Structure–function analysis of a novel member of the LIV-1 subfamily of zinc transporters, ZIP14

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Abstract Here, we report the first investigation of a novel member of the LZT (LIV-1 subfamily of ZIP zinc Transporters) subfamily of zinc influx transporters. LZT subfamily sequences all contain a unique and highly conserved metalloprotease motif (HEXPHEXGD) in transmembrane domain V with both histidine residues essential for zinc transport by ZIP (Zrt-, Irt-like Proteins) transporters. We investigate here whether ZIP14 (SLC39A14), lacking the initial histidine in this motif, is still able to transport zinc. We demonstrate that this plasma membrane located glycosylated protein functions as a zinc influx transporter in a temperature-dependant manner.

Keywords: ZIP transporter; LZT; Zinc transporter; LIV1; SLC39A14; ZIP14

1. Introduction

Zinc is essential to cells, a co-factor for more than 300 enzymes [1] and involved in many key aspects of normal cell growth [2]. Intracellular zinc levels are tightly regulated as zinc deficiency [3] and excess [4] can be detrimental to cells. Zinc transporters control movement of zinc into, out of and within cells, having a crucial role in maintaining the cellular balance between apoptosis and cell growth and disease prevention.

ZIP (Zrt-, Irt-like Proteins) transporters are an important group of proteins responsible for the control of zinc transport into the cell cytosol. They can be divided into 4 subfamilies, I, II, Gufa and LIV-1 [5,6]. The members of the LIV-1 subfamily, termed LZT (LIV-1 subfamily of ZIP zinc Transporters), are distinguished by their consensus sequence HEXPHEXGD in transmembrane (TM) domain V [6]. These LZT sequences, currently 53 from 12 species [7], now form part of the new solute carrier family 39 (SLC39A), which includes all the known ZIP transporters. There are currently 9 human LZT family members, few of which have been investigated to date. Oestrogen-regulated LIV-1 (SLC39A6), implicated in breast cancer, transports zinc into cells from its position on the plasma membrane [8]. HKE4 (SLC39A7) belongs to the LZT sub group containing KE4 sequences, is ubiquitously expressed, resides on internal membranes, particularly the endoplasmic reticulum, and transports zinc into the cytosol from intracellular stores [7]. BigM103 (SLC39A8, ZIP8) has been shown to reside in vesicular structures resembling lysosomes and endosomes and be capable of increasing intracellular zinc [9]. Defects in the hZIP4 gene (SLC39A4), another LZT family sequence, have been shown to lead to the zinc deficiency disease, acrodermatitis enteropathica, suggesting that this protein, which is expressed predominantly in the intestine, is responsible for the zinc uptake from that tissue [10,11]. Clearly, the LZT proteins have a comparable function to other ZIP transporters, such as hZIP1 and hZIP2 [12,13], and are similarly able to control intracellular zinc levels by transport of zinc into the cytosol.

LIV-1 subfamily of ZIP zinc Transporters sequences contain similarities to ZIP transporters, including the consensus sequence in TM IV which has been shown to be essential for zinc transport [14], as well as a histidine residue in TM V. This latter histidine is the initial residue in the HEXPHEXGD motif of LZT sequences and aligns with the quintessential histidine in TM V of ZIP transporters [6]. This motif fits the consensus sequence of the zincin and peptide deformylase groups of metalloproteases [15–17], where both histidines and the first glutamic acid residue are known to be essential [18].

Therefore a sequence, such as ZIP14 (SLC39A14), with an EEXPHEXGD motif and lacking the histidine-repeats common in LZT sequences [19] would be unlikely to transport zinc if the previous results with ZIP transporters hold fast. Here, we report the first investigation of recombinant human ZIP14 protein and test its ability to transport zinc into cells. We have engineered recombinant protein to examine location, glycosylation, abundance and zinc transport ability.

2. Materials and methods

2.1. Engineering ZIP14 cDNA

A PCR construct of ZIP14 (gene KIAA0062, clone HA1020, Accession No. XM_046677) was generated using Biotaq DNA polymerase from Bioline in conjunction with the following oligonucleotide primers, where ZIP14 overlap is underlined: 5′-CCCCACACCA TGAAGCTGCT GCTGCTGCAC CC-3′ and 5′-CCCAATCTGG ATCTGTCC-3′. This sequence differs from Q96BB3 [6] with 36 C-terminal residues replaced by 48, which align well with other LZT sequences and the mouse homolog (AAH21530) of ZIP14, LZT-Mm4 [6]. The PCR product was ligated with pcDNA3.1/V5-His-TOPO as described previously [8]. TM deletion mutants were constructed using 3′ oligonucleotide primers. 5′-CCCATCCTTC CTTTCATCCTC-3′ producing a TM7 domain protein (1–458 residues) and

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Abbreviations: LZT, LIV-1 subfamily of ZIP zinc Transporters; CHO, Chinese hamster ovary; ZIP, Zrt-, Irt-like Proteins; TM, Transmembrane; PNGaseF, peptide N-glycosidase F; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl) ethylenediamine

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5′-CCCATCCTTCTCGTCCTCCATGGG-3′ producing a TM3 domain protein (1–304 residues). The production of the LIV-1 and HKE4 constructs has already been described [7,8].

2.2. Western blotting and deglycosylation

Chinese hamster ovary (CHO) cells (Invitrogen) transfected with ZIP14 constructs were prepared for Western blot analysis as described previously [8]. Samples were reduced by addition of 5% β-mercaptoethanol. To investigate deglycosylation, CHO cell lysates were incubated with 2 units of endoglycosidase PNGaseF (peptide N-glycosidase F (Boehringer Ingelheim, Bracknell, UK) overnight at 37 °C prior to Western blot in non-reducing conditions.

2.3. FACS analysis and fluorescent microscopy

Chinese hamster ovary cells expressing recombinant proteins were loaded with Newport Green as described previously [8] and mean fluorescence measured by FACS analysis. The intracellular zinc concentration was calculated using $[Zn^{2+}]_i = Kd(F - F_{min})/(F_{max} - F)$, where F, F_{min} and F_{max} are the mean fluorescence obtained from the sample, 50 μM zinc chelator TPEN (*N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl) ethylenediamine) and 100 μM zinc with 10 μM zinc ionophore sodium pyrithione, respectively. Cells for fluorescent microscopy were fixed with 4% formaldehyde for 15 min, blocked with 10% normal goat serum, incubated with anti-V5 antibody (1/2000) for 1 h and Alexa Fluor 488-conjugated anti-mouse antibody (1/1000, Molecular Probes) for 1 h and assembled onto slides using Vectorshield with propidium iodide (Vector Laboratories).

2.4. ZIP14 expression in human tissues

A commercially produced Multiple Tissue Expression array (MTE™, Clontech), containing poly A^+ RNA from 68 normal human tissues and 8 cancer cell lines, was hybridised with a ZIP14-specific cDNA probe according to the manufacturer's instructions.

3. Results

3.1. Computer prediction of ZIP14 secondary structure

Secondary structure prediction of ZIP14 suggests 8 TM domains, a core size of 54 kDa and a cleavable signal peptide between residues 30 and 31 (Fig. 1). This was achieved using the combination of computer software described previously [8]. ZIP14 (SLC39A14) belongs to the LZT subgroup of ZIP transporters [6], which have an HEXPHEXGD signature motif. ZIP14 contains a glutamic acid replacement of the initial histidine in this motif (Fig. 2B, residues 375–384) and, in contrast to most other family members, contains few histidine residues throughout the sequence (Fig. 2C). Interestingly, ZIP14 shares differences in sequence with another molecule ZIP8, shown by asterisks in Fig. 2A and B.

3.2. Expression of recombinant ZIP14 proteins

Western blotting of recombinant proteins with the anti-V5 antibody demonstrated a double band (60 kDa) compatible with the predicted core size of 54 kDa for ZIP14 (WT) and an additional 5 kDa due to the V5 tag (Fig. 3A, WT). However, we also observed a band consistent with a trimer and a high molecular mass band, which increased in non-reducing conditions (NR). The different mutants produced bands of expected size (Fig. 3A), 57 kDa for TM7 mutant and 42 kDa for TM3 mutant (predicted 54 and 34 kDa, respectively), allowing 5 kDa for the V5 tag. We confirmed the presence of some or all of the predicted N-linked glycan chains (residues 77, 87, and

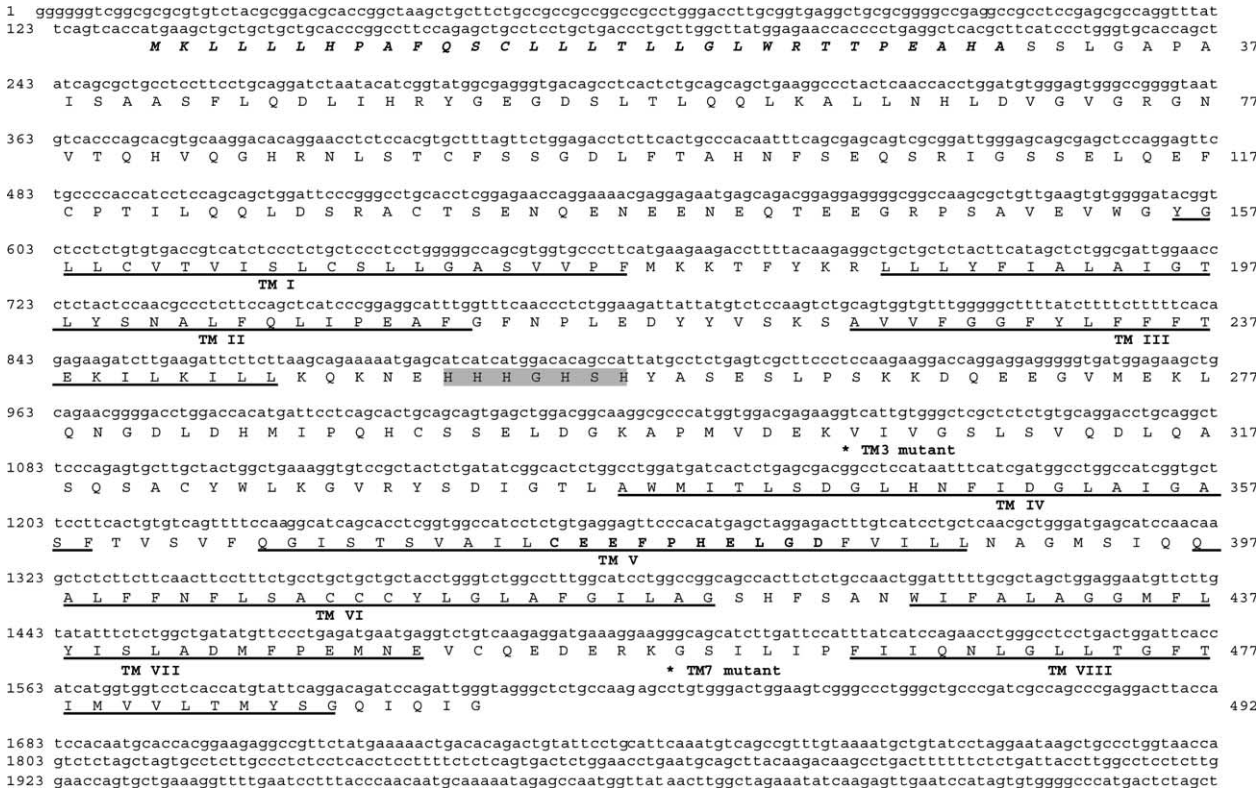

Fig. 1. *cDNA sequence of ZIP14*: cDNA and amino acid sequence of ZIP14 (Accession No. XM_046677, SLC39A14). Potential TM domains are underlined, histidine-rich region is shaded, signal peptide is in bold italics, LZT consensus motif (CEXPHEXGD, residues 375–384) is bold and an asterisk indicates the last residue of the mutants TM3 and TM7. Numbers on right refer to amino acid sequence and numbers on left refer to the cDNA sequence.

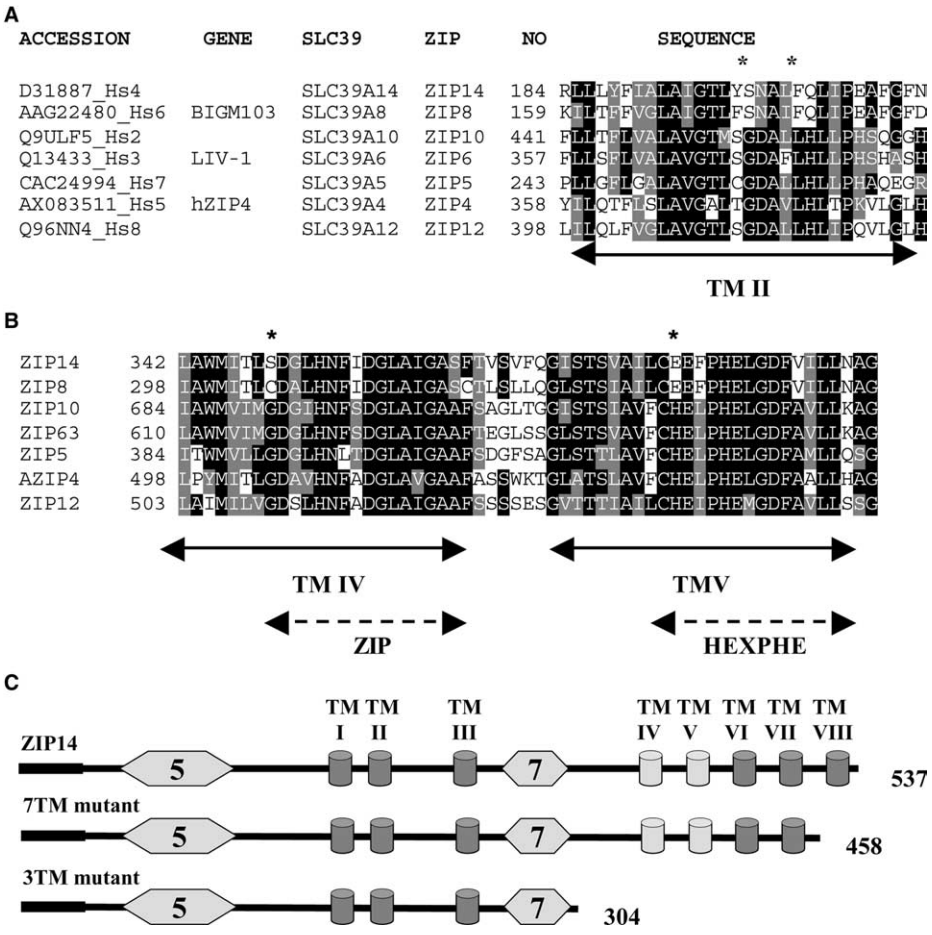

Fig. 2. *Alignment of human LZT sequences across three TM domains*: (A) Alignment across TM II with accession numbers in the left-hand column, followed by LZT family name, gene name, SLC39 name, ZIP name and residue numbers. (B) Alignment across TM IV and V, indicating positions of ZIP and LZT consensus sequence with dotted arrows. Residues coloured black and grey correspond to identical or complementary residues, respectively. Asterisks highlight differences shared by ZIP14 and ZIP8 in otherwise well conserved residues. (C) Schematic of predicted secondary structure of mutant ZIP14 proteins. TM domains are shaded barrels, numbers in hexagons are histidine residues and total residues are given on the right side.

102) by observing a reduced mass of all protein bands, including the trimer bands, after treatment with PNGaseF (Fig. 3A).

3.3. Cellular location of ZIP14

In order for ZIP14 to act as a zinc influx transporter, it would have to reside on the plasma membrane. This was demonstrated (Fig. 3B) on coverslips containing non-permeabilised ZIP14-transfected CHO cells, with particular dense staining in regions of cell–cell contact. The TM7 and TM3 deletion mutants exhibited the same cellular location (results not shown), indicating that the loss of TM regions did not alter the cellular processing.

3.4. Zinc transport analysis

Transfected CHO cells in suspension were loaded with the cell permeant zinc-specific fluorescent indicator Newport Green diacetate [20] and tested for their ability to uptake zinc. We compared the ZIP14 expressing cells with those expressing LIV-1, another LZT family member previously shown to transport zinc [8]. The intracellular zinc concentration ($[Zn^{2+}]_i$) of cells transiently expressing either LIV-1 or ZIP14 increased in response to increasing extracellular zinc concentration (Fig. 4A) and was abolished by addition of the zinc chelator, TPEN (results not shown), confirming the zinc-specificity of the Newport Green. However, without addition of extracellular zinc only LIV-1 expressing cells had an $[Zn^{2+}]_i$ greater than the control. The expression level of ZIP14 is considerably greater than that of LIV-1 (Fig. 3B), therefore no quantitative comparison can be drawn between ZIP14 and LIV-1 zinc uptake ability. Interestingly, the ZIP14 mutants lacking 1 or 5 TM domains (TM7 and TM3) were similar to control cells, suggesting a requirement for all TM domains for zinc transport. This ability of ZIP14 expressing cells to increase intracellular zinc was temperature-dependant as there was no evidence of cellular zinc accumulation when these experiments were repeated at 4 °C (results not shown).

3.5. Tissue distribution of ZIP14 expression

The multiple tissue expression array (Fig. 4B) shows ubiquitous expression of ZIP14 with increased expression in Liver (A9), Pancreas (B9), Foetal liver (D11), Thyroid gland (D9), Left ventricle (E4), Right atrium (D4), Right ventricle (F4) and Foetal heart (B11). Of particular interest is the general expression of ZIP14 in the heart (Lanes 4A–H), intestine

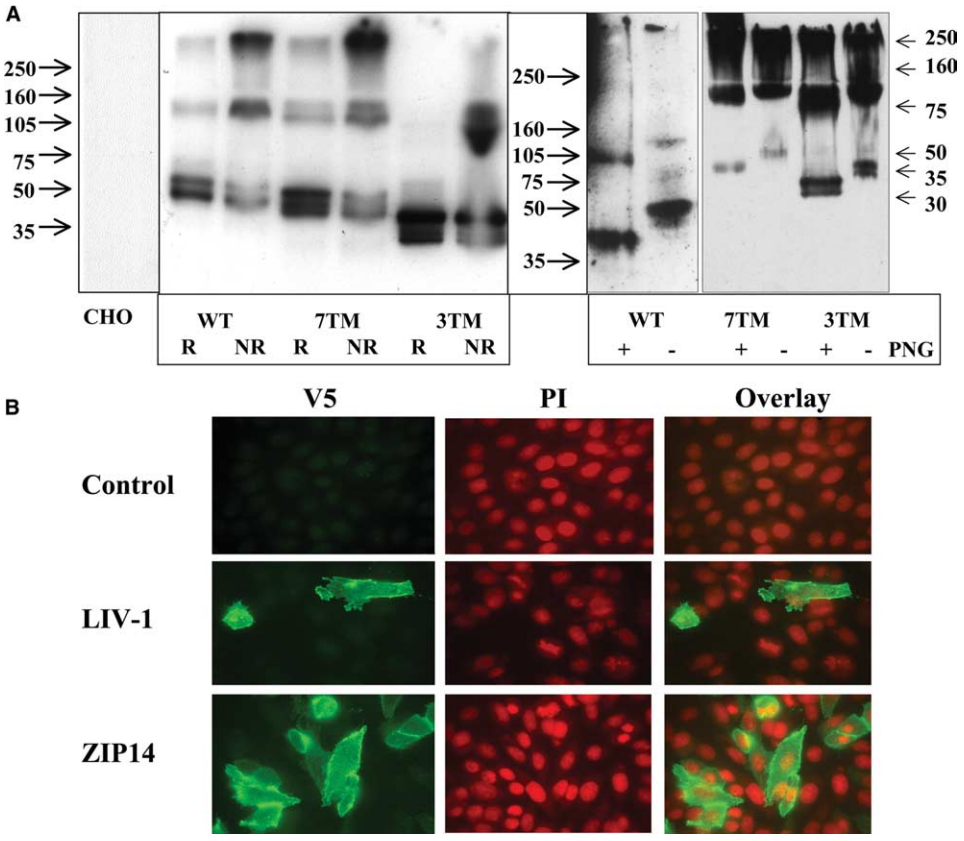

Fig. 3. *Western blot and fluorescent microscopy of recombinant ZIP14*: (A) Western blot with V5 antibody of CHO cells transiently expressing ZIP14 wild type (WT) and mutant (TM7 and TM3) proteins compared to control CHO cells (CHO) in non-reducing (NR) or reducing (R) conditions. Treatment with PNGaseF (+) or not (−) is indicated (PNG). (B) Comparison of fluorescent microscopy of CHO cells stained with propidium iodide (red) and transfected with LIV-1 or ZIP14 probed with anti-V5 antibody (green).

(Lanes 5A–H and 6A–C) and liver (Lane A9) which were negative for LIV-1 [8] and general low expression in the brain (Lanes 1–3) which was positive for LIV-1 [8].

4. Discussion

Expression of human ZIP14 protein in mammalian cells has enabled us to show that, firstly, ZIP14 is closely related to the previously described ZIP transporters [5,6], known as zinc influx transporters. Secondly, ZIP14 expression in cells increases the zinc uptake ability in a temperature-dependant manner from its location on the plasma membrane. These results are compatible with ZIP14 transporting zinc into cells via a carrier-mediated transport process.

This is the first demonstration of zinc influx ability of a human LZT protein containing an altered signature motif, namely EEXPHEXGD, in place of HEXPHEXGD. This result agrees with BIGM103, another human LZT family member, ZIP8 [9], containing the same motif as ZIP14 in TM V (Fig. 2B) located on intracellular membranes and transporting zinc into the cytosol. This result suggests that the histidine residue in TM V, thought to be quintessential for other zinc transporters [14], may not be necessary for zinc transport function in LZT proteins, although a definite conclusion is not possible without now mutating individual residues. Interestingly, the glutamic acid residue replacing the histidine in TM V (Fig. 2B, residue 382) is a residue capable of co-ordinating zinc [18]. This result confirms the inclusion of ZIP14 in the LZT subfamily of ZIP transporters by its ability to transport zinc into cells and justifies the inclusion of other proteins with the signature motif EEXPHEXGD, such as ZIP8 [9]. These results show increased intracellular zinc-dependant fluorescence in cells transfected with ZIP14, which is not present in the CHO controls. Although this suggests the ability to uptake zinc across the plasma membrane, especially in the presence of increased extracellular zinc, we are unable to rule out other effects such as an ability of these proteins to reorganise the pool of intracellular zinc available to the Newport Green dye. The previous judgement by us that ZIP14 did not have the ability to transport zinc [6] is also consistent with the result reported here. These previous experiments did not investigate the effect of increasing extracellular zinc and, as demonstrated in Fig. 4A, in the absence of extracellular zinc, the ZIP14 expressing cells do not show any increased fluorescence above controls.

The observed intracellular zinc concentrations were calculated to be 90 and 350 nM for control and LIV-1 or ZIP14 transfected cells, respectively. The Newport Green dye that was used for these experiments has a Kd of 1 μM. Therefore, further detailed investigations of zinc concentrations may be enhanced by the use of new zinc dyes with lower Kd for zinc.

It is noteworthy that both ZIP14 and ZIP8, apart from the above variation in TM V, also have a glutamine in place of a conserved histidine in TM II, and a serine and cysteine in place of two otherwise conserved glycine residues in TM II

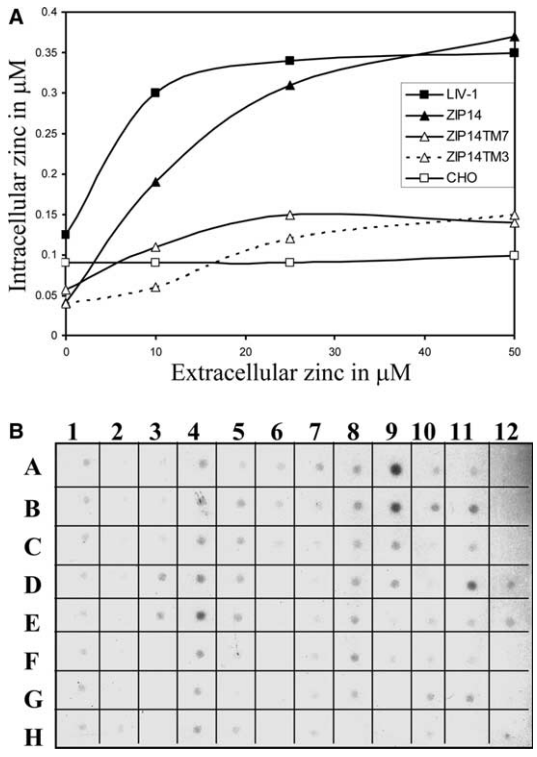

Fig. 4. *Zinc transport ability and tissue distribution of recombinant ZIP14*: (A) The variation of intracellular zinc concentration with extracellular zinc concentration in CHO cells transiently transfected with no DNA (CHO, hollow squares), LIV-1 (solid squares), or ZIP14 (solid triangles) and mutants (hollow triangles with full line (TM7) and dotted line (TM3)). Cells were loaded with Newport Green and mean fluorescence was read by FACS. (B) Autoradiograph of a human multi-tissue expression array hybridised with a ZIP14 specific cDNA probe. Highest levels of expression are observed in Liver (A9), Pancreas (B9), Foetal liver (D11), Thyroid gland (D9), Left ventricle (E4), Right atrium (D4), Right ventricle (F4) and Foetal heart (B11), though it is observed that ZIP14 is ubiquitously expressed. Tissues represented on the human multi-tissue expression array have been described previously [8].

and IV (Fig. 2, asterisks). This is consistent with our previous suggestion that the conserved histidine in TM II may act in concert with the conserved histidine in TM V in transporting zinc [6].

Interestingly, the ability of ZIP14 to transport zinc suggests minimal involvement of histidine-rich repeats. Unlike other human LZT sequences, ZIP14 only contains $(HX)_4$ and BIGM103 only contains $2(HX)_2$ [6] in the loop between TM III and TM IV. This is in keeping with ZIP transporters other than LZT sequences that only require 3 histidines in a (HX) repeat to transport zinc [5,14]. The failure of ZIP14 mutant lacking only TM8 to transport zinc suggests an important role for all TM domains in this process. Although these mutated proteins located to the plasma membrane, we cannot rule out alterations in protein processing caused by the absent residues.

The observed plasma membrane location and presence of glycan chains on ZIP14 are consistent with an extracellular topology for the N-terminus. The remaining presence of double bands after PNGaseF treatment (Fig. 3A) discounts glycosylation variation as the cause. There are no alternative methionine translation start sites in the N-terminus of ZIP14, however, translation can rarely initiate at leucine residues of which there are 12 in the initial 200 residues of this sequence [21].

The observed tissue expression of ZIP14, in combination with observations of other LZT proteins [6–11], is consistent with the tissue specific expression of these family members. Recently, LIV-1 has been shown to be essential for the epithelial–mesenchymal transition (EMT) required in zebrafish development [22]. Particularly, LIV-1 was the downstream target of STAT3 and also essential for the nuclear localisation of Snail, a prerequisite of EMT. The other human LIV-1 family members, such as ZIP14, should now be investigated to determine if they also have such a role.

In conclusion, we demonstrate the zinc influx ability of a novel human LZT family member, ZIP14, which lacks a histidine residue, previously thought to be quintessential, raising the question of which residues are actually essential for zinc transport. Given the increasing evidence of an important role for dysregulation of zinc and involvement of LZT family members in disease states (notably, LIV-1 in breast cancer and hZIP4 in acrodermatitis enteropathica), it is essential that any relationship of their expression to aberrant cell biology is now addressed.

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