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

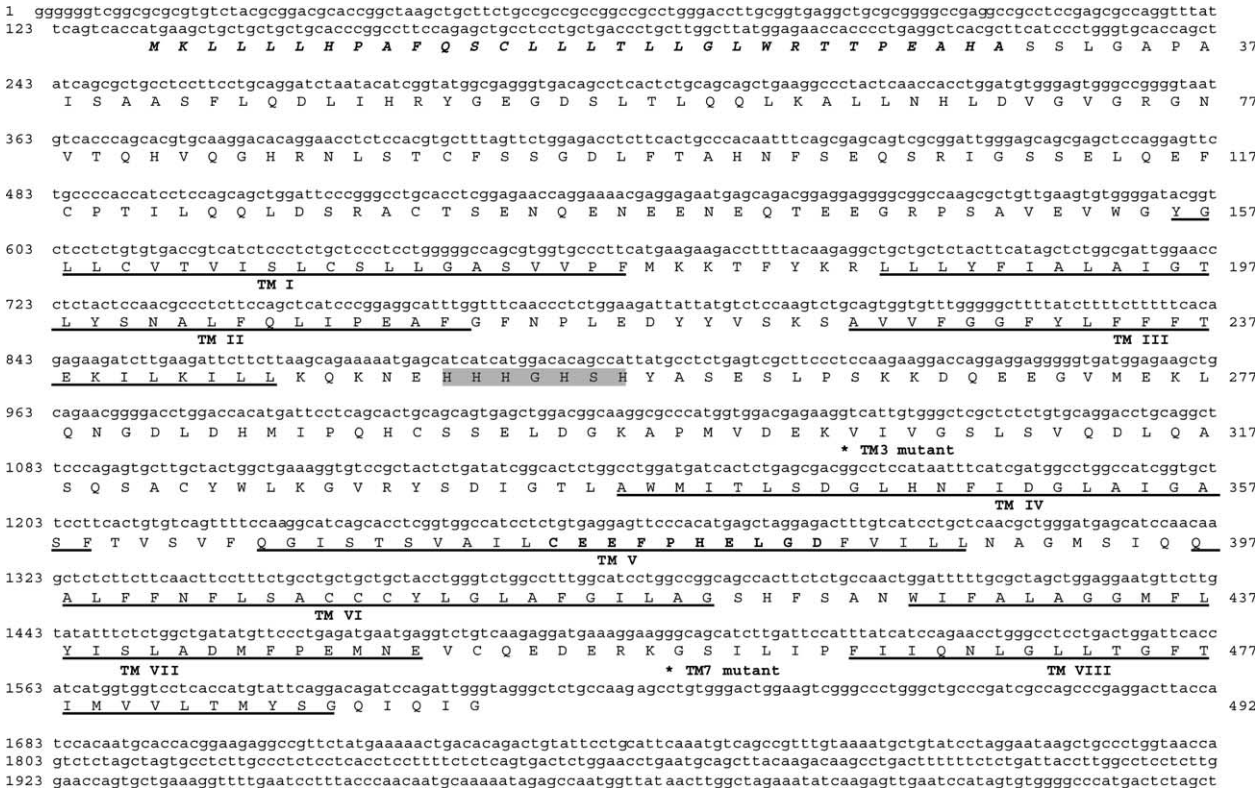

Fig. 1. *cDNA sequence of ZIP14*: cDNA and amino acid sequence of ZIP14 (Accession No. XM_046677, SLC39A14). Potential TM domains are underlined, histidine-rich region is shaded, signal peptide is in bold italics, LZT consensus motif (CEXPHEXGD, residues 375–384) is bold and an asterisk indicates the 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.