FEBS Letters 581 (2007) 2599–2604

Analysis of the rainbow trout solute carrier 11 family reveals iron import ⩽ pH 7.4 and a functional isoform lacking transmembrane domains 11 and 12

C.A. Cooper[a,*], M. Shayeghi[a], M.E. Techau[b], D.M. Capdevila[c], S. MacKenzie[c], C. Durrant[a], N.R. Bury[a]

[a] *Nutritional Sciences Research Division, King's College London, 150 Stamford Street, London SE1 9NH, UK*
[b] *Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, UK*
[c] *Unitat de Fisiologia Animal, Dept. de Biologia Cel.lular de Fisiologia i d'Immunologia, Facultat de Ciències, Universitat Autonoma de Barcelona, 08193 Bellaterra (Cerdanyola del Valles), Barcelona, Spain*

Received 20 March 2007; revised 27 April 2007; accepted 29 April 2007

Available online 8 May 2007

Edited by Barry Halliwell

Abstract A *Xenopus* oocyte heterologous expression system was used to characterise iron transport properties of two members of the solute carrier 11 (slc11) protein family isolated from rainbow trout gills. One cDNA clone differed from the trout Slc11α containing an additional 52 bp in the exon between transmembrane domains (TM) 10 and 11. The 52 bp contained a stop codon, resulting in a novel isoform lacking the last two TM (termed slc11γ). Slc11γ and another isoform slc11β, import Fe^{2+} at external pHs ⩽ to 7.4. Trout slc11β Fe^{2+} import was more sensitive to inhibition by divalent metals. The novel vertebrate slc11γ isoform functions without TM11 and 12.

Keywords: Divalent metal transporter 1 (DMT1); Iron; Natural resistance-associated macrophage protein (Nramp); Fish; Transmembrane domains; Rainbow trout

1. Introduction

There are two functional homologs of the mammalian solute carrier 11 (Slc11) family of proteins that transport both ferrous iron (Fe^{2+}) and protons (H^+). Both Slc11 homologs are hydrophobic proteins consisting of 10–12 putative transmembrane (TM) spanning domains [1–3]. Slc11a1 (formerly known as Natural resistance-associated macrophage protein1, Nramp1) was discovered by positional cloning and was identified as the single gene linked to the leishmania-, salmonella-, and mycobacteria-susceptibility (Lsh, Ity, Bcg) identified on mouse chromosome 1 [1]. Slc11a1 is expressed at the membrane of acidic phagosomes and is associated with the ability of macrophages to destroy bacterial pathogens [1]. Goswami et al. [4] using a *Xenopus* oocytes expression system showed that Slc11a1 Fe^{2+} uptake increases as extracellular pH rises suggesting Fe^{2+}/H^+ antiporter activity. The resulting elevated phagosomal iron concentration contributes to the antimicrobial activity by the generation of toxic free radical via the Fenton/Haber-Weiss reaction [5]. However, recently, Forbes and Gros [6] demonstrated that Slc11a1 Fe^{2+} (as well as Mn^{2+} and Co^{2+}) transport is pH-dependent which suggests that the antimicrobial activity may in fact result from the removal of essential metals from the phagosome.

The functional characteristics of the mammalian Slc11a2 (formerly known as Nramp 2) suggests that this protein preferentially acts as a pH dependant Fe^{2+}/H^+ symporter whose main role is thought to aid the uptake of non-haem bound iron from the diet [3] and the transfer of iron from the endosome to the cytosol following transferrin mediated iron uptake by cells [7]. However, Slc11a2 has also been located on the phagosome membrane suggesting a potential antimicrobial role [8]. A number of divalent metals have been shown to induce an inward current in *Xenopus* oocytes expressing Slc11a2 [3], and radiotracer or fluorophore experiments have confirmed that Slc11a2 can transport $^{54}Mn^{2+}$, $^{60}Co^{2+}$ [9], Ni^{2+} [5], $^{109}Cd^{2+}$ [10] and Cu^+ [11]. The promiscuous transport property of Slc11a2 has meant it is often referred to as Divalent Metal Transporter1 (DMT1) [3].

Slc11 homologs have been identified in a number of teleost fish species [12–18] and play an important role in the immune function of fish [17,18]. All teleost slc11 identified to date share a greater sequence homology to mammalian Slc11a2 rather than Slc11a1, but only one study has confirmed that a piscine slc11 homolog transports iron [15]. In rainbow trout (*Oncorhynchus mykiss*) there are two *slc11* homologs derived from separate loci termed Nrampα and β [12]. Northern blot analysis reveals differential mRNA expression of *Nramp*α (referred to as *slc11*α) and *Nramp*β (slc11β), which suggests that slc11α is involved in antimicrobial activity and slc11β in non-haem bound iron transport [15]. Our study identified a third trout slc11 isoform, slc11γ, whose predicted translated protein is truncated, lacking TMs 11 and 12. Polymorphisms in mammalian *Slc11A1 and A2* can have profound effects on the ability of the translated protein to transport iron, disrupting iron homeostasis and increasing susceptibility to bacterial infection [4,19–22]. Due to the lack of functional transport data for teleost fish slc11 proteins, the present study sets out to characterise the iron transport properties of the rainbow trout slc11β isoform and the novel slc11γ isoform.

*Corresponding author. Present address. School of Biosciences, University of Exeter, Hatherly Laboratories, Exeter EX4 4PS, UK. Fax: +44 1392 263000.
E-mail address: chris.cooper@ex.ac.uk (C.A. Cooper).

0014-5793/$32.00
doi:10.1016/j.febslet.2007.04.081

2. Materials and methods

2.1. Trout Nramp isoform isolation

Slc11 cDNAs were generated from total RNA extracted from Rainbow trout (*O. mykiss*) double seeded primary gill cell cultures (see [23] for cell culture methodology). Specific primers were designed to ensure the whole open reading frame (ORF) for both trout s*lc11* (s*lc11*α or Nrampα) (AF048760) For: CACAACC ATG AAG ACA GCG CGG GAA GCA; Rev: GCA CTG CAT CCT CCA TGT TAG; s*lc11* β or Nramp β (AF048761) For: CACAACCATG GAG ACA GAG CGG GAA GC, Rev: GGT GAT CTG CTG TGC ACA GC). These ORF primers included a 'kozak' sequence at the 5′ end (underlined). Proof reading Platinum® *Taq* DNA polymerase (Invitrogen, UK) was used to amplify target DNA sequences for ORF derivation, and resulting product ligated into TOPO 2.1® vector (Promega, UK) and transformed into chemically competent *Escherichia coli* cells (Promega, UK). Preparation of clones for sequencing was performed following the manufacturers guidelines (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, UK).

2.2. Trout Nramp functional analysis in Xenopus oocytes

African clawed toads (*Xenopus laevis*; 140 ± 30 g) were housed at King's College London in 100 L containers filled with dechlorinated water maintained at 18 °C with a photoperiod 12:12 h light:dark. *Xenopus* oocytes preparation, cRNA synthesis and injection followed the protocol of Mckie et al. [24], and control oocytes were injected with an equal volume of water.

Uptake studies were carried out at room temperature in MBS and repeated three times with different batches of oocytes. The pH of the MBS was altered with HCl and Tris-Base prior to the assay, depending on the experiment and 1 mmol L^{-1} ascorbic acid was included to reduce the metal into a divalent state prior to experimentation. For the dose-dependent uptake studies oocytes were exposed to 2, 4, 15, 20, 40 and 70 μmol Fe L^{-1}, ⩽15 μmol Fe L^{-1} iron was added as ^{59}Fe (specific activity 110 MBq/mg ^{59}Fe, Amersham, UK), ⩾ 15 μmol Fe L^{-1} the iron concentrations were made up by the addition of cold $FeCl_3$ (made freshly on the day) to 10 μmol ^{59}Fe L^{-1}. For the metal inhibition studies 100 μmol L^{-1} of each of the divalent metals tested ($CdCl_2$, $MnSO_4$, $ZnCl_3$, $CoCl_2$ and $Pb(NO_3)_2$) was added to an MBS containing 16 μmol L^{-1} of $^{59}FeCl_3$. For the experiments at pH, 5.5, 6.0, 7.4, 8.0 and 9.0, pH was verified following the addition of 5 μmol ^{59}Fe L^{-1} and was stable throughout the 4 h experiment (data not shown). Ferrous iron concentrations were measured using the phenanthroline method [25] at the beginning and end of the experiment. Oocyte uptake of cadmium was also measured at 10 μmol $^{109}CdCl_3$ L^{-1} (24.9 MBq/μg ^{109}Cd, Amersham, UK). All assays were carried out over 3–4 h, after which the oocytes were washed three times in ice cold MBS to remove loosely bound radiolabel. Radioactivity in each individual oocyte was measured using a gamma counter (LKB Wallac 1282 compugamma), metal uptake rate per oocyte being derived from the following equation:

$$\text{Oocyte Fe or Cd uptake} = \text{cpm}/(\text{SA} \times t)$$

where 'cpm' represents counts per minute measured in the oocyte, 'SA' the specific activity added to the media water (cpm $pmol^{-1}$), and '*t*' is the duration of the experiment in hours. For the temporal pattern of iron uptake '*t*' was removed.

2.3. Statistical analysis

Data are expressed as means ± S.E.M. and figures represent typical results from one of the repeated experiments. Sigma Plot (version 8) was used to plot graphs and identify the best fit uptake kinetic equations to the data. Analysis of variance (ANOVA) followed by a Tukey–Kramer multiple comparisons test was used to assess significant differences between treatments (GraphPad Instat statistical package). In all cases a minimum of $P < 0.05$ was accepted as a statistically significant difference.

3. Results

Sequencing confirmed the isolation of *slc11*β, however, the other cDNA isolated from the gill cells of rainbow trout was found to be a novel isoform termed *slc11*γ. *Slc11*γ cDNA (GenBank Accession number EF495162) is similar in sequence to *slc11*α (0.28% difference in nucleotide sequence) but contains a 52 bp insert between the coding region between TM10 and TM11. The 52 bp sequence starts with a stop codon and thus the predicted slc11γ open reading frame (1419 bp) encodes a protein of 473 amino acids, which lacks TM11 and 12; TM that are present in other vertebrate Slc11 proteins [2].

Xenopus oocytes injected with either *slc11*γ *or* β cRNA showed a temporal and dose-dependent uptake of ^{59}Fe (Fig. 1). Oocytes expressing slc11γ imported twice as much Fe^{2+} than those expressing slc11β over a 300 min flux experiment (Fig. 1A). A sigmoidal, three parameters, equation was used to best fit the dose-dependent uptake data. The maximum

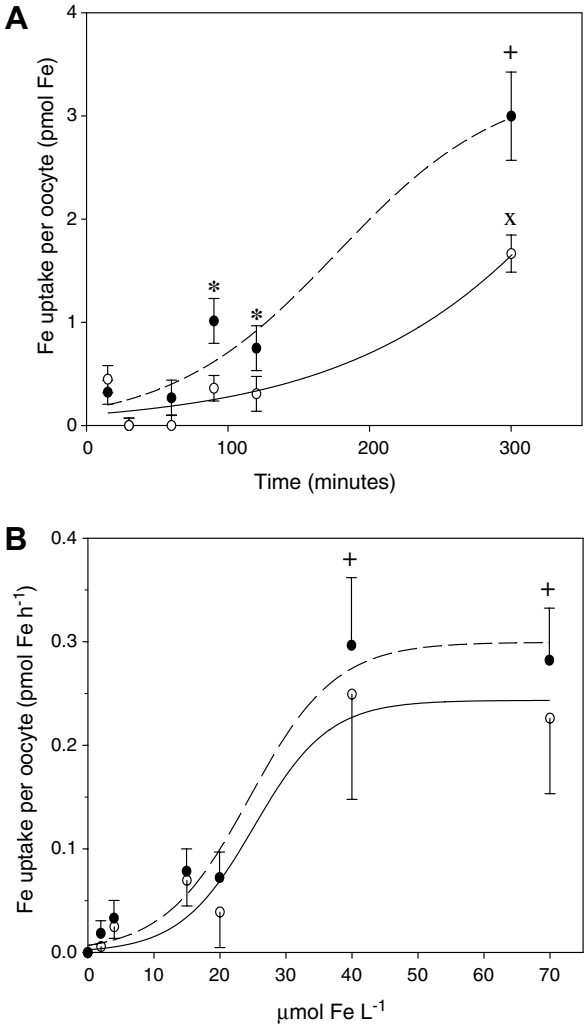

Fig. 1. (A) Temporal pattern of iron uptake in oocytes exposed to 17 μmol ^{59}Fe L^{-1} and (B) oocyte dose dependent uptake of ^{59}Fe during a 3 h exposure period, both at pH 6. Oocytes were either injected with *slc11*γ (filled circles with dashed line) or *slc11*β cRNA (open circles with solid line), whereas control oocytes were injected with water. Values are means ± S.E.M. ($N = 8$–13). A sigmoidal, three parameters, equation was used to best fit the dose-dependent uptake data (slc11γ: $y = 0.3/1 + e^{-((x - xo)/24.6)}$; slc11β: $y = 0.24/1 + e^{-((x - xo)/25.2)}$). An 'asterisk' indicates a significant difference in uptake rates between slc11γ and the control, a 'cross' denotes that both slc11γ and β are significantly different when compared to the control, and an 'X' shows a significant difference between slc11γ and β ($P < 0.05$).

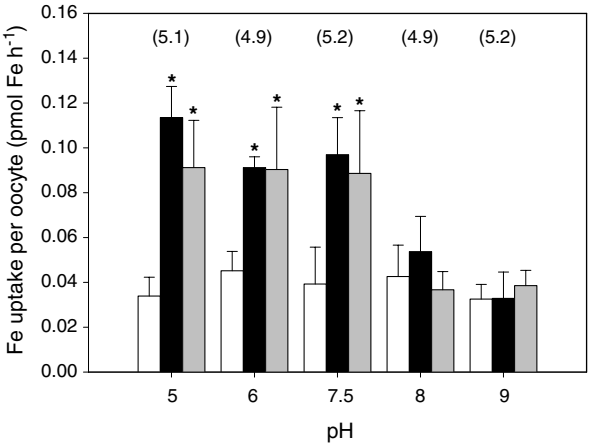

Fig. 2. pH-dependent iron uptake (5 μmol $^{59}Fe\ L^{-1}$) in control oocytes (white bars) or expressing slc11γ (black bars) or slc11β (grey bars). Values are means ± S.E.M. ($N = 7$). An 'asterisk' indicates a significant difference from controls ($P < 0.05$). Values in parentheses represent average measured Fe^{2+} concentration (μmol L^{-1}) at each pH.

iron transport capacity (J_{max}) and half maximal uptake rate (K_m) for iron for both slc11γ or slc11β were similar (Fig. 1B): J_{max} of 0.3 pmol Fe h^{-1} and 0.24 pmol Fe h^{-1} and K_m of 24.6 μmol Fe L^{-1} and 25.2 μmol Fe L^{-1}, respectively.

Iron uptake by oocytes expressing slc11γ or β was pH dependent (Fig. 2) being significantly greater at pH 5, 6 or 7.4 compared to uptake rates at pH 8 or 9 (Fig. 2). Results confirm that the 1 mmol L^{-1} ascorbic acid was sufficient to keep 5 μmol $^{59}Fe\ L^{-1}$ in the ferrous state throughout the experimental period at each pH concentration tested (Fig. 2). There was no difference in the uptake rate observed between the trout Slc11 isoforms.

Iron uptake, at an external concentration of 16 μmol $^{59}Fe\ L^{-1}$, by *Xenopus* oocytes expressing slc11γ or β was significantly inhibited by various divalent metals added at a concentration of 100 μmol L^{-1} (Fig. 3). Oocytes expressing slc11γ exhibited higher uptake rates of ^{59}Fe when compared to those expressing slc11β (Fig. 3A). Thus, to compare the degree of inhibition between the two isoforms we expressed the results as a percentage of the uptake in the absence of competing metal (Fig. 3B). When expressed in this way slc11β is more sensitive to inhibition by metal than slc11γ. The hierarchy of inhibition for slc11γ being Cd > Mn > Co > Zn > Pb, whereas all metals inhibited iron uptake to a similar degree in oocytes expressing slc11β. *Xenopus* oocytes injected with *slc11*β significantly accumulated ^{109}Cd when compared to those injected with water or *slc11*γ (Fig. 4).

4. Discussion

Sequence analysis of the Slc11 family typically predicts a protein with 12 putative transmembrane (TM) domains [2] and this is the case for the original Slc11 protein family members isolated from the rainbow trout [12]. However, anomalies exist in the eukaryote kingdom and within yeast and *Drosophila* the Slc11 homologs are predicted to have 11 and 10 TM domains, respectively [2]. In the current study we have identified a trout *slc11* isoform that is similar to *slc11*α (AF048760) except

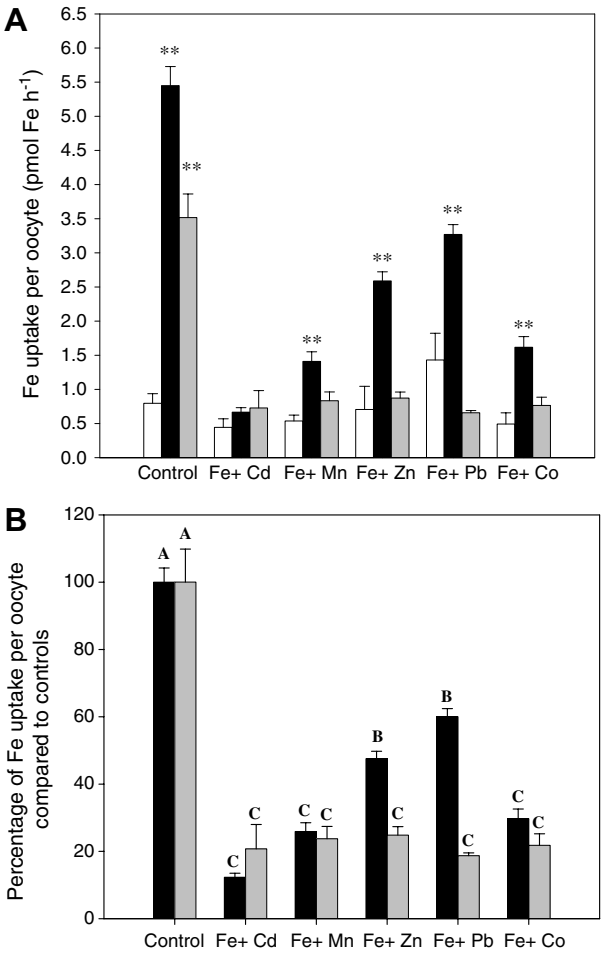

Fig. 3. (A) The uptake of Fe (16 μmol L^{-1} Fe; 1 mmol L^{-1} ascorbic acid) by control oocytes (white bars) and oocytes expressing slc11γ (black bars) or slc11β (grey bars) in the presence metals (100 μmol L^{-1}) at pH 5. A 'double asterisk' represents significantly higher uptake rates of Fe by slc11γ and slc11β when compared to the control for each separate metal treatment ($P < 0.01$). (B) Data expressed as a % of control Fe uptake in the absence of metal. Bars labelled with different letters are significantly different from each other ($P < 0.05$). All values are means ± S.E.M., $N = 6$.

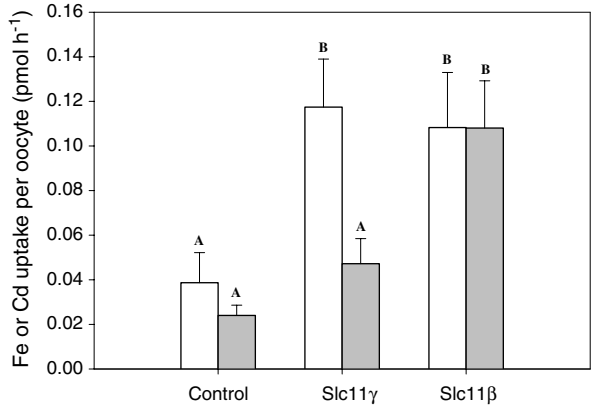

Fig. 4. The uptake of ^{59}Fe (white bars) and ^{109}Cd (grey bars) by control oocytes and oocytes expressing slc11γ and slc11β. Oocytes were exposed to 15 μmol $^{59}FeCl_3\ L^{-1}$ and 10 μmol $^{109}CdCl_3\ L^{-1}$ for a duration of 3 h at pH 6. Values are means ± S.E.M. ($N = 5$–8). Bars labelled with different letters are significantly different from each other ($P < 0.05$).

it possesses an additional 52 bp sequence between the region encoding for TM domain 10 and 11. The start of the insert contains a nucleotide sequence encoding a stop codon which results in the predicted translated *slc11* isoform being truncated and lacking 112 amino acids including the last 2 TM domains (termed slc11γ). Using a reverse primer specific to *slc11γ* and a forward primer the other side of a predicted intron (intron 13 of the human *SLC11A2* [26]) we used PCR to show that the additional 52 bp is not an additional exon (data not shown). Thus, we predict that s*lc11α* and *slc11γ* are likely products of gene duplication that may have arisen following the ancestral tetraploidisation known to have occurred in the salmonid lineage [27]. Thus, in rainbow trout there are at least 3 slc11 proteins and potentially other yet to be identified isoforms or splice variants.

The observation that slc11γ expressed in *Xenopus* oocytes imports iron (Figs. 1 and 2) suggests that the C-terminal region containing the last 2 TM domains is not essential for transport function. This contrasts to the observation that various amino acid deletions and mutations in other TM domains of the mammalian protein greatly affect iron transport capabilities of Slc11 members [21,22]. For example, recently a CTT deletion in intron 4 of Slc11a2 was shown to disrupt normal splicing causing mRNA transcripts to lack part of exon 5 [20]. The translated protein lacked 40 amino acids including the whole of TM2; the consequence is a loss of transport function and microcytic anaemia [20].

The dose-dependent uptake curve for both transporters exhibited similar maximum iron transport capacity and half maximal uptake rates for iron (Fig. 1B) (J_{max} of 0.3 pmol Fe h^{-1} and a K_m of 24.6 μmol Fe L^{-1} for slc11γ whereas slc11β had a J_{max} of 0.24 pmol Fe h^{-1} and a K_m of 25.2 μmol Fe L^{-1}). The dose-dependent shape of the uptake curve differs to that observed for other DMTs studied in oocyte expression systems [9]. The reason for this is unclear, but resembles the iron uptake profile observed in vivo for rainbow trout [29]. These K_m values are higher than other Slc11a2 homologs; for example, *Xenopus* oocytes expressing rat and yeast Slc11a2 both generate K_m values of below 5 μmol Fe L^{-1} [9,28]. These data suggest that both *O. mykiss* slc11 isoforms isolated from gill tissue have a relative low affinity for iron. This is in stark contrast with the observed branchial iron uptake rate affinity of 6.5 nmol Fe L^{-1} for *O. mykiss* when exposed to 16 nmol $^{59}FeCl_3$ L^{-1} for 3 h in circumneutral freshwater [29]. The conflicting uptake rate affinity data would indicate that other proteins present on the fish gill apical surface (e.g. mucus or membrane bound ferrireductases) are capable of binding iron with greater affinity than slc11, and are responsible for the measured in vivo K_m values.

Optimal iron uptake in *Xenopus* oocytes expressing slc11 γ or β occurs in acidic pHs. Because the internal pH of oocytes is reported to be approximately 7.35 [30] Fe transport by Slc11a2 is thus H^+-coupled (Fe^{2+}/H^+ symporter). Iron transport rates of *Xenopus* oocytes expressing trout slc11γ or β are elevated at an extracellular media pH of 5 or 6 compared to pH 8 or 9 (Fig. 2). The ferrous iron concentration at each pH condition remained constant in the presence of 1 mmol L^{-1} ascorbic acid indicating that the lower iron uptake rates observed at pH 8.0 and pH 9.0 is due to a reduction in the proton gradient. This supports the hypothesis that the trout slc11, which shows a higher sequence homology to mammalian Slc11a2, functions as a Fe^{2+}/H^+ symporter. One of the important roles of slc11 is in the immune function of fish [12–14,17,18], however, the mechanism by which these proteins exert their antimicrobial activity is uncertain. The observation that these proteins transport Fe^{2+} down a proton gradient suggests that their antimicrobial activity is due to the removal of Fe^{2+} (or other divalent metals) from the acidic phagosome and bacterial death due to essential micronutrients starvation. This corroborates the findings of Forbes and Gros [6] who demonstrated that the mammalian Slc11a1 might act as a metal efflux protein at the phagosome membrane. One cannot exclude the possibility that the full-length slc11α is a Fe^{2+}/H^+ antiporter, however, this is unlikely given that pH-dependency of the slc11 Fe^{2+} transport is due to histidine resides in TM 6 [28].

In addition to the observation that the trout slc11 can act as Fe^{2+}/H^+ symporters we also observed iron uptake rates at pH 7.4 that were similar to the rates measured at pH 5 or 6 (Fig. 2). This result supports the observation by Mackenzie et al. [31] that a component of iron transport by slc11a2 may be H^+-uncoupled. However, in contrast to the trout slc11, Fe^{2+} transport by the mammalian slc11a2 at pH 7.4 is at a substantially lower rate if compared to transport in acidic environments [31]. It has been hypothesised that a slc11 protein that transports iron efficiently at circumneutral pH is beneficial for the acquisition of iron by teleost fish [32–37]. This is because the pH of the external medium from which the fish extracts iron will often be mildly alkaline, hindering iron transport by a Fe^{2+}/H^+ symporter. For example, freshwaters are typically circumneutral, and can be as a high as pH 10.5 in the summer months during cyanobacterial blooms [38], whereas the intestinal lumen of freshwater fish has been measured at pH 7.3 (proximal region of rainbow trout intestine, [39]), and in the marine fish intestine, due to the secretion of bicarbonate, lumen can be considerably alkaline (∼pH 9) [40]. However, a caveat has to be added; mucus secreted by the transport epithelia creates a barrier between the epithelium and the surrounding medium. The chemistry of the microclimate close to the branchial or intestinal epithelium created by this mucosal covering has not been accurately assessed, but may well affect both the metal availability and transport conditions close to the site of import.

Gunshin et al. [3] were the first to show that a range of divalent metals could evoke an inward current in *Xenopus* oocytes expressing slc11a2. Confirmation that slc11a2 can transport other divalent metals has been ascertained from the use of radiotracer and flourophores (e.g. [9,10,41,42]). Iron uptake, by *Xenopus* oocytes expressing slc11γ and β were significantly inhibited by divalent metals (Fig. 4A). Slc11β was found to be more sensitive to inhibition by metals than slc11γ (Fig. 4B). However, it is important to note that inhibition of Fe^{2+} uptake does not in itself mean that these metals are being imported by the piscine slc11. This was demonstrated in the ^{109}Cd uptake experiment where slc11β was found to import ^{109}Cd, whereas oocytes injected with *slc11γ* cRNA did not accumulate the radiolabel (Fig. 4A). It is uncertain why slc11β shows greater affinity for metals, presumably this is due to the presence of the last two TM domains. However, the extracellular loop between TM11 and 12 does not contain cystine or histidine (sequence VTSLSSVAL for slc11β (Nrampβ) [12]), amino acids normally associated with metal interactions.

This study has characterised the iron transport properties of teleost fish slc11 proteins. *Xenopus* oocytes expressing slc11β and the novel slc11γ efficiently transports Fe^{2+} at pH 7.4, sug-

gesting an uncoupling Fe^{2+} transport to the proton gradient. In addition, slc11γ lacks TM 11 and 12 showing that this region does not play a role in Fe^{2+} import.

Acknowledgements: This work was supported by a UK Natural Environmental Research Council Studentship (NER/S/A/2002/10390). We thank Andy Mckie (King's College London) for the supply of *Xenopus laevis*.

References

[1] Vidal, S.M., Malo, D., Vogan, K., Skamene, E. and Gros, P. (1993) Natural resistance to infection with intracellular parasites: Isolation of a candidate for *Bcg*. Cell 73 (3), 469–485.
[2] Cellier, M., Prive, G., Belouchi, A., Kwan, T., Rodrigues, V., Chia, W. and Gros, P. (1995) Nramp defines a family of membrane proteins. Proc. Natl. Acad. Sci. USA 92 (22), 10089–10093.
[3] Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L. and Hediger, M.A. (1997) Cloning and characterisation of a mammalian proton coupled metal ion transporter. Nature 388, 842–888.
[4] Goswami, T., Bhattacharjee, A., Babal, S., Moores, E., Li, M. and Blackwell, J.M. (2001) Natural-resistance associated macrophage protein 1 is an H^+/bivalent cation transporter. Biochem. J. 354, 511–519.
[5] Mackenzie, B. and Hediger, M.A. (2004) SLC11 family of H^+-coupled metal–ion transporters NRAMP1 and DMT1. Pflugers Arch. 447 (5), 571–579.
[6] Forbes, J.R. and Gros, P. (2003) Iron, manganese, and cobalt transport by Nramp1 (SLC11a1) and Nramp2 (SLC11a2) expressed at the plasma membrane. Blood 102 (5), 1884–1892.
[7] Gruenheid, S., Canonne-Hergaux, F., Gauthier, S., Hackam, D.J., Grinstein, S. and Gros, P. (1999) The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. J. Exp. Med. 189 (5), 831–841.
[8] Jabado, N., Canonne-Hergaux, F., Gruenheid, S., Picard, V. and Gros, P. (2002) Iron transporter Nramp2/DMT-1 is associated with the membrane of phagosomes in macrophages and Sertoli cells. Blood 100 (7), 2617–2622.
[9] Sacher, A., Cohen, A. and Nelson, N. (2001) Properties of the mammalian and yeast metal-ion transporters DCT1 and Smf1p expressed in *Xenopus laevis* oocytes. J. Exp. Biol. 204 (6), 1053–1061.
[10] Bannon, D.I., Abounader, R., Lees, P.S.J. and Bressler, J.P. (2003) Effect of DMT1 knockdown on iron, cadmium and lead uptake in Caco-2 cells. Am. J. Physiol. 284, C44–C50.
[11] Arredondo, M., Munoz, P., Mura, C.V. and Nunnez, M.T. (2003) DMT1, a physiologically relevant apical Cu transporter of intestinal cells. Am. J. Physiol. 284, C1525–C1530.
[12] Dorschner, M.O. and Phillips, R.B. (1999) Comparative analysis of two Nramp loci from rainbow trout. DNA Cell Biol. 18 (7), 573–583.
[13] Saeij, J.P.J., Wiegertjes, G.F. and Stet, R.J.M. (1999) Identification and characterisation of a fish natural resistance associate macrophage protein (Nramp) cDNA. Immunogenetics 50, 50–60.
[14] Chen, H., Waldbieser, G.C., Rice, C.D., Elibol, B., Wolters, W.R. and Hanson, L.A. (2002) Isolation and characterisation of channel catfish natural resistance associated macrophage protein gene. Dev. Comp. Immunol. 26, 517–531.
[15] Donovan, A., Brownlie, A., Dorschner, M.O., Zhou, Y., Pratt, S.J., Paw, B.H., Phillips, R.B., Thisse, C., Thisse, B. and Zon, L.I. (2002) The zebrafish mutant gene chardonnay (*cdy*) encodes divalent metal transporter 1 (DMT1). Blood 100 (13), 4655–4659.
[16] Sibthorpe, D., Baker, A.M., Gilmartin, B.J., Blackwell, J.M. and White, J.K. (2004) Comparative analysis of two SLC11 (NRAMP) loci in *Takifugu rubripes*. DNA Cell Biol. 23 (1), 45–58.
[17] Chen, S.L., Xu, M.Y., Ji, X.S. and Yu, G.C. (2004) Cloning and characterisation of natural resistance associated macrophage protein (Nramp) cDNA from red sea bream (*Pagrus major*). Fish Shellfish Immunol. 17 (4), 305–313.
[18] Burge, E.J., Gauthier, D.T., Ottinger, C.A. and Van Veld, P.A. (2004) Mycobacterium – Inducibale NRAMP in Striped Bass (*Morone saxatilis*). Infect. Immun. 72 (3), 1626–1636.
[19] Chen, Y.J., Lin, C.H., Ou, T.T., Wu, C.C., Tsai, W.C., Liu, H.W. and Yen, J.H. (2007) Solute carrier family 11 member A1 gene polymorphisms in reactive arthritis. J. Clin. Immunol. 27, 46–52.
[20] Iolascon, A., d'Apolito, M., Servedio, V., Cimmino, F., Piga, A. and Camaschella, C. (2006) Microcytic anaemia and hepatic iron overload in a child with compound heterozygous mutations in DMT1 (SCL11A2). Blood 107 (1), 349–354.
[21] Touret, N., Martin-Orozco, N., Paroutis, P., Furuya, W., Lam-Yuk-Tseung, S., Forbes, J., Gros, P. and Grinstein, S. (2004) Molecular and cellular mechanisms underlying iron transport deficiency in microcytic anaemia. Blood 104 (5), 1526–1533.
[22] Lam-Yuk-Tseung, S., Camaschella, C., Iolascon, A. and Gros, P. (2006) A novel R416C mutation in human DMT1 (SLC11A2) displays pleiotropic effects on function and causes microcytic anaemia and hepatic iron overload. Blood Cells Mol. Dis. 36, 347–354.
[23] Chung, M.J., Walker, P.A., Brown, R.W. and Hogstrand, C. (2005) Zinc-mediated gene expression offers protection against H_2O_2-induced cytotoxicity. Toxicol. Appl. Pharmacol. 205, 225–236.
[24] Mckie, A.T., Marciani, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T.J., Farzaneh, F., Hediger, M.A., hentze, M.W. and Simpson, R.J. (2000) A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol. Cell 5, 299–309.
[25] Sandell, E.D. (1959) Colourmetric Determination of Trace metals, 3rd ed, Inter Science publishers, New York and London.
[26] Kishi, F. and Tabuchi, M. (1998) Human natural resistance-associated macrophage protein 2: gene cloning and protein identification. Biochem. Biophys. Res. Commun. 251, 775–783.
[27] Allendorf, F. and Thorgaard, G.H. (1984) Tetraploidy and the evolution of salmonid fishes in: Evolutionary genetics of fishes (Turner, B.J., Ed.), pp. 1–53, Plenum Press, New York.
[28] Lam-Yuk-Tseung, S., Govoni, G., Forbes, J. and Gros, P. (2003) Iron transport by Nramp2/DMT1: pH regulation of transport by 2 histidines in transmembrane domain 6. Blood 101, 3699–3707.
[29] Cooper, C.A. and Bury, N.R. (2007). The gills as an important uptake route for the essential nutrient iron in freshwater rainbow trout *Oncorhynchus mykiss*. J. Fish Biol., doi:10.1111/j.1095-8649.2007.01474.
[30] Kim, K.H., Shcheynikov, N., Wang, Y. and Muallem, S. (2005) SLC26A7 is a Cl^- channel regulated by intracellular pH. J. Biol. Chem. 280 (8), 6463–6470.
[31] Mackenzie, B., Ujwal, M.L., Chang, M.H., Romero, M.F. and Hediger, M.A. (2006) Divalent metal-ion transporter DMT1 mediates both H^+-coupled Fe^{2+} transport and uncoupled fluxes. Pflugers Arch. 451, 544–558.
[32] Roeder, M. and Roeder, R.H. (1966) Effect of iron on the growth rate of fishes. J. Nutr. 90, 86–90.
[33] Bury, N.R., Grosell, M., Wood, C.M., Hogstrand, C., Wilson, R.W., Rankin, J.C., Busk, M., Lecklin, T. and Jensen, F.B. (2001) Intestinal iron uptake in the European flounder (*Platichthys flesus*). J. Exp. Biol. 204, 3779–3787.
[34] Bury, N. and Grosell, M. (2003) Iron acquisition by teleost fish. Comp. Biochem. Physiol. 135C, 97–105.
[35] Bury, N. and Grosell, M. (2003) Waterborne iron acquisition by a freshwater teleost fish, zebrafish *Danio rerio*. J. Exp. Biol., 206.
[36] Cooper, C.A., Handy, R.D. and Bury, N.R. (2006) The effects of dietary iron on intestinal and branchial assimilation of both essential and toxic metals in vivo via iron transporters in a freshwater teleost fish species, zebrafish (*Danio rerio*). Aquat. Toxicol. 79, 167–175.
[37] Cooper, C.A., Bury, N.R. and Grosell, M. (2006) The effects of pH and the iron redox state on iron uptake in the intestine of a marine teleost fish, gulf toadfish (*Opsanus beta*). Comp. Biochem. Physiol. 143A, 292–298.
[38] Scott, D.M., Lucas, M.C. and Wilson, R.W. (2005) The effect of high pH on ion balance,nitrogen metabolism and behaviour in freshwater fish from a eutrophic lake: a laboratory and field study. Aquat. Toxicol. 73, 31–43.

[39] Vielma, J. and Lall, S.P. (1997) Dietary formic acid enhances apparent digestibility of minerals in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aqua. Nutr. 3, 265–268.
[40] Wilson, R.W. and Grosell, M. (2003) Intestinal bicarbonate secretion in marine teleost fish-source of bicarbonate, pH sensitivity, and consequences for whole animal acid–base and calcium homeostasis. Biochim. Biophys. Acta 1618, 163–174.
[41] Bressler, J.P., Olivi, L., Cheong, J.H., Kim, Y. and Bannon, D. (2004) Divalent metal transporter 1 in lead and cadmium transport. Ann. NY Acad. Sci. 1012, 142–152.
[42] Toyohara, H., Yamamoto, S., Hosoi, M., Takagi, M., Hayashi, I., Nakao, K. and Kaneko, S. (2005) Scallop DMT functions as a Ca^{2+} transporter. FEBS Lett. 579 (12), 2727–2730.