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PROPERTIES OF THE MAMMALIAN AND YEAST METAL-ION TRANSPORTERS DCT1 AND Smf1p EXPRESSED IN *XENOPUS LAEVIS* OOCYTES

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Summary

Transition metals are essential for many metabolic processes, and their homeostasis is crucial for life. Metal-ion transporters play a major role in maintaining the correct concentrations of the various metal ions in living cells. Little is known about the transport mechanism of metal ions by eukaryotic cells. Some insight has been gained from studies of the mammalian transporter DCT1 and the yeast transporter Smf1p by following the uptake of various metal ions and from electrophysiological experiments using *Xenopus laevis* oocytes injected with RNA copies (c-RNA) of the genes for these transporters. Both transporters catalyze the proton-dependent uptake of divalent cations accompanied by a 'slippage' phenomenon of different monovalent cations unique to each transporter. Here, we further characterize the transport activity of DCT1 and Smf1p, their substrate specificity and their transport properties. We observed that Zn^{2+} is not transported through the membrane of *Xenopus laevis* oocytes by either transporter, even though it inhibits the transport of the other metal ions and enables protons to 'slip' through the DCT1 transporter. A special construct (Smf1p-s) was made to enhance Smf1p activity in oocytes to enable electrophysiological studies of Smf1p-s-expressing cells. $^{54}Mn^{2+}$ uptake by Smf1p-s was measured at various holding potentials. In the absence of Na^+ and at pH 5.5, metal-ion uptake was not affected by changes in negative holding potentials. Elevating the pH of the medium to 6.5 caused metal-ion uptake to be influenced by the holding potential: ion uptake increased when the potential was lowered. Na^+ inhibited metal-ion uptake in accordance with the elevation of the holding potential. A novel clutch mechanism of ion slippage that operates *via* continuously variable stoichiometry between the driving-force pathway (H^+) and the transport pathway (divalent metal ions) is proposed. The possible physiological advantages of proton slippage through DCT1 and of Na^+ slippage through Smf1p are discussed.

Key words: metal ion, transporter, ion current, ion uptake, microinjection, yeast, *Saccharomyces cerevisiae*, oocyte, *Xenopus laevis*.

Introduction

Metal-ion homeostasis is maintained through highly regulated processes of uptake, storage and secretion (Hediger, 1997; Eide, 1998; Radisky and Kaplan, 1999; Gunshin et al., 1997; Fleming et al., 1997; Andrews et al., 1999). The discovery that the yeast homologue Smf1p of the mammalian natural-resistance-associated macrophage protein (NRAMP) is a metal-ion transporter paved the way for the advancement of our knowledge about these transporters (Supek et al., 1996). It was subsequently demonstrated that the two mammalian homologues of Smf1p are broad-range metal-ion transporters and may play a crucial role in the absorption of iron from the duodenum and in the low-pH-dependent release of iron in the endosomes. The NRAMP family encoding metal-ion transporters is ubiquitous and is present in bacteria, fungi, plants and animals (Supek et al., 1996; Supek et al., 1997; Nelson, 1999). A member of this family, DCT1 (or Nramp2), was also the first metal-ion transporter whose activity was demonstrated by mRNA expression in *Xenopus laevis* oocytes, and its mechanism of action has been analyzed using electrophysiological techniques (Gunshin et al., 1997).

DCT1 cotransports Fe^{2+} together with H^+ with a stoichiometry of 1 (Gunshin et al., 1997). Replacing Na^+ with choline and Cl^- with NO_3^- or SCN^- had no effect on Fe^{2+} transport. Later, I observed that substituting Cl^- in the transport solution with gluconate or isethionate resulted in a drastic reduction in the rate of Fe^{2+} transport into oocytes (Nelson, 1999). Therefore, metal-ion transport is dependent on the presence of Cl^- or other small anions such as NO_3^- or SCN^-. Recently, it has been shown that Cl^- affects the absorption of copper into the intestine of catfish (Handy et al., 2000). We proposed that the metal ion is cotransported with Cl^- and that the steady-state current results from the transport of the positively charged H^+ (Nelson, 1999). At physiological membrane potentials of −90 to −30 mV, the apparent affinity constant for H^+ was approximately 1 μmol l^{-1} for DCT1 expressed in *Xenopus laevis* oocytes. In these oocytes, under

certain conditions, a proton leak was observed at low pH (Gunshin et al., 1997).

Yeast cells possess three genes (*SMF1*, *SMF2* and *SMF3*) that encode homologues of the NRAMP proteins with 51–54 % identity in amino acid sequence to each other and 33–36 % identity to DCT1 (Cohen et al., 2000). Studies using a specific antibody have demonstrated that Smf1p is located in the yeast plasma membrane, where it mediates the uptake of Mn^{2+} into the cytoplasm (Supek et al., 1996). There is indirect evidence that other divalent metal ions, such as Cd^{2+}, Co^{2+} and Cu^{2+}, are also substrates of Smf1p (Liu et al., 1997; Liu and Culotta, 1999). Recently, we demonstrated that Smf1p expressed in *Xenopus laevis* oocytes mediates H^+-dependent transport of Mn^{2+} and Fe^{2+}, and competition experiments with other divalent cations indicated that it might also transport Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} (Chen et al., 1999). We also observed that Smf1p expressed in *Xenopus laevis* oocytes exhibited a metal-ion-independent Na^+ flux. The Smf1p-mediated Fe^{2+} transport exhibited saturation kinetics, but the Na^+ flux did not saturate at concentrations up to 150 mmol l^{-1}. Expressed Smf1p also induced permeability to Li^+, Rb^+, K^+ and Ca^{2+}, which may share the same uncoupled pathway (Chen et al., 1999). In the present study, we show that Zn^{2+}, an inhibitor of Mn^{2+} transport, is not transported by DCT1 or by Smf1p expressed in *Xenopus laevis* oocytes. Using a two-electrode voltage-clamp technique and measuring the rate of transport of radioactive divalent cations, we studied the relationship between coupled metal-ion transport into oocytes and the proton and Na^+ 'slips' that accompany this transport process.

Materials and methods

Oocyte preparation

Yeast *SMF1*, *SMF2* and *SMF3* cDNAs were cloned by the polymerase chain reaction (PCR) from yeast genomic DNA as described previously (Supek et al., 1996; Chen et al., 1999; Cohen et al., 2000). The short version of *SMF1* (Smf1p-s) was constructed by PCR as described previously (Cohen et al., 2000) and cloned into a plasmid containing the 5′ and 3′ untranslated regions of DCT1 together with codons for 49 and 18 amino acid residues, respectively, from the N and C termini of DCT1. *Xenopus laevis* oocytes were handled as described previously (Liu et al., 1993).

Rat *DCT1* cDNA was cloned into pSPORT1 plasmid. Capped *SMF* and *DCT1* cRNAs were synthesized by *in vitro* transcription from their cDNAs. Oocytes were extracted from stage V–VI *Xenopus laevis* and defolliculated using a Ca^{2+}-free solution (100 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 2 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} Hepes, pH 7.5) containing 2 mg ml^{-1} collagenase (Roche Molecular Biochemicals, Mannheim, Germany) for approximately 2 h at 18 °C. Oocytes were injected, on the following day, with 50 nl of water containing 15 ng of *SMF1*, *SMF2* or *SMF3* cRNA or 25 ng of *DCT1* cRNA. Equal amounts of water were injected into control oocytes. Injected oocytes were incubated at 18 °C in a solution containing 100 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 2 mmol l^{-1} $MgSO_4$, 1 mmol l^{-1} $CaCl_2$, 2.5 mmol l^{-1} sodium pyruvate, 5 mmol l^{-1} Hepes (pH 7.6) and 50 μg ml^{-1} gentamicin.

Radiotracer measurements

Uptake experiments were performed 3–7 days following injection. Uptake solutions for radiotracer experiments contained 100 mmol l^{-1} NaCl or choline chloride, 10 mmol l^{-1} Hepes, 2 mmol l^{-1} Mes, 2 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2$ and 2 mmol l^{-1} L-ascorbic acid (where indicated), and the pH was adjusted to 5.5–7.5 with Tris base. L-Ascorbic acid was added to solutions to maintain iron in the Fe(II) form. Oocytes (5–10) were incubated in 0.5 ml of a solution containing $^{55}FeCl_2$, $^{54}MnCl_2$, $^{65}ZnCl_2$ or $^{60}CoCl_2$. The radioactive chemicals were purchased from NEN Life Sciences Products, Inc. Boston, USA, or Amersham Pharmacia Biotech, and the specific activities of the radioactive tracers were as follows: $^{65}ZnCl_2$, 2.1 mCi mg^{-1}; $^{60}CoCl_2$, 75 mCi mg^{-1}; $^{55}FeCl_2$, 3 mCi mg^{-1}; $^{54}MnCl_2$, 20 mCi mg^{-1} (1 mCi=3×10^7 Bq). The radioactive tracer was usually mixed with 0.5–2 μmol l^{-1} unlabelled metal ion. Uptake was followed for 20–30 min and was terminated by washing the oocytes five times in uptake solution from which the tested metal ion had been omitted.

Electrophysiological experiments

Experiments utilizing the two-microelectrode voltage-clamp technique were performed as described previously (Loo et al., 1993; Loo et al., 1998; Chen et al., 1998). The resistance of microelectrodes filled with 3 mol l^{-1} KCl was 0.5–2 MΩ. In experiments involving holding potentials, the oocyte was clamped to the holding potential indicated. Solutions used in electrophysiology were the same as in the tracer assays except that they contained no L-ascorbic acid when metal ions other than Fe^{2+} were present.

Experimental results are expressed as means ± S.E.M. (*N*), where *N* indicates the number of oocytes used. Data analysis was performed as described previously (Chen et al., 1998).

Simultaneous voltage-clamped tracer and current measurements

Before starting tracer uptake, the oocyte was clamped at −25 or −50 mV and perfused with substrate-free solution. The membrane potential was then held at another test value. After the perfusion had stopped, the uptake solution (50 μl) was added manually using a pipette to wash out the substrate-free solution. Uptake was followed for 5–10 min in the chamber (volume approximately 50 μl) and was terminated by perfusing (washing) the oocyte for 3 min with 2.7 ml of substrate-free solution. The oocyte was then dissolved in 50 μl of 1 % SDS and mixed with 4.5 ml of scintillation mixture.

Results

DCT1 was the first metal-ion transporter whose mRNA expression in *Xenopus laevis* oocytes activity was demonstrated by large pre-steady-state currents, suggesting

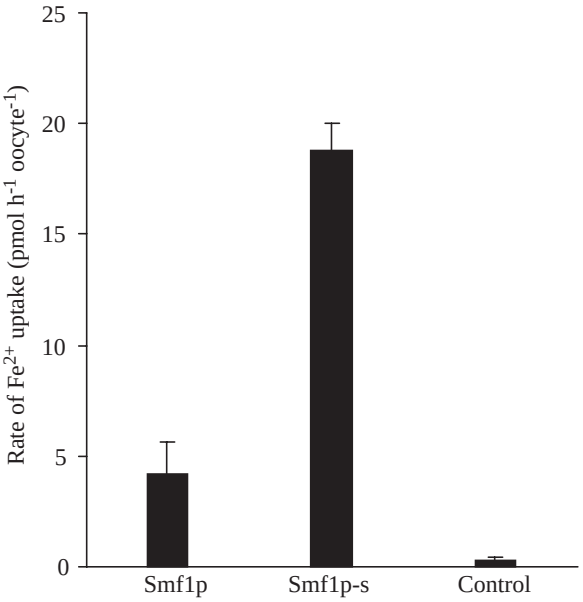

Fig. 1. Rate of uptake of $^{55}Fe^{2+}$ into *Xenopus laevis* oocytes expressing *SMF1* and *SMF1*-short. *Xenopus laevis* oocytes were injected with the synthetic RNAs indicated, and the $^{55}Fe^{2+}$ uptake was assayed as described in Materials and methods (*N*=5). Transport activity was measured with $^{55}Fe^{2+}$ in the presence of 1 μmol l^{-1} cold Fe^{2+} and 100 mmol l^{-1} choline chloride at pH 5.5. The control was uninjected oocytes. Short Smf1p is designated Smf1p-s. Values are means + S.E.M.

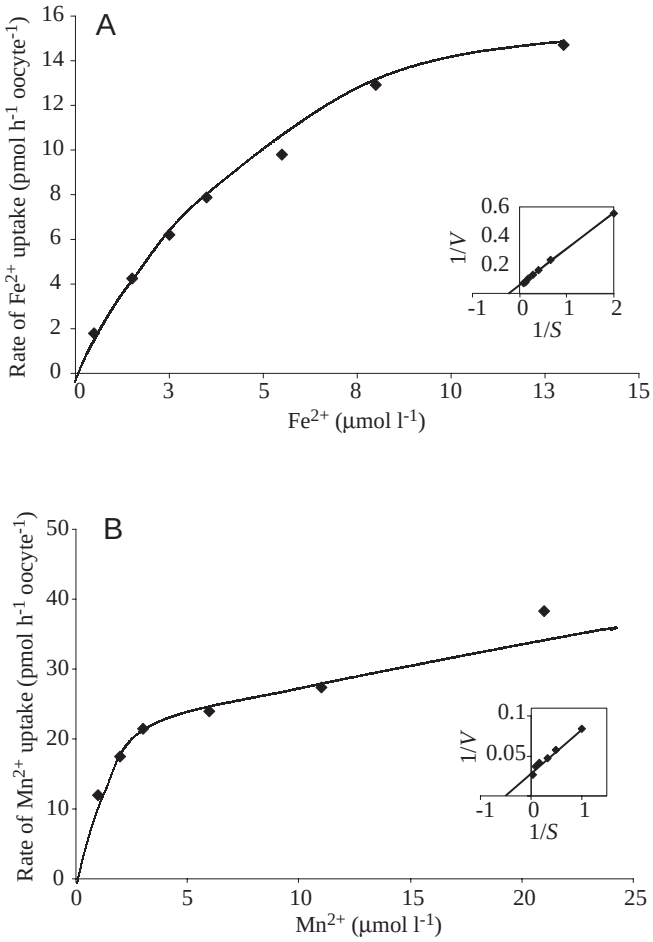

Fig. 2. Kinetics of $^{55}Fe^{2+}$ (A) and $^{54}Mn^{2+}$ (B) uptake into *Xenopus laevis* oocytes expressing the short Smf1p (Smf1p-s). The uptake experiment was performed as described in Materials and methods (*N*=5). (A) Uptake by Smf1p-s as a function of Fe^{2+} concentration assayed in the presence of 100 mmol l^{-1} choline chloride at pH 5.5. The curve is a Michaelis–Menten fit with K_m=4.6±0.25 μmol l^{-1}. (B) Uptake by Smf1p-s as a function of Mn^{2+} concentration assayed in the presence of 100 mmol l^{-1} choline chloride (pH 5.5). The curve represents a Michaelis–Menten fit with K_m=1.9±0.01 μmol l^{-1}. *V*, rate of uptake; *S*, substrate concentration. Values are means ± S.E.M.

that a considerable number of transporters are expressed in the oocyte plasma membrane (Gunshin et al., 1997; Wright et al., 1994). Yeast Smf1p, expressed in *Xenopus laevis* oocytes, exhibited a much lower activity than its mammalian homologue DCT1 (Chen et al., 1999). Electrophysiological measurements require higher levels of expression, so we constructed a chimeric protein of *SMF1* cDNA and some of the amino acid residues from the 5′ and 3′ ends of the open reading frame of DCT1 as well as its 3′ untranslated region (see Materials and methods). Equivalent constructs were prepared for *SMF2* and *SMF3*. *Xenopus laevis* oocytes expressing the chimeric Smf1p exhibited an increase in Fe^{2+} uptake compared with the native Smf1p, but no detectable change was observed for the Smf2 and Smf3 chimeras (data not shown).

There are significant differences between the amino acid sequence of the N terminus of Smf1p and those of the other two homologous yeast genes. The fact that only oocytes expressing the chimera Smf1p exhibited much higher activity prompted us to modify the N termini of the other constructs. Only one of the constructs gave a positive result, the control in which 68 amino acid residues had been deleted from the N-terminus of Smf1p to give Smf1p-s. Surprisingly, as shown in Fig. 1, Fe^{2+} uptake mediated by Smf1p-s was three times that of the original Smf1p; with some oocytes, in the presence of 10 μmol l^{-1} Fe^{2+}, the rate of uptake of Fe^{2+} reached 100 pmol h^{-1} $oocyte^{-1}$ (not shown). Smf1p-s was also effective in complementing the growth arrest of the Δ*SMF1* yeast mutant in the presence of EGTA and an even higher $^{54}Mn^{2+}$ uptake activity in yeast mutants in which the three SMF genes had been inactivated (Cohen et al., 2000).

The increased activity of Smf1p-s did not alter the other transport properties of the protein. The K_m values of Smf1p-s for Fe^{2+}, Mn^{2+} and Co^{2+} were not significantly different from those of Smf1p expressed in *Xenopus laevis* oocytes (not shown) and, therefore, we continued our work using this construct. Fig. 2 shows that the transport of both Fe^{2+} and Mn^{2+} by Smf1p-s was saturable and followed Michaelis–Menten kinetics. The apparent affinity constant (K_m) was 4.6±0.25 μmol l^{-1} (*N*=5) for Fe^{2+} (Fig. 2A), 1.9±0.01 μmol l^{-1} (*N*=5) for Mn^{2+} (Fig. 2B) and 8.6±1.2 μmol l^{-1} for Co^{2+} (not shown). It is important to note that *FET3* and *FET4* constitute the main iron-transport system of yeast, with their proteins

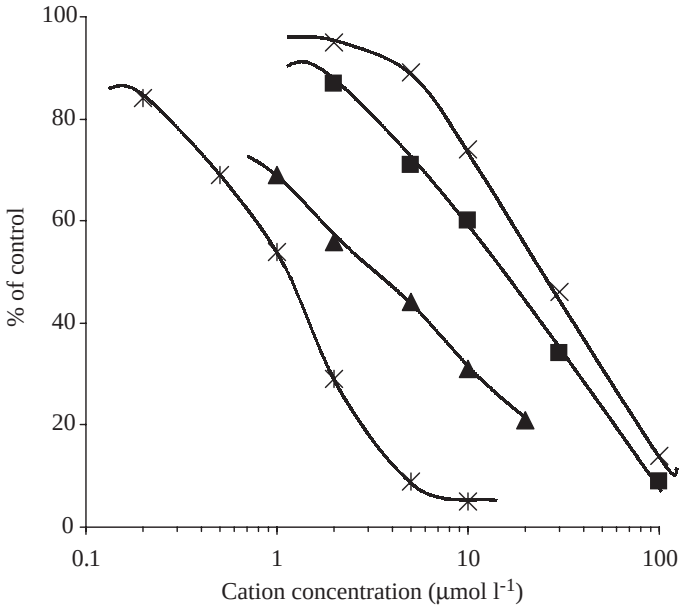

Fig. 3. Dose-dependent inhibition of $^{54}Mn^{2+}$ uptake by various divalent cations into *Xenopus laevis* oocytes expressing the short Smf1p (Smf1p-s). The rate of transport of 1 μmol l^{-1} Mn^{2+} in the presence of various concentrations of divalent metal ions was compared with the control rates of transport (Mn^{2+} alone, 100 %). Mn^{2+} uptake was measured in the presence of 100 mmol l^{-1} choline chloride (pH 5.5). ×, Zn^{2+}; ■, Fe^{2+}; ▲, Mn^{2+}; *, Cu^{2+}.

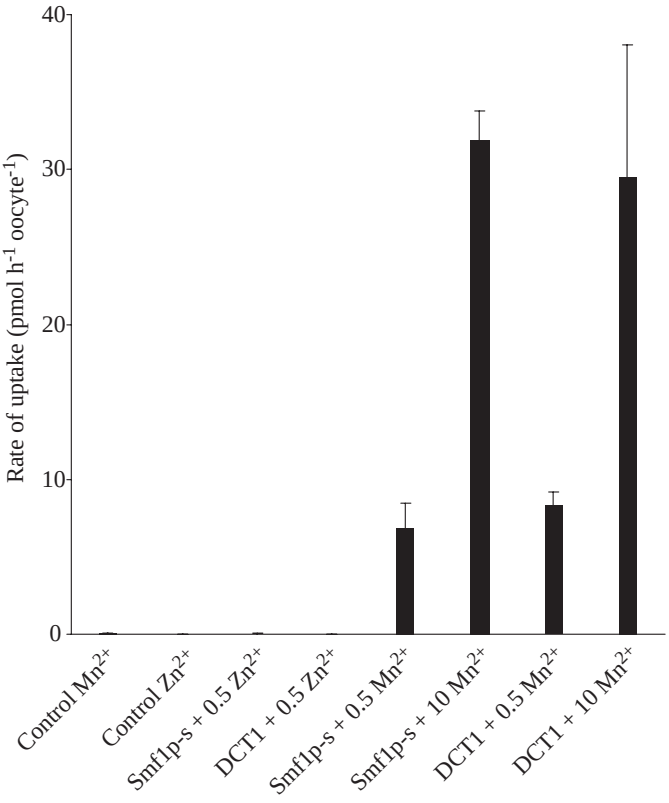

Fig. 4. Transport of Zn^{2+} and Mn^{2+} into *Xenopus laevis* oocytes expressing the short Smf1p (Smf1p-s) and DCT1. The c-RNAs of Smf1p-s and DCT1 were injected into *Xenopus laevis* oocytes as described in Materials and methods. The rates of uptake of $^{65}Zn^{2+}$ and $^{54}Mn^{2+}$ were measured in the presence of the indicated concentrations (in μmol l^{-1}) of unlabelled metal ions in a medium containing 100 mmol l^{-1} choline chloride (pH 5.5). The metal ions were added to the uptake medium in their chloride form. Values are means + S.E.M. (*N*=5).

having high and low affinities, respectively (Dix et al., 1994), whereas Smf1p mainly mediates the uptake of Mn^{2+} and other metal ions rather than Fe^{2+}, hence its highest measured affinity for Mn^{2+}. Competition of the divalent cations Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} with $^{54}Mn^{2+}$ uptake is shown in Fig. 3. The IC_{50} for $^{54}Mn^{2+}$ uptake was 3.5 μmol l^{-1}; for Fe^{2+} it was 15 μmol l^{-1}, for Zn^{2+} it was 25 μmol l^{-1} and for Cu^{2+} it was 1.2 μmol l^{-1}, demonstrating that Cu^{2+} has the highest affinity for the transporter. Since radioactive Cu^{2+} is not available to us, we were unable to demonstrate its uptake by Smf1p.

Zn^{2+} has been shown to inhibit Fe^{2+} and Mn^{2+} uptake by both DCT1 and Smf1p (Gunshin et al., 1997; Supek et al., 1996). Interestingly, as shown in Fig. 4, there is no detectable transport of $^{65}Zn^{2+}$ into *Xenopus laevis* oocytes expressing either Smf1p-s or DCT1. Moreover, elevation of the Zn^{2+} concentration from 0.5 to 10 μmol l^{-1} had no detectable effect on uptake activity (not shown). Recent studies with DCT1 expressed in Caco-2 TC7 cells also suggest that Zn^{2+} is not transported by DCT1 (Tandy et al., 2000). However, the same study failed to show inhibition of Fe^{2+} uptake by Zn^{2+}. This inhibition is readily detected both in oocytes expressing Smf1p-s and DCT1 and in yeast cells expressing Smf1p.

The uptake of Co^{2+}, Mn^{2+} and Fe^{2+} into *Xenopus laevis* oocytes expressing DCT1 and Smf1p-s was analyzed under identical conditions. Fig. 5 shows that, in a solution containing 100 mmol l^{-1} choline chloride (pH 5.5), Co^{2+} uptake by DCT1 was greater than Mn^{2+} or Fe^{2+} uptake. Replacing choline chloride with NaCl had no effect on the uptake activity of DCT1 (not shown). The yeast homologue Smf1p-s exhibited a different metal-ion specificity: Mn^{2+} uptake was faster than Fe^{2+} uptake, and Co^{2+} was taken up at a much lower rate.

The transport of metal ions by all the members of the NRAMP family investigated is driven by protons (Nelson, 1999). In the case of Smf1p exclusively, Fe^{2+} uptake mediated by its c-RNA expressed in oocytes was inhibited by Na^+ (Chen et al., 1999). Na^+ inhibits $^{55}Fe^{2+}$ and $^{54}Mn^{2+}$ uptake by *Xenopus laevis* oocytes expressing Smf1p-s (see Figs 6, 7). To investigate how Na^+ inhibits metal-ion uptake, we tested the effects of NaCl on $^{55}Fe^{2+}$ uptake by Smf1p-s at various pH values (Fig. 6). The results show that Na^+ inhibition of Fe^{2+} uptake is pH-dependent. The higher the pH in the medium, the stronger the inhibition by Na^+, suggesting that Na^+ and H^+ compete for the same binding site and/or transport pathway. This was further supported by the experiment depicted in Fig. 7, in which rates of $^{54}Mn^{2+}$ uptake in the absence and presence of NaCl at different Mn^{2+} concentrations are compared. The percentage inhibition by Na^+ was not affected by Mn^{2+} concentration. From Figs 6 and 7, we can conclude

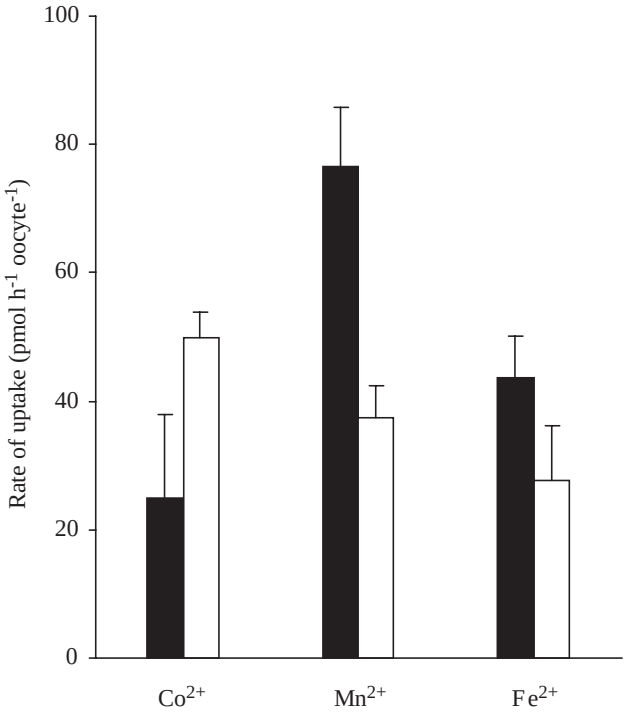

Fig. 5. Rates of uptake of metal ions into *Xenopus laevis* oocytes expressing DCT1 and the short Smf1p (Smf1p-s). The uptake experiment was performed as described in Materials and methods. The uptake solution contained 100 mmol l^{-1} choline chloride (pH 5.5), 2 mmol l^{-1} ascorbic acid and 11 μmol l^{-1} of the indicated metal ion in its chloride form. Filled columns, Smf1p-s; open columns, DCT1. Values are means + S.E.M. (*N*=5).

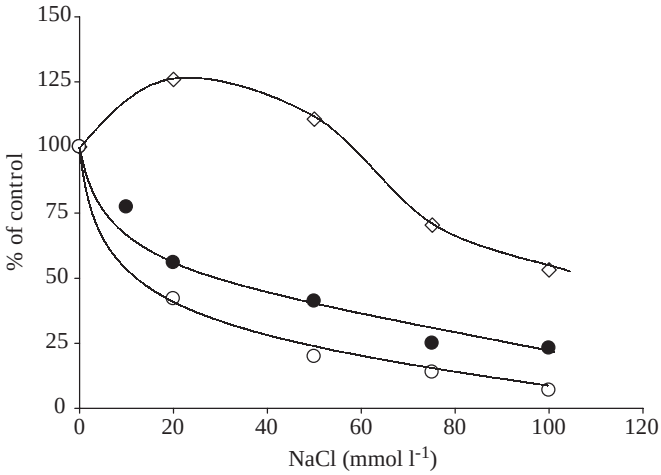

Fig. 6. Effects of pH on the inhibition by Na^+ of Fe^{2+} uptake by *Xenopus laevis* oocytes expressing the short Smf1p (Smf1p-s). Fe^{2+} uptake was assayed in a medium containing 1 μmol l^{-1} Fe^{2+} and the indicated NaCl concentrations that, together with choline chloride, gave a concentration of 100 mmol l^{-1}. The medium was buffered by Tris-base to give the following pH values; pH 5.5 (open diamonds), pH 6.5 (filled circles) and pH 7 (open circles).

that inhibition by Na^+ was affected by H^+ concentration but not by Mn^{2+} concentration. This finding strengthens the hypothesis that the inhibition by Na^+ is associated with the H^+ binding site and not with the metal-ion binding site.

A two-electrode voltage-clamp analysis was employed to gain further understanding of the mechanism of metal-ion uptake and its inhibition by Na^+. The pre-steady-state and steady-state currents generated by metal ions or Na^+ in *Xenopus laevis* oocytes expressing Smf1p-s were recorded. As reported previously (Chen et al., 1999), expression of Smf1p generates major Na^+-uncoupled currents. Current were recorded in oocytes injected with Smf1p-s cRNA and bathed in choline chloride buffer (pH 5.5) in the absence of metal ions. Stepping the membrane potential from the holding potential of –25 mV to the test potential of +50 mV induced a transient current (pre-steady-state current). Addition of 50 μmol l^{-1} $MnCl_2$ resulted in the disappearance of the pre-steady-state current and induced an inward positive current of approximately 20 nA when the potential was stepped to –130 mV. The presence of Na^+ in the bath medium, buffered at pH 5.5, abolished the pre-steady-state current and generated an even bigger leak current of approximately 250 nA at –50 mV. As reported previously, the magnitude of the Na^+ leak current is proportional to the pH of the medium (Chen et al., 1999), indicating that Na^+ is transported into the oocyte. Na^+ also inhibits metal-ion transport in the same pH-dependent manner (Fig. 6). These observations suggest that Na^+ competes with protons in the driving-force (H^+) pathway and that the

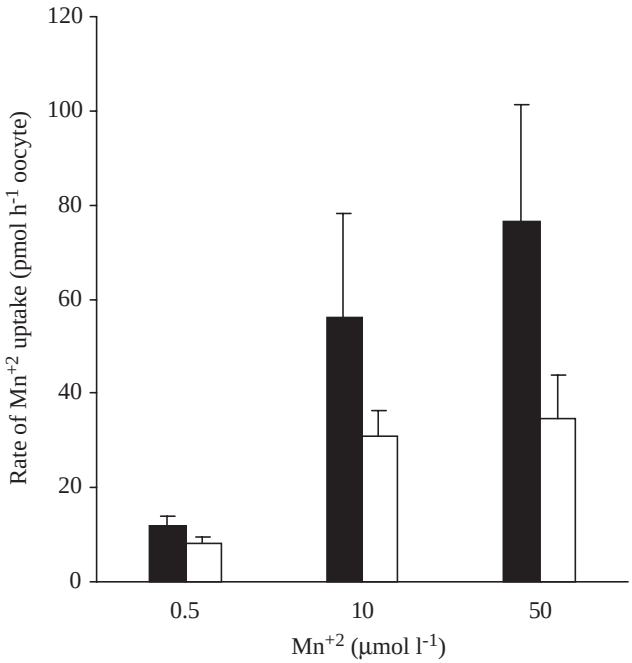

Fig. 7. Competition between Na^+ and Mn^{2+} for $^{54}Mn^{2+}$ uptake by *Xenopus laevis* oocytes expressing the short Smf1p (Smf1p-s). Experimental conditions were as described in Fig. 6, except that the uptake medium was buffered to pH 5.5. Filled columns, medium containing 100 mmol l^{-1} choline chloride; open columns, medium containing 100 mmol l^{-1} NaCl. Values are means + S.E.M. (*N*=5).

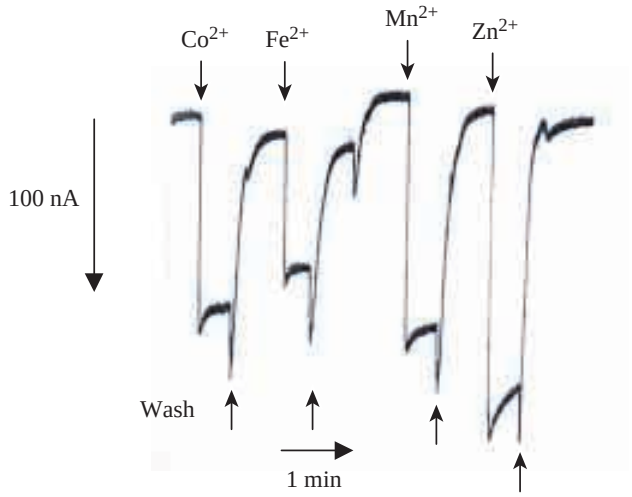

Fig. 8. Currents generated in *Xenopus laevis* oocytes expressing DCT1 in the presence of different metal ions. Currents generated by the addition of the various metal ions were measured in NaCl solution at pH 5.5. Metal ion (1 mmol l^{-1}) in its chloride form was added where indicated. L-Ascorbic acid (2 mmol l^{-1}) was added to all solutions to maintain iron and cobalt in the II form. A downward movement of the trace indicates an increase in current.

Na^+-uncoupled current is essentially a slippage that is intimately involved in the mechanism of metal-ion transport by Smf1p. The Na^+ leak currents generated by Smf1p are not dependent on the presence of metal ions; in contrast, DCT1-generated proton leak currents depend on the presence of the metal ion in the medium (Gunshin et al., 1997). Fig. 8 shows metal-ion-dependent currents generated by DCT1 expressed in *Xenopus laevis* oocytes. Remarkably, the proton currents were generated not only by Mn^{2+}, Co^{2+} and Fe^{2+} but also by Zn^{2+}, which is not transported by DCT1. Thus, the stoichiometry between H^+ and the metal ion transported by DCT1 can vary from 1 to infinity. This phenomenon represents a built-in slippage in the mechanism of metal-ion transport.

Even though metal-ion uptake is driven by H^+, the energy source for this transport is not clear. It has been shown previously that Fe^{2+} uptake into oocytes mediated by DCT1 was not affected by the membrane potential of the oocytes (Nelson, 1999). To determine the dependency of Smf1p-s transport activity on membrane potential, we measured $^{54}Mn^{+2}$ uptake into oocytes expressing Smf1p-s at three different membrane potentials. As shown in Fig. 9A, similar to the results for DCT1, at pH 5.5 and in the presence of choline chloride, the holding potential had little effect on the transport activity of Smf1p-s. Between −75 and −10 mV, uptake activity was more or less unchanged. Moreover, at pH 5.5 and a holding potential of −75 mV, no significant inhibition by 100 mmol l^{-1} NaCl was observed (Fig. 9A). Changing the holding potential to −10 mV did not change the transport activity in medium containing choline chloride, but replacing the choline chloride with NaCl resulted in approximately 40 % inhibition of Mn^{2+} uptake at this holding potential. An increase in the holding potential to +20 mV drastically reduced the

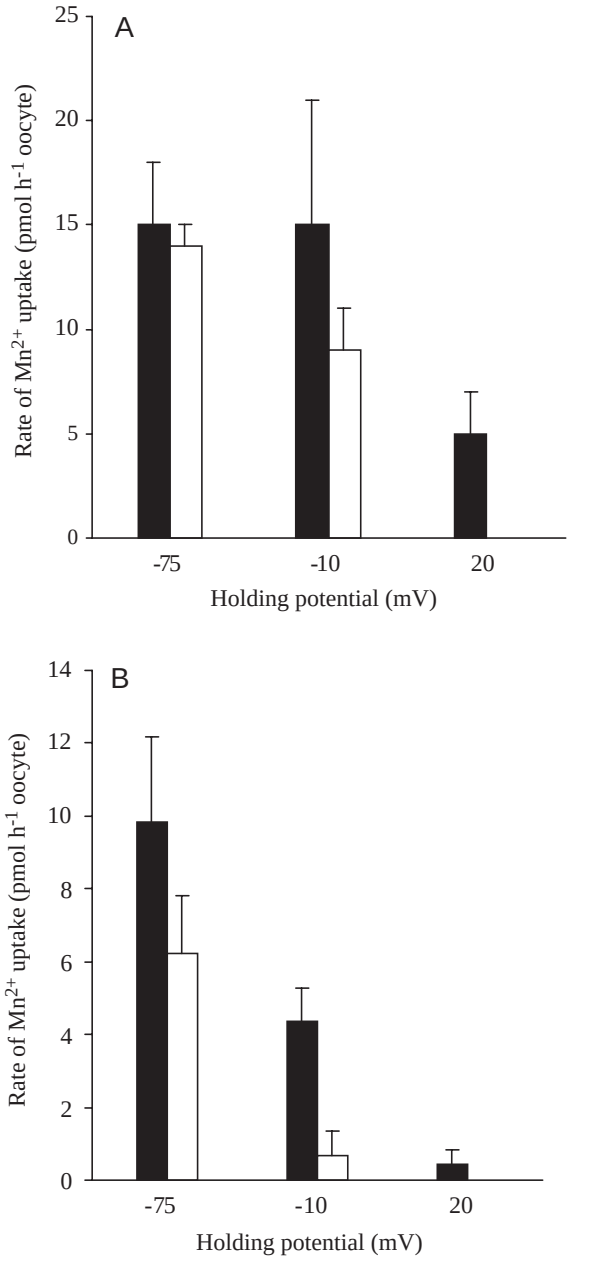

Fig. 9. Rate of Mn^{2+} uptake by *Xenopus laevis* oocytes expressing the short Smf1p (Smf1p-s) at different holding potentials, at different pH values and in the presence or absence of NaCl. Mn^{2+} uptake at a constant holding potential was measured as described in Materials and methods and in Fig. 8 in medium containing either choline chloride or NaCl. (A) $^{54}Mn^{2+}$ uptake (1 µmol l^{-1}) into oocytes expressing Smf1p-s was measured at three different holding potentials, −75, −10 and +20 mV, in the presence (open columns) and absence (filled columns) of NaCl at pH 5.5. At each potential indicated, $^{54}Mn^{2+}$ uptake was assayed for 10 min, and uptake was terminated by a 2 min wash (2 ml) with the same buffer containing no radioactive tracer. (B) The same experiment as in A, but at pH 6.5. Values are means + S.E.M. (*N*=5).

transport activity of Smf1p-s. Elevating the medium pH to 6.5 resulted in significant inhibition by Na^+ even at −75 mV (Fig. 9B; *P*=0.06). A change in the holding potential to

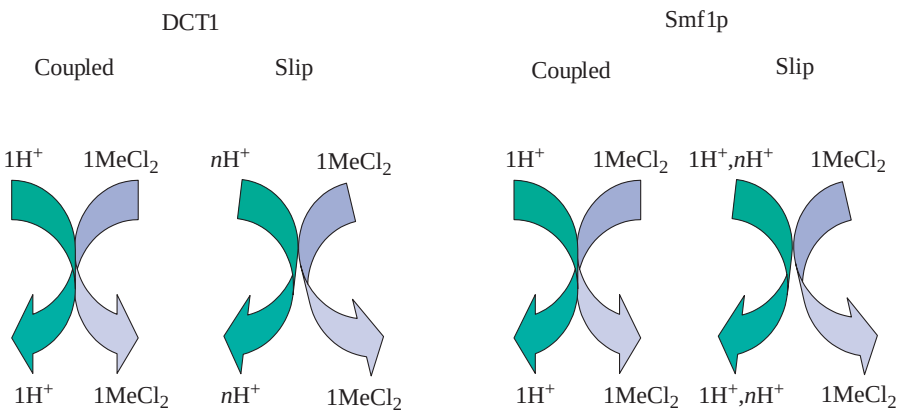

Fig. 10. Schematic representation of slippage by a 'clutch' mechanisms. A mechanistic 'clutch' regulates a variable stoichiometry between the ion-motive pathway (blue) and the substrate-transport pathway. High concentrations of H^+ for DCT1 or of Na^+ for Smf1p result in slippage through the driving-force (ion-motive) pathway and loose coupling with the divalent metal-ion transport pathway. Me, metal ion.

–10 mV in the presence of choline chloride reduced the transport activity twofold, and addition of Na^+ almost abolished the transport activity of Smf1p-s. Switching to a holding potential of +20 mV almost abolished the transport of Mn^{2+}. From these results, we conclude that the driving force for metal-ion transport does not behave in a pure Mitchellian fashion (Mitchell and Moyle, 1967; Mitchell, 1968).

Discussion

Metal-ion transport by the members of the NRAMP family of transporters is driven by protons (Supek et al., 1997; Gunshin et al., 1997). However, the molecular mechanism of this process is largely obscure and it is not known whether the transport is driven by a classical proton-motive force (Mitchell, 1968; Nelson, 1999). DCT1 has been shown to cotransport Fe^{2+} together with H^+ with a stoichiometry of 1:1 (Gunshin et al., 1997). The substitution of Cl^- with gluconate or isethionate results in a drastic reduction in Fe^{2+} transport into oocytes expressing DCT1 (Nelson, 1999), and it was proposed that the metal ion is cotransported with Cl^- (or other small anions such as NO_3^- or SCN^-; see Gunshin et al., 1997) and that the steady-state current results from the transport of positively charged protons. In the presence of Cl^-, metal-ion transport was unaffected by an imposed membrane potential (Nelson, 1999). However, when Cl^- was replaced by gluconate, Fe^{2+} uptake by DCT1 was affected by the membrane potential, with imposed positive potentials drastically inhibiting metal-ion uptake. Metal-ion transport is therefore dependent on the concentration of protons on the external side of the membrane because protons are cotransported with Fe^{2+} (Tandy et al., 2000), but the nature of the driving force for this transport is obscure.

At physiological membrane potentials of –90 to –30 mV, the apparent affinity constant for H^+ was approximately 1 μmol l^{-1}, suggesting that, at neutral pH, proton binding is the rate-limiting step in the transport process. At low pH, DCT1 expressed in *Xenopus laevis* oocytes induces a proton leak into the oocytes and, under certain conditions, the transporter may operate as a H^+ uniporter (Gunshin et al., 1997). This proton leak is influenced by the membrane potential: it increases as the imposed potentials become more negative and is absolutely dependent on the presence of metal ions in the medium (Gunshin et al., 1997; see also Fig. 8). The binding constant of the metal ions is closely related to their affinity for the transporter (results not shown). The involvement of Zn^{2+} in the transport process and proton leak distinguishes this transport from that of all the other metal ions tested. We observed that Zn^{2+} is not transported by DCT1, yet induces the proton leak as effectively as the other metal ions (Fig. 4; A. Sacher and N. Nelson, unpublished results). Zn^{2+} is an inhibitor of Fe^{2+} transport by DCT1 and Smf1p (Fig. 3; see also Gunshin et al., 1997) and is likely to bind to the same site as the other metal ions. The proton leak in DCT1 is, therefore, a genuine slip that is intimately involved in the mechanism of metal-ion transport by the transporter.

The mechanism of metal-ion transport by Smf1p is closely related to that of the mammalian DCT1. The yeast transporter exhibits similar affinities for the various metal ions and, while Zn^{2+} is a competitive inhibitor, it is not transported by Smf1p (Fig. 4; see also Supek et al., 1996; Cohen et al., 2000). The most striking difference between DCT1 and Smf1p is in the uncoupled leak that is specific for Na^+ in Smf1p and for H^+ in DCT1 (Chen et al., 1999). The Na^+ slip in Smf1p is not dependent on the presence of metal ions and increases with increasing pH. Since Na^+ abolishes the pre-steady-state current, it is likely to bring about a conformational change similar to that obtained in response to the binding of metal ions. However, it is unlikely to be bound to the metal-ion transporting site, because metal ions do not compete with Na^+ for the leak current and elevation of the metal ion concentration did not affect the inhibition of metal-ion transport by Na^+ (Fig. 7). Therefore, Na^+ is likely to compete with protons on the proton-binding site and to generate a Na^+ slippage through the proton transport pathway.

The phenomenon of proton and Na^+ slip by metal-ion transporters could be explained by two distinct mechanisms: one involves the formation of selective ion channels during the transport process, and the other involves a variable coupling stoichiometry between the pathway of the driving force (proton) and the transport pathway (metal ion). When changes in the stoichiometry of the driving ions *versus* the substrate were discovered in sugar and neurotransmitter transporters, it was suggested that these membrane proteins occasionally open up channels that account for the ion slippage (Mager et al., 1994; DeFelice and Blakely, 1996; Kavanaugh, 1998). For

neurotransmitter transporters, the existence of a Cl^- channel activity for only 0.1% of the time could explain the slippage phenomenon. To explain the slippages observed in DCT1 and Smf1p in terms of the occasional formation of a proton or Na^+ channel, the channel activity should occur for 1–5% of the transport time. Moreover, while the proton slippage in DCT1 is dependent on the presence of a substrate (as in the case of neurotransmitter transporters), the slippage of Na^+ through Smf1p is independent of the presence of a substrate.

An alternative explanation for the slip phenomenon could involve a novel 'clutch' mechanism (Fig. 10). This mechanism could be explained in terms of two unique but interconnected ion pathways, one dominated by the ion utilized for driving the transport, and the other by the transported metal ions. In the coupled state of DCT1, the stoichiometry of proton to metal ion is close to 1. Increasing the driving force by lowering the pH or imposing a highly negative potential generates a proton slip that maintains transport of the metal ion at a rate close to that of the coupled state, while increasing the rate of proton movement across the transporter. A similar mechanism applies for Smf1p, except that the slipping ion is Na^+ and not H^+. The Na^+ competes with protons for a common binding site and slips across the transporter in the proton pathway.

The evolutionary and physiological virtues of this phenomenon could be explained in terms of a protection mechanism against overloading of metal ions. It has recently become apparent that DCT1 functions as the entrance port for Fe^{2+} into the duodenum, and mutations in the gene encoding this protein cause anaemia (Fleming et al., 1997). Apparently, the evolution of the system could not provide an alternative driving force for the proton electrochemical gradient. Given that excess iron and other metal ions are toxic, a protection mechanism against overtransport of these elements had to be developed. Several kinds of food product, such as blood sausages and liver, are highly enriched in iron and eating too much of them can also cause heartburn. The excess acid may reach the duodenum together with high iron concentrations, and this combination of very high driving force and substrate abundance may be deleterious. Uncoupling by a built-in proton slip could protect the organism from overuptake of metal ions. It was suggested that a similar protection function might be performed by the Na^+ slip through the yeast Smf1p (Chen et al., 1999). In this case, the yeast cells may have been protected against excessive influxes of toxic metal ions by evolving a Na^+ slippage that competes with metal-ion uptake under conditions of increasing salt concentration in the medium. This hypothesis should be rigorously tested in future experiments.

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