \) transport in the oocyte system, we compared the properties of DMT1-mediated \( \text{Fe}^{2+} \) transport in the oocyte system by using PGSK fluorescence with those determined by using radiotracer assays. As we have observed previously (2), we found that \( ^{55}\text{Fe}^{2+} \) uptake at pH 5.5 in oocytes expressing DMT1 was saturable (Fig. 4A); the \( \text{Fe}^{2+} \) concentration at which transport was half-maximal (\( K_{\text{Fe}}^{\text{e}} \)) was 5.5 \( \pm \) 1.0 \( \mu \text{M} \) (S.E.). Likewise, estimates of \( k \) as a function of \( \text{Fe}^{2+} \) concentration were saturable and could be fit by a Michaelis-Menten function (Fig. 4B); the \( \kappa_{0.5} \) of 4.4 \( \pm \) 1.7 \( \mu \text{M} \) predicted from fluorescence quenching did not differ from that determined from \( ^{55}\text{Fe}^{2+} \) uptake (\( p = 0.97 \), by Student’s t test).

DMT1-mediated iron transport is energized by the \( \text{H}^{+} \) electrochemical potential gradient (1–3, 23). As expected, uptake of 20 \( \mu \text{M} ^{55}\text{Fe}^{2+} \) was accelerated at low pH (5.2) compared with pH 6.2 and 7.2 in oocytes expressing DMT1 (Fig. 4C). We found that the rates of PGSK quenching induced by 20 \( \mu \text{M} ^{55}\text{Fe}^{2+} \) in oocytes expressing DMT1 (Fig. 4D) were pH-dependent in the same manner as for \( ^{55}\text{Fe}^{2+} \) uptake. Therefore, because the properties of DMT1-mediated iron transport (\( K^{\text{e}}_{\text{Fe}} \) and pH dependence) determined by PGSK-quenching rates matched those determined by radiotracer assay, we conclude that PGSK fluorescence quenching can serve as a reporter of DMT1-mediated \( \text{Fe}^{2+} \) transport in oocytes.

**Metal-ion Substrate Profile of DMT1 Determined by Continuous Monitoring of Transport by Fluorescence**—We tested the substrate profile of DMT1 by superfusing RNA-injected oocytes with a range of transition metal ions, group-12 metal ions, or \( \text{Ga}^{3+} \), while continuously monitoring intracellular PGSK fluorescence. The ionic species present in our system (pH 5.5, in the absence or presence of 1 \( \text{mm} \) \( \text{L}^{-} \)-ascorbic acid, as indicated, as reducing agent) were predicted from an atlas of Eh-pH (Pourbaix) diagrams (24) constructed using the SUPCRT/FLASK-AQ model. Thus, all metal ions are assumed to be in the form \( \text{M}^{2+} \) (where \( \text{M} \) is metal) with these exceptions: chromium in the absence of reducing agent, predicted to be in the form \( \text{CrO}^{2+} \), \( \text{CrOH}^{2+} \), or \( \text{Cr}^{3+} \) (for simplicity described as
Cr\(^{3+}\)); gallium, predicted to be in the form HGaO\(_2\)(aq), GaO\(^+\), GaOH\(^+\), or Ga\(^{3+}\) (for simplicity described as Ga\(^{3+}\)); mercury, predicted to be in the form HgO\(_2\)(aq) or Hg\(^{2+}\) (for simplicity described as Hg\(^{2+}\)); vanadium in strongly reducing conditions, VO\(^+\) (or possibly V\(^2+\)); and vanadium in the absence of reducing agent VO\(^2+\).

Superfusion with 100 μM Cd\(^{2+}\), Co\(^{2+}\), Fe\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), VO\(^2+\), or Zn\(^{2+}\) (at pH 5.5) resulted in more rapid quenching of PGSK fluorescence in oocytes expressing DMT1 than in control oocytes (Table 1). The rate constants for PGSK fluorescence quenching in DMT1-expressing oocytes superfused with Cd\(^{2+}\), Co\(^{2+}\), Fe\(^{2+}\), or Mn\(^{2+}\) at 10 μM were similar to the rate constants at 100 μM, suggesting that these metal ions were transported by DMT1 with high affinity (k\(^{D}_{0.5}\) < 10 μM). In contrast, rate constants for 10 μM Ni\(^{2+}\), VO\(^2+\), and Zn\(^{2+}\) were much lower than those at 100 μM, suggesting that these three metal ions were transported by DMT1 with lower affinity (k\(^{D}_{0.5}\) > 10 μM). We concluded that Cd\(^{2+}\), Co\(^{2+}\), Fe\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), VO\(^2+\), and Zn\(^{2+}\) are transported substrates of DMT1.

We have not compared rates (k) of fluorescence quenching between different metal ions in Table 1 because we cannot predict how k may depend upon the PGSK-metal-ion dissociation constants, which differ among the metal ions tested (supplemental Table 1). In preliminary experiments, we found that superfusion with Pb\(^{2+}\) induced a slow-onset, modest increase in PGSK fluorescence in oocytes expressing DMT1 but not control oocytes (data not shown). Because this unexpected result was difficult to interpret, we have discontinued the use of Pb\(^{2+}\) in the PGSK assay (see “Discussion”). Neither Cr\(^{3+}\) nor Cr\(^{2+}\) quenched PGSK fluorescence in oocytes expressing DMT1, consistent with our previous data revealing that 53Cr\(^{3+}\) and 51Cr\(^{3+}\) are not transported by DMT1 (3). We found that fluorescence quenching resulting from superfusion with Cu\(^{+}\), Cu\(^{2+}\), Ga\(^{3+}\), Hg\(^{2+}\), or VO\(^2+\) did not differ between control oocytes and oocytes expressing DMT1 (Table 1). Based on these fluorescence data, we conclude that Cr\(^{2+}\), Cr\(^{3+}\), Cu\(^{+}\), Cu\(^{2+}\), Ga\(^{3+}\), Hg\(^{2+}\), and VO\(^2+\) are not transported substrates of DMT1.

In preliminary experiments, we had observed strong quenching of PGSK fluorescence in control oocytes superfused with Hg\(^{2+}\), possibly a result of Hg\(^{2+}\)-induced membrane damage. Given the resulting variability, we increased the sample size (n = 16–18) to conserve statistical power, but expression of DMT1 did not affect quenching induced by 100 μM Hg\(^{2+}\) (Table 1). Similarly, we observed strong quenching of PGSK

### TABLE 1
Fluorescence-based assay of metal-ion transport in oocytes expressing human DMT1

| Metal Ion | Conditions | 100 μM metal ion | P<sub>s</sub> significant by FDR? | 10 μM metal ion | P<sub>s</sub> significant by FDR? |
|-----------|-------------|------------------|---------------------------------|------------------|---------------------------------|
| Cd\(^{2+}\) | Ascorbic acid | 0.00 ± 0.02 (3) | 1.63 ± 0.72 (13) | 0.002, Yes | 0.00 ± 0.02 (3) | 1.67 ± 0.69 (11) | 0.002, Yes |
| Co\(^{3+}\) | l-His | 0.02 ± 0.01 (3) | 3.53 ± 1.44 (17) | < 0.001, Yes | 0.2 ± 0.02 (3) | 3.20 ± 1.34 (16) | 0.002, Yes |
| Cr\(^{3+}\) | l-Asc, l-His | 0.00 ± 0.00 (3) | 0.00 ± 0.01 (4) | 0.44, No | 0.00 ± 0.00 (3) | 0.00 ± 0.01 (4) | 0.44, No |
| Cu\(^{+}\) | l-Asc, l-His | 0.21 ± 0.28 (6) | 0.11 ± 0.15 (6) | 0.50, No | 0.21 ± 0.28 (6) | 0.11 ± 0.15 (6) | 0.50, No |
| Cu\(^{2+}\) | l-His | 1.09 ± 1.42 (5) | 1.12 ± 1.19 (6) | 0.97, No | 1.09 ± 1.42 (5) | 1.12 ± 1.19 (6) | 0.97, No |
| Fe\(^{3+}\) | l-Asc | 0.06 ± 0.10 (3) | 3.78 ± 1.49 (18) | < 0.001, Yes | 0.06 ± 0.10 (3) | 3.78 ± 1.49 (18) | < 0.001, Yes |
| Ga\(^{3+}\) | l-Asc | 0.27 ± 0.49 (4) | 0.04 ± 0.05 (6) | 0.33, No | 0.27 ± 0.49 (4) | 0.04 ± 0.05 (6) | 0.33, No |
| Hg\(^{2+}\) | l-Asc | 2.05 ± 0.77 (18) | 1.66 ± 0.68 (16) | 0.13, No | 2.05 ± 0.77 (18) | 1.66 ± 0.68 (16) | 0.13, No |
| Mn\(^{2+}\) | l-Asc | 0.00 ± 0.00 (3) | 0.07 ± 0.30 (6) | 0.004, Yes | 0.00 ± 0.00 (3) | 0.07 ± 0.30 (6) | 0.004, Yes |
| Ni\(^{2+}\) | l-Asc | 0.78 ± 0.14 (3) | 0.97 ± 0.15 (4) | 0.87, No | 0.78 ± 0.14 (3) | 0.97 ± 0.15 (4) | 0.87, No |
| VO\(^3+\) | l-Asc | 0.05 ± 0.07 (5) | 2.10 ± 0.78 (4) | 0.001, Yes | 0.05 ± 0.07 (5) | 2.10 ± 0.78 (4) | 0.001, Yes |
| VO\(^2+\) | l-Asc | 0.09 ± 0.20 (5) | 2.18 ± 1.10 (7) | 0.002, Yes | 0.09 ± 0.20 (5) | 2.18 ± 1.10 (7) | 0.002, Yes |

Where indicated, media contained 1 mM L-ascorbic acid (l-Asc) or 1 mM L-histidine (l-His). (Data for Fe\(^{2+}\) are independent of those used in Fig. 4B.) Data were analyzed by using Student’s t test to obtain P, and significance was determined using the false discovery rate (FDR) procedure (see “Experimental Procedures”).
fluorescence in control oocytes superfused with Cu²⁺ (in the presence of histidine) or, to a lesser extent, with VO⁺. That 100 μM copper also was inducing membrane damage is suggested by the following observations: (i) either Cu⁺⁺ or Cu³⁺ resulted in stronger quenching when they were presented in the absence of histidine (which might otherwise minimize nonspecific binding of copper by membrane proteins, data not shown), and (ii) the onset of copper-induced quenching was delayed 3–4 min (data not shown), in contrast to the immediate onset observed for Fe²⁺ (see Fig. 3, B, C, and G). In any event, DMT1 expression did not increase PGSK quenching in oocytes superfused with 100 μM Cu²⁺, Cu³⁺, or VO⁺ nor did expression of DMT1 stimulate the uptake of 2 μM ⁶⁴Cu²⁺ or ⁶⁴Cu³⁺ (Fig. 2B) in experiments in which the use of radiotracer Cu-⁶⁴ permitted us to use much lower concentrations of these copper ions, with no evidence of membrane damage.

**Competitive Inhibition among DMT1 Substrates—Transport of ⁵⁵Fe²⁺ in oocytes expressing DMT1 at pH 5.5 was inhibited by Co²⁺, Mn²⁺, and Zn²⁺ (each at 20 μM) at all except higher concentrations of ⁵⁵Fe²⁺ (Fig. 5A). We found that Co²⁺ and Mn²⁺ increased the apparent Kᵣ for ⁵⁵Fe²⁺, with 6.5 μM in the absence of metal, without effect on Vₘₐₓ (Table 2), and therefore we concluded that Co²⁺ and Mn²⁺ are competitive inhibitors of DMT1-mediated Fe²⁺ transport. The inhibition constants (Kᵣ) computed by using Equation 2 for both Co²⁺ and Mn²⁺ were ~10 μM, whereas that for Zn²⁺ (Kᵣ = 26 μM) was significantly higher than either Kᵣ for either Co²⁺ or Mn²⁺ and much higher than the Kᵣ obtained by measuring ⁵⁵Fe²⁺ transport (Table 2) (p < 0.006).

Transport of ⁶⁵Zn²⁺ in oocytes expressing DMT1 at pH 5.5 was saturable (Fig. 5B), and data were fit by a Michaelis-Menten function (Equation 1). The half-maximal Zn²⁺ concentration (Kᵣ = 32 μM, Table 2) was considerably higher than the Kᵣ for Mn²⁺, which we had estimated for ⁵⁵Fe²⁺ transport (6.5 μM) (p < 0.001). Kᵣ was identical to the Kᵣ for Mn²⁺ inhibition of ⁶⁵Zn²⁺ transport (p = 0.54). We found that 10 μM Fe²⁺ strongly inhibited ⁶⁵Zn²⁺ transport (Fig. 5B), increasing the apparent Kᵣ to 144 μM without effect on the estimated Vₘₐₓ (Table 2), again consistent with competition between Fe²⁺ and Zn²⁺. The Kᵣ for inhibition of ⁶⁵Zn²⁺ transport by Fe²⁺ was ~3 μM (Table 2) and was not significantly different from the Kᵣ for ⁵⁵Fe²⁺ transport (p = 0.085).

Collectively, these data indicate the following: (i) Fe²⁺, Co²⁺, Mn²⁺, and Zn²⁺ compete with one another for a single homogeneous transport pathway (25) through DMT1, and (ii) DMT1 binds Zn²⁺ at much lower affinity than it does the other three metal ions.

**Vanadyl-evoked Currents in Oocytes Expressing DMT1—**By using the voltage clamp, we observed metal-ion-evoked currents in oocytes expressing DMT1 (Fig. 6). The addition of 100 μM VO(SO₄)²⁻ evoked inward currents that were identical to the currents evoked by VCl⁻ in the absence of reducing agent (Fig. 6A). This observation supports our interpretation of the Eh-pH relationship for vanadium (24), i.e. that the major species present in our system in the absence of reducing agent should be the vanadyl, or oxovanadium(IV), cation (VO²⁻) irrespective of

### TABLE 2

**Competitive inhibition among DMT1 substrates**

Data from the measurement of ⁵⁵Fe²⁺ or ⁶⁵Zn²⁺ transport at pH 5.5 in oocytes expressing DMT1 (Fig. 5) in the absence or presence of candidate inhibitor metal ions were fit by Equations 1 and 2. Fit parameters for ⁵⁵Fe²⁺ transport saturation kinetics (Fig. 5A) were compared by using Student’s t tests to obtain P, and significance was tested by using the false discovery rate (FDR) procedure (see under “Experimental Procedures”). Fit parameters for ⁶⁵Zn²⁺ transport saturation kinetics (Fig. 5B) were compared by using Student’s t tests.

**Saturation kinetics (Eq. 1) for ⁵⁵Fe²⁺ transport (data in Fig. 5A)**

| Inhibitor metal ion | Goodness of fit | Vₘₐₓ ± S.E. (pmol·min⁻¹) | Vₘₐₓ comparison with ‘None’ | Kᵣ (or Kᵣ) ± S.E. (μM) | Kᵣ comparison with ‘None’, Significant by FDR? | Kᵣ ± S.E. (μM) |
|---------------------|-----------------|--------------------------|-----------------------------|-------------------------|---------------------------------------------|-----------------|
| None                | 0.99            | 4.9 ± 0.2                | NS (P = 0.45)               | 65 ± 0.9                | P = 0.004, Yes                              | 10.2 ± 2.3      |
| Co²⁺                | 0.98            | 5.1 ± 0.3                | NS (P = 0.38)               | 19.1 ± 3.3              | P < 0.001, Yes                              | 10.2 ± 1.9      |
| Mn²⁺                | 0.99            | 5.2 ± 0.3                | NS (P = 0.32)               | 19.2 ± 2.5              | P < 0.001, Yes                              | 25.9 ± 5.5      |
| Zn²⁺                | 0.98            | 4.6 ± 0.2                | NS (P = 0.32)               | 11.4 ± 1.9              | P = 0.02, Yes                               | 25.9 ± 5.5      |

**Saturation kinetics (Eq. 1) for ⁶⁵Zn²⁺ transport (data in Fig. 5B)**

| Inhibitor metal ion | Goodness of fit | Vₘₐₓ ± S.E. (pmol·min⁻¹) | Vₘₐₓ comparison with ‘None’ | Kᵣ (or Kᵣ) ± S.E. (μM) | Kᵣ comparison with ‘None’, Significant by FDR? | Kᵣ ± S.E. (μM) |
|---------------------|-----------------|--------------------------|-----------------------------|-------------------------|---------------------------------------------|-----------------|
| None                | 0.99            | 2.4 ± 0.2                | NS (P = 0.89)               | 32 ± 5                  | P = 0.016                                   | 2.8 ± 1.3       |
Metal-ion Substrate Profile and Selectivity of Human DMT1

We used the voltage clamp in oocytes expressing DMT1 to determine the metal-ion selectivity for those metal ions demonstrated in radiotracer or CMTF assays to be transported substrates of DMT1. Because the currents evoked by Fe$^{2+}$ and other metal ions are strongly voltage-dependent (2,3), the voltage clamp is superior to radiotracer or CMTF assays (in which membrane potential is not controlled) for comparing under fixed conditions the saturation kinetic parameters of several metal ions. In experiments in which oocytes expressing DMT1 were voltage-clamped at $-70$ mV at pH 5.5 (Table 3), DMT1 exhibited the highest affinity for Cd$^{2+}$ and Fe$^{2+}$ ($K_{D}^{m} \approx 1 \mu M$). As anticipated, the $K_{D}^{m}$ estimates obtained in voltage clamp experiments at $-70$ mV (Table 3) were lower than those obtained in radiotracer and CMTF assays (Figs. 3 and 4 and Table 2). In these last two assays, oocytes were not voltage-clamped and became depolarized, whereas $K_{D}^{m}$ in DMT1 is known to be voltage-dependent (3). DMT1 exhibited moderately high affinity for Co$^{2+}$ and Mn$^{2+}$ ($K_{D}^{m}$ in the range 3–4 $\mu M$), whereas DMT1 reacted with Ni$^{2+}$, VO$^{2+}$, and Zn$^{2+}$ at lower affinity ($K_{D}^{m}$ in the range 10–20 $\mu M$).

We compared the DMT1 selectivity for these seven metal ions by using the ratio $P_{max}/K_{D}^{m}$ (sometimes called the “specificity constant”) as an index of substrate selectivity (Fig. 7). At $-70$ mV and at pH 5.5, the selectivity of DMT1 metal-ion substrates were ranked Cd$^{2+} >$ Fe$^{2+} >$ Co$^{2+} >$ and Mn$^{2+} \gg$ Zn$^{2+}$, Ni$^{2+}$, VO$^{2+}$ (where $\gg$ represents a statistically significant difference of magnitude $\approx 0.3 \log_{10}$ units and $\approx$ represents $0.6 \log_{10}$ units).

**DISCUSSION**

Establishing the Metal-ion Substrate Profile of DMT1—We present a comprehensive substrate–profile analysis by utilizing two direct approaches for measuring metal-ion transport in oocytes expressing DMT1 as follows: a novel fluorescence-based assay and conventional radiotracer assays. Our data validate the use of the PGSK fluorophore as a reporter of cellular iron transport with sufficient fidelity to be used in estimating functional properties of DMT1-mediated Fe$^{2+}$ transport ($K_{D}^{m}$ and pH dependence) that matched those properties determined by radiotracer assay. The application of PGSK is therefore not limited to reporting steady-state intracellular concentration or accumulation of the metal, and this fluorophore may be widely useful in studying metal-ion transport in cultured cells or tissues.

From our fluorescence assay (CMTF), we concluded that DMT1 is capable of transporting Cd$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, VO$^{2+}$, and Zn$^{2+}$. We found no evidence that DMT1 transports Cr$^{3+}$, Cr$^{2+}$, Cu$^{2+}$, Ga$^{3+}$, Hg$^{2+}$, or VO$^{2+}$. Gallium was tested because of its applications in medicine. Gallium is generally

**TABLE 3**

Half-maximal concentrations ($K_{D}^{m}$) for DMT1 metal-ion substrates

| Metal ion | Mean $K_{D}^{m}$ (M) | 95% CI | $n$ |
|-----------|-------------------|-------|-----|
| Cd$^{2+}$ | 1.06             | 0.93, 1.20$^{a}$ | 9   |
| Fe$^{2+}$ | 1.22             | 0.99, 1.44$^{a}$ | 9   |
| Co$^{2+}$ | 2.56             | 2.00, 3.11    | 10  |
| Mn$^{2+}$ | 4.18             | 3.18, 5.18    | 9   |
| Ni$^{2+}$ | 10.7             | 8.8, 12.5$^{c}$ | 6   |
| VO$^{2+}$ | 16.9             | 14.0, 19.9$^{c}$ | 7   |
| Zn$^{2+}$ | 19.1             | 17.2, 20.9$^{c}$ | 7   |

$^{a}$ $p = 0.029$ (pairwise comparison).

$^{b}$ $p = 0.087$ (pairwise comparison).

$^{c}$ $p = 0.56$ (pairwise comparison).

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**FIGURE 6.** Vanadyl-evoked currents. A, currents evoked by 100 $\mu$M vanadyl compounds added to pH 5.5 medium as VCl$_{2}$ (empty circles) or VOSO$_{4}$ (empty diamonds), in the absence of l-ascorbic acid, in three oocytes expressing DMT1. Data are mean ± S.D. (n = 3). Repeated measures two-way ANOVA revealed a main effect of $V_{m}$ ($p < 0.001$) but no difference between VCl$_{2}$ and VOSO$_{4}$ ($p = 0.09$). B, currents evoked by 100 $\mu$M vanadyl ion (VO$^{2+}$, where v represents variable valence) added as VCl$_{2}$ in three oocytes expressing DMT1 as a function of l-ascorbic acid concentration: 0 (black circles), 70 $\mu$M (gray circles), or 1 $\mathrm{m}$M (empty circles), along with the currents evoked by 10 $\mu$M Fe$^{2+}$ in the presence of 1 $\mathrm{m}$M l-ascorbic acid (filled triangles), all at pH 5.5. Data are means ± S.D. (n = 3). Repeated measures two-way ANOVA revealed an interaction between metal-ion/ascorbic acid and $V_{m}$ ($p < 0.001$); Holm–Sidak all-pairwise multiple comparisons revealed that currents differed between all metal-ion/ascorbic acid combinations ($p \leq 0.042$) except for vanadyl/no ascorbic acid versus vanadyl, 70 $\mu$M ascorbic acid ($p = 0.27$). Data in A and B are from independent preparations.

the salt added. Moreover, we found that 100 $\mu$M VO$^{2+}$ added as VOSO$_{4}$ induced more rapid quenching of PGSK fluorescence in oocytes expressing DMT1 than in control oocytes ($p = 0.028$) and that the rate constants for DMT1 did not differ whether VO$^{2+}$ was added as VOSO$_{4}$ or as VCl$_{2}$ in the absence of reducing agent ($p = 0.20$) ($n = 3$, data not shown; independent of data in Table 1).

In oocytes expressing DMT1, superfused in the presence of 1 $\mathrm{m}$M l-ascorbic acid, 10 $\mu$M Fe$^{2+}$ evoked large inward currents that exhibited a curvilinear dependence on membrane potential ($V_{m}$) (Fig. 6B), as we have observed previously (1–3). The Fe$^{2+}$-evoked inward currents did not saturate with hyperpolarization (up to $-150$ mV) and did not reverse with depolarization (up to $+50$ mV). In the absence of l-ascorbic acid, 100 $\mu$M vanadyl ion evoked a current that was much smaller than the Fe$^{2+}$-evoked current but was qualitatively similar, as we have observed previously (3). The vanadyl-evoked currents were no different in the presence of 70 $\mu$M l-ascorbic acid, equivalent to the approximate physiological concentration of l-ascorbic acid in plasma. These findings suggest that VO$^{2+}$ is the transported species even when l-ascorbic acid is present at a concentration within the physiological range. Much stronger reducing conditions are expected to generate VO$^{+}$ (24), and notably, 1 $\mathrm{m}$M l-ascorbic acid abolished the vanadyl-evoked currents (Fig. 6B). The latter observation is consistent with the lack of PGSK fluorescence quenching in oocytes superfused with vanadyl under the same conditions (Table 1) and support the conclusion that VO$^{+}$ is not transported by DMT1.

Metal-ion Selectivity Determined from Evoked Currents—We used the voltage clamp in oocytes expressing DMT1 to determine the metal-ion selectivity for those metal ions demonstrated in radiotracer or CMTF assays to be transported substrates of DMT1. Because the currents evoked by Fe$^{2+}$ and other metal ions are strongly voltage-dependent (2,3), the voltage clamp is superior to radiotracer or CMTF assays (in which membrane potential is not controlled) for comparing under fixed conditions the saturation kinetic parameters of several metal ions. In experiments in which oocytes expressing DMT1 were voltage-clamped at $-70$ mV at pH 5.5 (Table 3), DMT1 exhibited the highest affinity for Cd$^{2+}$ and Fe$^{2+}$ ($K_{D}^{m} \approx 1 \mu M$). As anticipated, the $K_{D}^{m}$ estimates obtained in voltage clamp experiments at $-70$ mV (Table 3) were lower than those obtained in radiotracer and CMTF assays (Figs. 3 and 4 and Table 2). In these last two assays, oocytes were not voltage-clamped and became depolarized, whereas $K_{D}^{m}$ in DMT1 is known to be voltage-dependent (3). DMT1 exhibited moderately high affinity for Co$^{2+}$ and Mn$^{2+}$ ($K_{D}^{m}$ in the range 3–4 $\mu M$), whereas DMT1 reacted with Ni$^{2+}$, VO$^{2+}$, and Zn$^{2+}$ at lower affinity ($K_{D}^{m}$ in the range 10–20 $\mu M$).

We compared the DMT1 selectivity for these seven metal ions by using the ratio $P_{max}/K_{D}^{m}$ (sometimes called the “specificity constant”) as an index of substrate selectivity (Fig. 7). At $-70$ mV and at pH 5.5, the selectivity of DMT1 metal-ion substrates were ranked Cd$^{2+} >$ Fe$^{2+} >$ Co$^{2+} >$ and Mn$^{2+} \gg$ Zn$^{2+}$, Ni$^{2+}$, VO$^{2+}$ (where $\gg$ represents a statistically significant difference of magnitude $\approx 0.3 \log_{10}$ units and $\approx$ represents $0.6 \log_{10}$ units).
thought to be handled like iron, but although transferrin binds gallium (26), DMT1 does not appear to transport the metal.

Radiotracer assays demonstrated that DMT1 transports ferrous iron (Fe$^{2+}$) but not ferric iron (Fe$^{3+}$). Expression of DMT1 also stimulated the uptake of $^{59}$Cd$^{2+}$, $^{54}$Mn$^{2+}$, and $^{65}$Zn$^{2+}$ but not of $^{64}$Cu$^{2+}$ or $^{64}$Cu$^{3+}$; in addition, we have previously shown (3) that human DMT1 does not transport $^{51}$Cr$^{3+}$ (II or III). This agreement between CMFT and radiotracer assays suggests a lack of kinetic isotope effects in DMT1, i.e. DMT1 is similarly reactive (or unreactive) with the radioisotopes of cadmium, (chromium), (copper), iron, manganese, and zinc as it is with the common isotopes of those metals.

Our data reveal that Fe$^{2+}$, Co$^{2+}$, Mn$^{2+}$ and, more weakly, Zn$^{2+}$ compete with one another for DMT1, which is the first demonstration that inhibition of DMT1 transport activity by these metal ions is competitive in nature. Previous studies (27–33) have shown that these metal ions, with the general exception of Zn$^{2+}$, are capable of inhibiting cellular cadmium, iron, or manganese transport activity attributed to DMT1, but competitive and noncompetitive models were not tested.

We have provided the first analysis of DMT1 metal-ion selectivity, previously only estimated from apparent inhibition constants. To do so, we took advantage of the sizable metal-ion-evoked currents associated with expression of DMT1 in oocytes and the controlled conditions afforded by the voltage clamp, necessary because DMT1 is strongly voltage-dependent (1–3). Metal-ion-evoked currents in each individual were normalized by the current evoked by 50 μM Mn$^{2+}$ in the same oocyte, a maneuver not afforded by radiotracer assays, permitting a robust statistical comparison of $I_{\text{max}}^M/K_{0.5}^M$ (the selectivity constant) between DMT1 substrates.

Whereas our study is the first to demonstrate DMT1-mediated transport of vanadyl ion (VO$^{2+}$), an Nramp homolog (distantly related to DMT1) from the marine invertebrate Ascidia sydneiensis samea was found to be a H$^+/\text{VO}^{2+}$ antiporter and may serve a critical role in the vacuolar accumulation of vanadium in vanadocytes (34). That DMT1 should bind metal ions of differing valences might seem improbable. Metal ions determined to be DMT1 substrates in our assays were all in the oxidation state 2$,^+$, with the exception of vanadium(IV), because we expect VO$^{2+}$ to be the predominant vanadium species under nonreducing or weakly reducing conditions (24). Such conditions may be encountered in plasma (in which [L-ascorbic acid] $\approx$70 μm) or the intestinal lumen. Therefore, whereas DMT1 appears capable of transporting metals of differing oxidation states, the presumptive ionic species all bear a charge of 2$^+$. 

**Use of the Xenopus Oocyte Heterologous Expression System to Study DMT1-mediated Metal-ion Transport**—In this study, we obtained very efficient expression of DMT1 in RNA-injected Xenopus oocytes, e.g. $^{55}$Fe$^{2+}$ transport activity was increased 700-fold over background (Fig. 1A). Thus, the oocyte system offers a degree of sensitivity that is far superior to transfection or knockdown of DMT1 in mammalian cell lines, and no high affinity blocker of DMT1 is currently available. DMT1-mediated transport of Cd$^{2+}$, Fe$^{2+}$, and Zn$^{2+}$ has been previously demonstrated in oocytes by us and others (2, 35, 36). This study extends the confirmed substrate profile of human DMT1 to include Co$^{2+}$, Ni$^{2+}$, and Mn$^{2+}$ and that of DMT1 of any species to include vanadium, predicted to be transported as VO$^{2+}$.

These observations add to previous findings of metal-ion transport in mammalian cell lines expressing DMT1 and in brush-border membrane vesicles isolated from the DMT1-deficient Belgrade rat (27, 28, 32, 33, 36–39). The importance of also determining which metal ions are not DMT1 substrates has been stressed elsewhere (40), and this study considerably extends for DMT1 the catalog of nonsubstrates.

**Physiological Substrates of DMT1**—DMT1 plays critical roles in iron homeostasis (4). We found that DMT1 favors Fe$^{2+}$ over any of its other physiological substrates and that Co$^{2+}$ and Mn$^{2+}$ are also transported with moderately high affinity. These last two metal ions are, in turn, strongly favored over the remaining DMT1 substrates, because metal-ion selectivity was ranked Cd$^{2+}$ > Fe$^{2+}$ > Co$^{2+}$, Mn$^{2+}$ > Zn$^{2+}$, Ni$^{2+}$, VO$^{2+}$ (see Fig. 7). Predictions of the multiple roles of DMT1 should take into account the metal-ion selectivity in relation to extracellular (or endosomal) metal-ion concentrations. For example, DMT1 transports VO$^{2+}$ at significantly lower affinity than it does Fe$^{2+}$, and iron is more abundant in the diet than is vanadium, such that DMT1-mediated vanadium absorption is probably extremely low; nevertheless, DMT1 may suffice vanadium absorption because the metal is required in only trace amounts. Deficiency of vanadium results in growth retardation and altered metabolism of glucose and lipids (41). An intestinal anionic transport system may additionally serve in the absorption of vanadate (oxoanions of vanadium) (41).

The DMT1-deficient Belgrade rat exhibits impaired intestinal absorption of Mn and its uptake into reticulocytes (42); however, substantial activity remains suggesting that alternative Mn-transport systems (in addition to DMT1) are expressed in those tissues. Whether DMT1 serves the physiological absorption or cell-specific transport of Co, Zn, or V remains to be established using specific cell preparations or rodent models. Co and Fe appear to share a common absorptive pathway in the rat (43, 44); however, nutritional requirements for Co (aside from cobalamin) are trivial. We found that Zn$^{2+}$ was poorly transported by DMT1 and only weakly inhibited $^{55}$Fe$^{2+}$ transport. We predict that DMT1 should not contribute to transport of Zn$^{2+}$ in normal physiological conditions because of the fol-
DMT1 and Heavy Metal Intoxication—DMT1 transports the toxic metals cadmium and nickel; meanwhile, every one of the physiological substrates of DMT1 are also toxic in excess. For example, chronic occupational exposure to manganese leads to a neurological disorder known as manganism, and DMT1 is implicated in its etiology (47). In addition to the intestinal absorption of heavy metals in contaminated water or food, several toxic metals can enter via olfactory neurons in nasal mucosa (leading directly to the brain) (48) or nasal and respiratory epithelia (to the blood). DMT1 expressed in those tissues may therefore play significant roles in heavy-metal intoxication (47, 49–53), although DMT1-mediated clearance of metals from the lung is thought to minimize metal-related injury in that tissue (51).

Notably, human DMT1 transports the toxic heavy metal Cd$$^{2+}$$ more efficiently than it does Fe$$^{2+}$$. This activity of DMT1 may provide an explanation for why iron-deficiency anemia (especially in children and pregnant or nursing women) is associated with cadmium intoxication (53–58), and evidence in rats implicates DMT1 (59–62). DMT1 is expressed in the renal proximal tubule and is thought to contribute to cadmium nephrotoxicity (63, 64). Evidence also exists for interactions between nickel and iron transport and metabolism in human cell lines (39).

We also tested whether mercury and lead are DMT1 substrates, given the toxicological importance of these metals. We found no evidence of DMT1-mediated mercury transport in our CMTF assay. Why superfusion of Pb$$^{2+}$$ should induce a modest increase in PGSK fluorescence in oocytes expressing DMT1 is difficult to explain, particularly because we found no evidence of a biphasic effect of Pb$$^{2+}$$ on PGSK quenching in the cell-free system. An alternative method or another fluorophore will be needed to definitively examine lead transport by DMT1. Whereas others have reported that transfection of DMT1 into a yeast line or the mammalian cell line HEK293 stimulated lead transport at low pH (65), the same group later found that mRNA knockdown of DMT1 expression in Caco2 cells inhibited transport of iron but not lead (32). In *Xenopus* oocytes expressing human DMT1, Pb$$^{2+}$$ evoked extremely small currents relative to the Fe$$^{2+}$$-evoked currents (3). From the evoked currents, we have estimated $k_{\text{Fe}}^{\text{p}}$ $\approx 46$ $\mu$M and found that the DMT1 selectivity for Pb$$^{2+}$$ was an order of magnitude lower than that for VO$$^{2+}$$.

Others have concluded that lead transport in Caco2 cells is not attributable to DMT1 (66). These observations suggest that, even if DMT1 were capable of transporting lead, the contribution of DMT1 to lead transport should be trivial, and DMT1 need not be considered in lead intoxication. Therefore, DMT1 is likely to be of toxicological significance in the absorption of cadmium, manganese, and nickel in intestinal, olfactory, or pulmonary tissues but not that of lead or mercury.

**No Evidence for DMT1-mediated Copper Transport**—We have found no evidence for DMT1-mediated copper (I or II) transport in assays of $^{64}$Cu uptake or PGSK fluorescence. Our conclusion that DMT1 does not transport copper contradicts two studies in which investigators measured iron and copper transport in the Caco-2 intestinal cell line and concluded that DMT1 is a physiologically relevant Cu$$^{2+}$$ transporter (67, 68), and a third study in which investigators measured copper uptake in intestinal brush-border membrane vesicles and concluded that DMT1 can transport Cu$$^{2+}$$ (69). Further studies may be warranted to resolve these important discrepancies. Although we have tested here only the 1A/IRE(+) isoform, believed to be the predominant DMT1 isoform expressed in intestinal cells (12), other DMT1 isoforms may potentially transport copper. It is also plausible, however unlikely, that a subunit normally expressed in mammalian cells and required for DMT1 to transport copper may be lacking in the *Xenopus* oocyte heterologous system. Copper transport by DMT1 therefore could be further tested in additional mammalian expression systems or in rodent models in which DMT1 is lacking or overexpressed. Meanwhile, there is strong evidence that absorption and cellular uptake of copper is served by alternative transport systems, among which are the copper transporter 1 (70, 71) and an anion transporter that appears capable of transporting copper-chloride complexes (72).

**Summary**—Our data reveal that DMT1 is an iron-prefering transporter that is a likely route of entry for the toxic heavy metal cadmium. Whereas DMT1 may also serve absorption or cellular uptake of cobalt, manganese, and vanadium, we predict that DMT1 should not contribute to the absorption or transport of zinc or copper, both of which are served by multiple transport systems other than DMT1.

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