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Review

The LZT proteins; the LIV-1 subfamily of zinc transporters

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Abstract

Zinc is an essential ion for cells with a vital role to play in controlling the cellular processes of the cell, such as growth, development and differentiation. Specialist proteins called zinc transporters control the level of intracellular zinc in cells. In mammals, the ZIP family of zinc transporters has a pivotal role in maintaining the correct level of intracellular zinc by their ability to transport zinc into cells from outside, although they may also transport metal ions other than zinc. There are now recognised to be four subfamilies of the ZIP transporters, including the recently discovered LIV-1 subfamily which has similarity to the oestrogen-regulated gene *LIV-1*, previously implicated in metastatic breast cancer. We call this new subfamily LZT, for *L*IV-1 subfamily of *Z*IP zinc *T*ransporters. Here we document current knowledge of this previously uncharacterised group of proteins, which includes the KE4 proteins. LZT proteins are similar to ZIP transporters in secondary structure and ability to transport metal ions across the plasma membrane or intracellular membranes. However, LZT proteins have a unique motif (HEXPHEXGD) with conserved proline and glutamic acid residues, unprecedented in other zinc transporters. The localisation of LZT proteins to lamellipodiae mirrors cellular location of the membrane-type matrix metalloproteases. These differences to other zinc transporters may be consistent with an alternative role for LZT proteins in cells, particularly in diseases such as cancer.

Keywords: LIV-1; ZIP transporter; Zinc; HEXXH; Metalloprotease; KE4; Molecular phylogeny

1. Introduction

Zinc is essential to cell growth and is a cofactor for more than 300 enzymes, representing over 50 different enzyme classes [1]. Zinc is involved in protein, nucleic acid, carbohydrate and lipid metabolism, as well as in the control of gene transcription and the coordination of other biological processes controlled by proteins containing DNA-binding zinc finger motifs [2], RING fingers and LIM domains (for review, see Ref. [3]). Many molecules associated with DNA and RNA synthesis are also zinc metalloenzymes such as RNA polymerase [4], reverse transcriptases and transcription factors [5]. Zinc is a non-redox active ion, like calcium, and is therefore targeted to transcription factors and other enzymes involved in DNA metabolism as the use of redox active metal ions for these tasks could lead to radical reactions and nucleic acid damage. However, these processes must be tightly regulated to ensure that the exact amount of zinc ions is present at all times. Zinc also associates with many macromolecules in cells that act to control growth, apoptosis, development and differentiation. Zinc is a component of hormones and part of the active site of numerous metalloenzymes, especially metallothionen, which acts as an intracellular pool of zinc [6]. Excess zinc can also be toxic to cells [7] and aberrant levels of zinc have been linked to various disease states thereby making it vital that the level of intracellular zinc is tightly controlled. For example, increases in zinc have been linked to neurodegeneration [8], whereas decreases have been associated with immunological impairment [9]. In order for zinc to have such a varied role in cells, and because it cannot passively diffuse cell membranes, it has to be transported into the intracellular compartments of a cell where it is required for these zinc-dependent processes. A group of proteins called zinc transporters is dedicated to this transport of zinc across biological membranes.

There are many zinc transporters in eukaryotes, which have now been implicated in this transport process, but not all of these have been fully characterised. The most well researched of these transporters belong to either the ZIP (for

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*Z*rt-, *I*rt-like *P*roteins) [10–12] or the CDF (for *C*ation *D*iffusion *F*amily) [13–18] transporter families. Both CDF and ZIP transporters, with members from both prokaryotes and eukaryotes, have a similar secondary structure and function, as clearly demonstrated by Gaither and Eide [19]. There are three subfamilies of the CDF transporters (I, II and III) which transport zinc either from the cytoplasm to intracellular organelles or from the cytoplasm to the extracellular space. In contrast, ZIP transporters are important for zinc uptake from the extracellular space into the cytoplasm as well as mobilising stored zinc from intracellular compartments. The ZIP family consists of at least 86 members and can be divided into four separate subfamilies [19]: subfamily I is mainly fungal and plant sequences, subfamily II consists of mammalian, nematode and insect genes [20], the gufA subfamily is related to the *gufA* gene of *Myxococcus xanthus* which has unknown function, and the LIV-1 subfamily is related to the oestrogen-regulated gene, *LIV-1* (Fig. 1). Clearly, it is important to determine the role of each of these subgroups in cellular zinc transport and how they co-operate in the management of cellular zinc homeostasis.

Here we document much that is currently known about the previously uncharacterised LIV-1 subfamily of ZIP transporters that we have termed LZT (for *L*IV-1 subfamily of *Z*IP zinc *T*ransporters). We intend to clarify not only the similarities of the LZT subfamily to ZIP transporters but also their unique differences and suggest a potential role for them in diseases such as cancer.

2. The discovery of the LIV-1 subfamily of ZIP transporters (LZT)

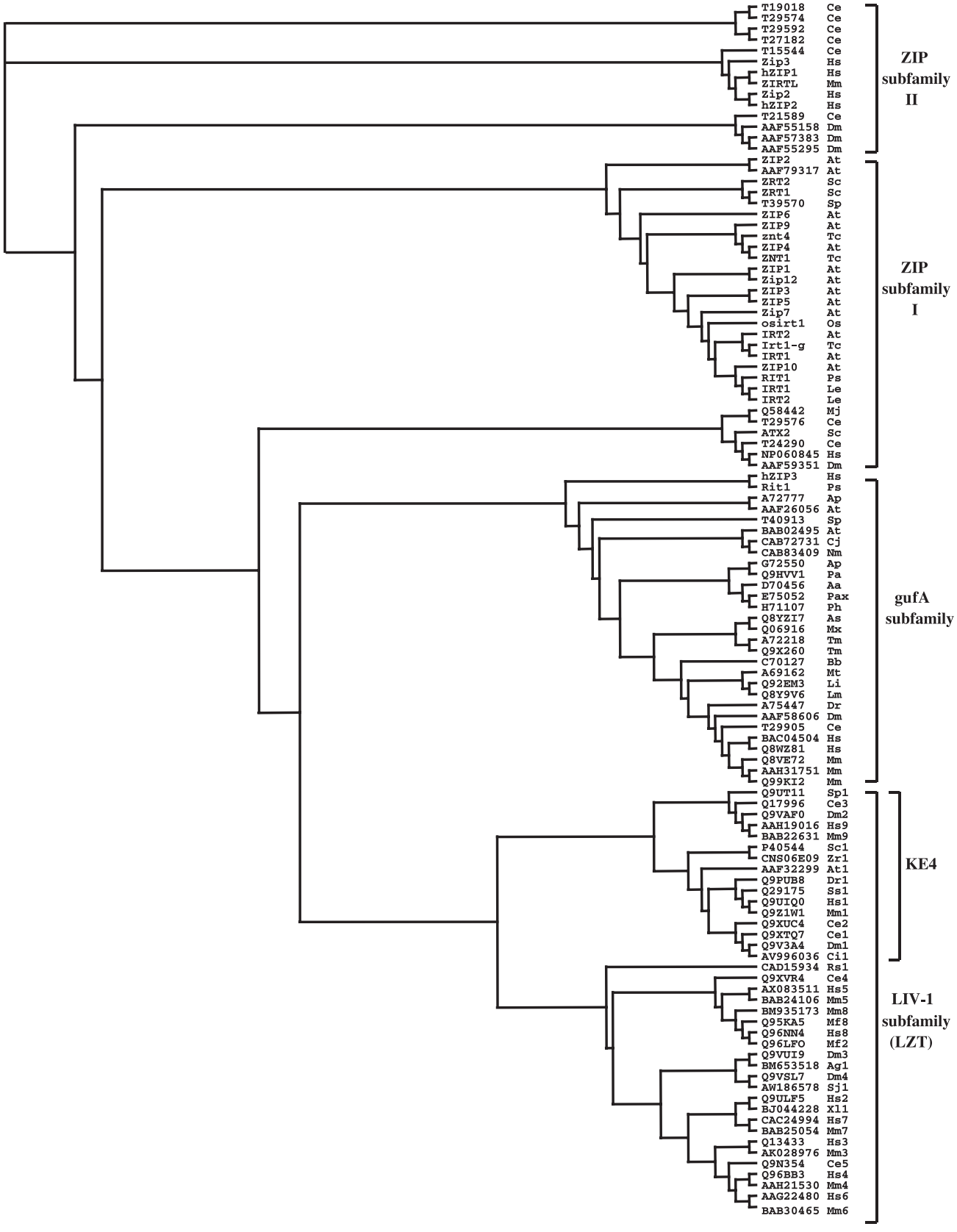
LIV-1 is an oestrogen-regulated gene that has been implicated in metastatic breast cancer [21]. Its detection was associated with oestrogen receptor-positive breast cancer [22] and with the metastatic spread of these cancers to the regional lymph nodes [23]. Subsequent computer searches of secondary structure predicted LIV-1 to contain six to eight transmembrane domains, a long extracellular N terminus, a short extracellular C terminus and a consensus sequence for the catalytic zinc-binding site of metalloproteases (HEXXH, where H = histidine, E = glutamic acid and X = any amino acid). This latter motif was unusual in LZT sequences in that it also contained two novel residues (HEX**P**H**E**), proline (**P**) and glutamic acid (**E**), previously unprecedented in these positions in any other metalloprotease motifs [24]. Originally, the non-redundant NCBI database was searched using BLAST [25] and the unique HEX**P**H**E**XGD motif of LIV-1 (U41060, residues 629–637) which was a restricted set within the consensus sequence HEXXHXXG present in zincin and PDF metalloproteases [26–28]. This procedure identified a number of sequences not only containing this unique potential metalloprotease motif but also containing six or eight transmembrane domains and numerous histidine-rich repeats, relating them to the ZIP family of zinc transporters. Initial searches found 11 sequences from five different species that all contained this unique sequence, suggesting that they belonged to a new family involved in zinc transport [24]. However, with recent progress in the sequencing of the various genomes, searches of the GenBank have to date uncovered 39 LZT sequences from 12 species, including human, mouse, *Caenorhabditis elegans*, *Drosophila*, yeast and bacteria, that contain this unique and highly conserved motif. Fig. 1 shows a phylogenetic tree of the ZIP family of transporters, which is similar to that in Gaither and Eide [19] but with additional LIV-1 subfamily members. It is noteworthy that although the KE4 proteins share the LIV-1 conserved HEXPHEXGD motif, they cluster together as a separate group. Clearly, all these sequences have been well conserved during evolution, implying an important role in zinc homeostasis.

We have aligned this exclusive LZT motif with the corresponding region of ZIP transporters, encompassing transmembrane (TM) domains IV and V (Fig. 2). It is clear that there is a high degree of identical (black) and complementary (grey) residues shared by both the LZT subfamily and other ZIP transporters, especially in TM IV. However, it is equally clear that the ZIP transporters do not contain the same degree of similarity in TM V, where the LZT specific motif (HEXPHEXGDFAXLLXXG) is situated. We suggest that the following consensus sequence in single amino acid code, where residues within brackets are allowed and X refers to any amino acid, GX_{9-10}(H,E)E(L,F)P(H,Q,A)E(L,I,V,M)(G,S)D(F,L,V)(M,A,V,G)XL(L,I,V)X_{2-4}G, is a good marker to identify those sequences belonging to the LZT subfamily of ZIP transporters.

Previously, computer software for prediction of secondary structure forecast that the HEXPHEXGD motif was situated adjacent to rather than within TM V [24]. However, this prediction may have been erroneous due to the presence of the proline residue in this sequence causing a premature termination of the TM prediction. When LIV-1 and other LZT sequences are aligned with other ZIP transporters (Fig. 2), it is apparent that the first H of the HEXPHEXGD motif lines up with the conserved H in TM V of the ZIP transporters. We suggest that, by similarity to conventional ZIP transporters, this motif is situated within TM V (Fig. 3), and therefore placed within the pore, able to form a metal binding site in a similar manner to conventional ZIP transporters [29].

3. Similarity of LZT subfamily to other ZIP transporters

The LZT subfamily of ZIP transporters exhibit considerable similarity to conventional ZIP transporters particularly in their secondary structure prediction, plasma membrane location and ability to transport zinc into cells.


T19018 Ce
T29574 Ce
T29592 Ce
T27182 Ce
T15544 Ce
Zip3 Hs
hZIP1 Hs
ZIRTL Mm
Zip2 Hs
hZIP2 Hs
T21589 Ce
AAF55158 Dm
AAF57383 Dm
AAF55295 Dm
ZIP subfamily II
ZIP2 At
AAF79317 At
ZRT2 Sc
ZRT1 Sc
T39570 Sp
ZIP6 At
ZIP9 At
znt4 Tc
ZIP4 At
ZNT1 Tc
ZIP1 At
Zip12 At
ZIP3 At
ZIP5 At
Zip7 At
osirt1 Os
IRT2 At
Irt1-g Tc
IRT1 At
ZIP10 At
RIT1 Ps
IRT1 Le
IRT2 Le
Q58442 Mj
T29576 Ce
ATX2 Sc
T24290 Ce
NP060845 Hs
AAF59351 Dm
ZIP subfamily I
hZIP3 Hs
Rit1 Ps
A72777 Ap
AAF26056 At
T40913 Sp
BAB02495 At
CAB72731 Cj
CAB83409 Nm
G72550 Ap
Q9HVV1 Pa
D70456 Aa
E75052 Pax
H71107 Ph
Q8YZI7 As
Q06916 Mx
A72218 Tm
Q9X260 Tm
C70127 Bb
A69162 Mt
Q92EM3 Li
Q8Y9V6 Lm
A75447 Dr
AAF58606 Dm
T29905 Ce
BAC04504 Hs
Q8WZ81 Hs
Q8VE72 Mm
AAH31751 Mm
Q99KI2 Mm
gufA subfamily
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Q17996 Ce3
Q9VAF0 Dm2
AAH19016 Hs9
BAB22631 Mm9
P40544 Sc1
CNS06E09 Zr1
AAF32299 At1
Q9PUB8 Dr1
Q29175 Ss1
Q9UIQ0 Hs1
Q9Z1W1 Mm1
Q9XUC4 Ce2
Q9XTQ7 Ce1
Q9V3A4 Dm1
AV996036 Ci1
KE4
CAD15934 Rs1
Q9XVR4 Ce4
AX083511 Hs5
BAB24106 Mm5
BM935173 Mm8
Q95KA5 Mf8
Q96NN4 Hs8
Q96LFO Mf2
Q9VUI9 Dm3
BM653518 Ag1
Q9VSL7 Dm4
AW186578 Sj1
Q9ULF5 Hs2
BJ044228 Xl1
CAC24994 Hs7
BAB25054 Mm7
Q13433 Hs3
AK028976 Mm3
Q9N354 Ce5
Q96BB3 Hs4
AAH21530 Mm4
AAG22480 Hs6
BAB30465 Mm6
LIV-1 subfamily (LZT)

3.1. Secondary structure prediction

ZIP transporters [19,29] are predicted to contain eight TM domains with extracellular N and C termini and a long variable region in the cytoplasmic loop between TM III and IV (Fig. 3). This variable loop contains a histidine-rich repeat with the general formula (HX)*n*, where H = histidine, X = any amino acid and *n* = 3–6 [20,21,30], which is considered to be a metal-binding domain with a role in metal ion transport [19,20]. Interestingly, the LZT subfamily sequences found by searching for the HEXPHEXGD motif also (i) contain this histidine-rich repeat seen in ZIP transporters and (ii) are predicted to contain between six to eight transmembrane domains. Some of the protein sequences of the LIV-1 subfamily are either identical or vary by a few amino acids, which may be due to inconsistencies in the sequencing, and therefore to prevent bias, only one representative protein for each type was included in the full alignment used for Figs 1 and 2. The additional accession numbers of the omitted sequences are included in Table 1. The exact number of TM domains predicted for each protein generally varies from six to eight and according to the software package used for the prediction. It appears that the discrepancy of TM number arises concerning the potential pore region across TM IV and V. These TM domains are not as hydrophobic as the other TM domains which results in some software packages failing to predict a TM domain in this region. Here we have used the prediction from six different software packages (SOSUI [31], Tmpred [32], SOPMA [33], HMMTOP, [34], PSORT [35–37] and DAS [38]), and allowing the prediction of TM IV and V as obtained by Tmpred and DAS only, we have obtained a general prediction of eight TM domains, similar to other ZIP transporters (Fig. 3). Ermelin, the mouse equivalent of LIV-1, has been predicted to contain six TM domains [39], yet it has been aligned with other members of the LZT family, especially the KE4 family members which have been predicted by us and others to contain eight TM domains [24,40,41].

LZT sequences also exhibit other similarities to ZIP transporters, especially in the region of TM IV and V. For example, they fit the single amino acid code consensus sequence for ZIP transporters across TM IV [29], where any residue in square brackets is allowed in that position, [LIVFA][GAS][LIVMD][LIVSCG][LIVFA]H[SAN] [LIVFA][LIVFMAT][LIVDE]G[LIVF][SAN][LIVF][GS]. Zip transporters have two adjacent conserved residues, HS (at positions 6 and 7 in this motif) which lie in TM IV and conserved residues (HX_3E) which lie in TM V (Figs. 2, asterisks, and 3). These residues have been shown to be crucial for function [29] as their removal from Irt1 prevents zinc transport [42]. This HS motif is thought to be part of the intramembrane heavy metal binding site, which forms the pore region of the ZIP transporters [29]. The LZT sequences contain a highly conserved HNF motif in place of the HS motif in TM IV of ZIP transporters (Figs. 2 and 3). The HXXXE motif in TM V of ZIP transporters (Fig. 1) is replaced in LZT transporters by a similar motif (HEXPHE) which is almost completely conserved (Fig. 2). This similarity of residues suggests a comparable mechanism and pore structure for both LZT and ZIP transporters. Another example of similarities in the protein sequences of ZIP transporters to the LZT family of proteins is the extremely short C termini that they share.

3.2. Cellular location of LZT family proteins

It is noteworthy that LZT sequences are predicted to be complex transmembrane proteins located in either endoplasmic reticulum or plasma membranes (Table 1). This endoplasmic reticulum prediction may be flawed as most of the LZT sequences contain endoplasmic reticulum retention signals in the first few residues of their signal peptides, for example, residues 2ARKL5 for LIV-1 which would be expected to be removed after synthesis were completed and before entering the secretory pathway. LIV-1 itself is predicted to be endoplasmic reticulum located (57%) but we have evidence of plasma membrane location (Fig. 4) in non-permeabilised cells.

To determine the cellular location of LZT family members, we have expressed four of the human sequences as recombinant V5-fusion proteins in CHO cells. This V5 tag enabled their detection by Western blot (Fig. 5) and immunofluorescent microscopy (Fig. 4). HKE4 is predicted to be 50 kDa with an additional N-linked carbohydrate side chain and LZT-Hs4 is predicted to be 53 kDa with up to three additional N-linked carbohydrate side chains. The bands obtained (Fig. 5) agree with this prediction in reducing conditions, but in non-reducing conditions, both proteins appeared as high molecular weight bands with an additional band in between that approximated to a trimer band. This ability to form complexes is consistent with the formation of

Fig. 1. Phylogenetic tree of the ZIP family sequences. The association of different ZIP family sequences was determined using the Clustal V [66] programme to perform an alignment. This dendrogram shows how the KE4 proteins form a separate section of the LIV-1 subfamily. The LZT family code is given after the accession number or gene name (see Table 1 for further details) and the two-letter species abbreviations are Ag = *Anopheles gambiae*, Ap = *Aeropyrum pernix*, As = *Anabaena* sp., At = *Arabidopsis thaliana*, Bb = *Borrelia burgdorferi*, Br = *Brachydanio rerio*, Ce = *Caenorhabditis elegans*, Ci = *Ciona intestinalis*, Cj = *Campylobacter jejuni*, Dm = *Drosophila melanogaster*, Dr = *Deinococcus radiodurans*, Hs = *Homo sapiens*, Le = *Lycopersicon esculentum*, Li = *Listeria innocua*, Lm = *Listeria monocytogenes*, Mf = *Macaca fascicularis*, Mj = *Methanococcus jannaschii*, Mm = *Mus musculus*, Mx = *Myxococcus xanthus*, Nm = *Neisseria meningitides*, Os = *Oryza sativa*, Pa = *Pseudomonas aeruginosa*, Pax = *Pyrococcus abyssi*, Ph = *Pyrococcus horikoshii*, Ps = *Pisum sativum*, Rs = *Ralstonia solanacearum*, Sc = *Saccharomyces cerevisae*, Sj = *Schistosoma japonicum*, Sp = *Schizosaccharomyces pombe*, Ss = *Sus scrofa*, Tc = *Thlaspi caerulescens*, Tm = *Thermotoga maritime*, Xl = *Xenopus laevii*, Zr = *Zygosaccharomyces rouxxii*.

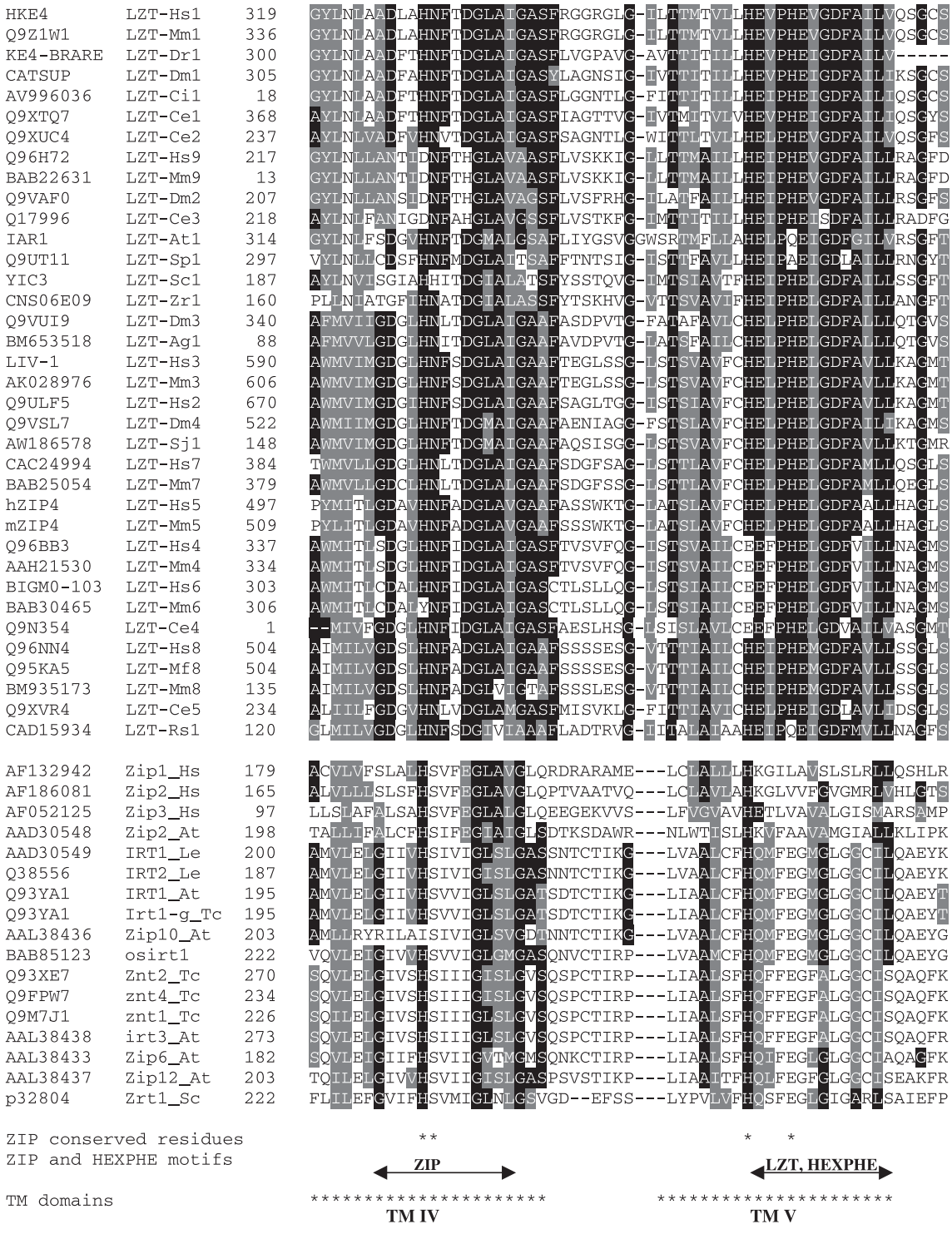

Fig. 2. Alignment of LZT subfamily and ZIP transporters across transmembrane domains IV and V. This alignment across transmembrane domains IV and V includes the conserved HEXPHE motif of the LZT subfamily and the consensus sequence for ZIP transporters (arrows) and the conserved residues (asterisks). Residues coloured black and grey correspond to identical or residues of the same group, respectively. Accession numbers (or gene names) are given in the left-hand column, followed by LZT or ZIP family nomenclature (Table 1) and residue number. Although there is similarity between the two subfamilies, it is clear that the ZIP family lacks the high degree of conservation encompassing the HEXPHEXGDFAXLLXXG motif. Alignment of sequences was achieved in Clustal V [66].

ion channels [43] especially the formation of trimers as a stable form [44]. Three of the four expressed proteins were plasma membrane located (LZT-Hs2, LZT-Hs4 and LIV-1), similarly to mouse ZIP4 [68] (Fig. 4). These proteins all co-localised with F-actin and concentrated on lamellipodiae, in an identical manner to the membrane-type matrix metal-

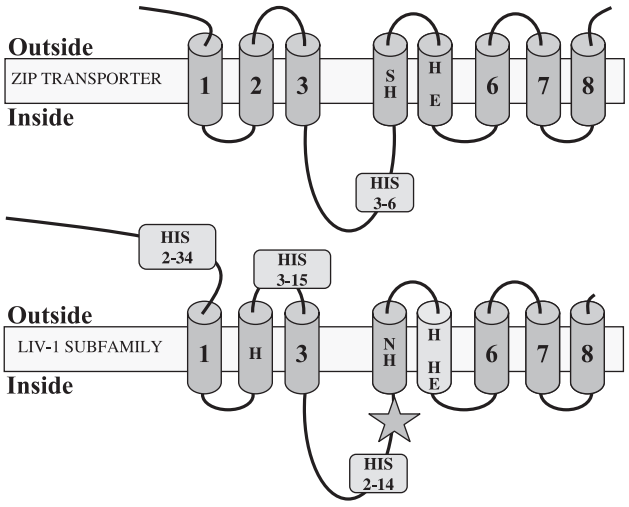

Fig. 3. Schematic comparing the predicted secondary structure of LZT and ZIP transporters. The predicted secondary structure of the LZT family is compared with that previously suggested for the ZIP family by Gaither and Eide [19]. His = Histidine-rich repeats (numbers are total histidines), Star = mixed charge region, Barrels = transmembrane domains, Pale barrel = conserved HEXPHE motif, letters in barrels represent the highly conserved residues known to be important for zinc transport in ZIP transporters and the corresponding residues in LZT sequences.

loproteases [45], which have a HEXXH motif in their active site. To our knowledge, no other mammalian zinc transporters contain such a potential metalloprotease motif, which could impart an alternative function for LZT transporters. It is noteworthy that LIV-1 has been implicated in metastatic breast cancer [22], while the relationship of membrane-type matrix metalloproteases with cancer invasion has been well documented [46–49].

3.3. Zinc transport of LZT family of proteins

The similarity of the LZT subfamily sequences to ZIP transporters suggests an ability to transport zinc into cells. To test this, we transiently expressed recombinant LZT proteins in CHO cells that had been loaded with Newport Green Diacetate, a membrane-permeable fluorescent zinc-specific indicator that increases in fluorescence when in contact with free zinc. We tested that the indicator did in fact respond to changes in intracellular zinc, for example, addition of 50 μM zinc or the membrane-permeable zinc-specific chelator TPEN to the medium produced a rise and fall, respectively, in the percentage of cells with high fluorescence. When LIV-1-expressing cells were tested, instead of the usual 10% basal level of highly fluorescent cells, 40% of the cells had high fluorescence. This indicated that those cells transfected with LIV-1 (usually 25–40%) were able to transport zinc into cells across the plasma membrane. Interestingly, the LZT protein lacking the initial H of the HEXXH motif (LZT-Hs4), which has been shown to be crucial for both zinc transport in ZIP transporters [29] and zinc-binding in metalloproteases [28], was not able to transport zinc, never varying from control cells. Expectedly, HKE4, which is present on intracellular membranes, did not transport zinc into cells.

Two recent reports support the involvement of one LZT sequence, the hZIP4 gene, in acrodermatitis enteropathica. This disease is caused by a defective uptake of zinc in the intestine which is believed to be due to a defect in hZIP4 [68,69]. These reports provide compelling evidence that mutations in the hZIP4 gene are responsible for this defective zinc uptake. Most of the mutations documented are either in transmembrane domains, cause premature termination of the protein sequence or are predicted to alter the tertiary structure of the protein. This has been clearly demonstrated [69] by observing the position of the mutations within the conserved regions when a number of other ZIP sequences were aligned together. These results, coupled with our observations of LIV-1, provide good evidence that LZT family members situated on the plasma membrane of cells can function as zinc influx transporters, with the possible exception of the KE4 subfamily, situated on intracellular membranes, and those sequences with a CEXXH motif.

3.4. Ubiquitination of LIV-1

Zrt1 is processed in cells by ubiquitin degradation via interaction with a lysine residue (K195) on the cytoplasmic loop between TM III and IV [30]. We have successfully immunoprecipitated recombinant LIV-1 with an ubiquitin antibody (unpublished results), indicating that it too binds ubiquitin in cells. Whether one of the 15 lysine residues on the cytoplasmic loop of LIV-1 is involved has not been determined.

3.5. Tissue distribution of LZT family of proteins

The tissue distribution of LIV-1 and two other human LZT family members, LZT-Hs4 and HKE4, has been investigated by probing a multi-tissue Northern array (unpublished results). The occurrence of LIV-1 is restricted to hormonal tissues such as breast, prostate, pituitary gland and brain. HKE4, however, has a different distribution to LIV-1 in that it is not expressed in the brain, but is expressed in liver, kidney and most hormonal tissues similarly to LIV-1. These arrays suggest that LZT family proteins are widely expressed in many different normal tissues as are ZIP zinc transporters. However, it is noteworthy that LIV-1 expression was increased further in any cancer samples included on the array, for example, HeLa cells and lung carcinoma.

4. Differences of LZT subfamily to ZIP zinc transporters

Although the LZT subfamily of proteins exhibit similarities to the conventional ZIP transporters, there are also a number of differences that suggest they may have a different role in cells.

Table 1
Current information about the LZT family sequences and their predicted locations

Table 1A Accession number	Family name	Species	Other sequences	Tissue	Given name	Length	Predicted location	Special features	Database description
Q9UIQ0	LZT-Hs1	*Homo sapiens* chromosome 6	CAA20238, AL031228, E12645 (fragment)	Kidney	HKE4	469	ER 56/PM 22	KE4 group	HLA class II region expressed gene KE4
Q92504	(Hs1)	*Homo sapiens*	D82060, BAA11528	Breast, embryo, embryonic carcinoma, heart, kidney, liver, lung, muscle, pancreas, placenta, thymus		429	ER	HKE4 protein with premature stop	membrane protein with histidine-rich repeats
Q9ULF5	LZT-Hs2	*Homo sapiens* chromosome 2	BAA86579, AB033091, KIAA1265, Q8NC35 (fragment)	Brain		835	PM 39/ER 35	N-term zinc finger, cytochrome *c* heme-binding site	
Q9Y3Z1	(Hs2)	*Homo sapiens*	AL050294, CAB43393			529		Fragment of Q9ULF5	
Q13433	LZT-Hs3	*Homo sapiens* chromosome 18	U41060, AX017261 (fragment), AU120027, Q96HP5, AAH39498 (fragment)	Numerous	LIV-1	749	ER 57/PM 30		Oestrogen regulated and implicated in metastatic breast cancer
Q96BB3	LZT-Hs4	*Homo sapiens* chromosome 8	Fragments D31887, Q15043, BC015770, AAH15770			537	PM 61/ER 35	CEEXXH, ABC transporter	Cell cycle and proliferation
Q9H6T8	LZT-Hs5	*Homo sapiens* chromosome 8	BAB15164, AK025537	hZIP4		647	ER 56/PM 22	Zinc uptake gene involved in acrodermatitis enteropathica [68,69]	
Q9NXC4	(Hs5)	*Homo sapiens*	BAA91200, BAA91091, AK000695, AK000334, Q9NX22, AAH01688, AX083511, AK025537	Fragments					
BAB21559	LZT-Hs6	*Homo sapiens* chromosome 4	AAG22480, AFL93052, BAA96442 (fragment), Q9C0K1, CAC38522 patent wo0129221	Bcg-induced, neuronal precursor cells, retinoic acid induction	BIGMO-103	460	ER 67/PM 22	Inhibit cancer cell growth	Bcg induced integral membrane protein
CAC24994	LZT-Hs7	*Homo sapiens*	AX061633, AAH27884, Q8N6Y3			540	ER 36/PM 28		
Q96NN4	LZT-Hs8	*Homo sapiens*	AK055061, BAB70848	Brain, retina		654	ER 56/PM 44	Similar to LIV-1	
Q96H72	LZT-Hs9	*Homo sapiens*	AAH19016, BC019016, Q8WV10, Q8N7C9	Fragment		364		KE4 group	ZIP MOTIF

Table 1B

Accession number	Family name	Species	Other sequences	Given name	Length	Predicted location	Special features
Q9GKV2	LZT-Mf2	*Macaca fascicularis*	AB051127; BAB18153		528	PM 39	Hllp motif
Q95KA5	LZT-Mf8	*Macaca fascicularis*			654	ER 44/PM 44	
Q9Z1W1	LZT-Mm1	*Mus musculus*	AF100956, AAC69903, BM054306, BM054306	KE4	476	ER 44	KE4 group
Q31125	(Mm1)	*Mus musculus*	M32010, AAA37747	KE4	436	ER	Fragment of Q9Z1W1, development, MHC section of genome
BM721841	LZT-Mm3	*Mus musculus*	Q8R518, BAB86300, ABO71697	Ermelin	>733	PM 47	ER located but possibly missing >228aa of N terminus (see text)
AAH21530	LZT-Mm4	*Mus musculus*			489	ER 35/PM 61	
BAB24106	LZT-Mm5	*Mus musculus*	Q9DAT9	mZIP4	660	ER 44/PM 33	Zinc uptake gene involved in acrodermatitis enteropathica
BAB30465	LZT-Mm6	*Mus musculus*	BAB29610, Q91W10, Q9D5V4, Q9D426		462	ER 67/PM 22	CEEXXH, ABC transporter
BAB25054	LZT-Mm7	*Mus musculus*	BAB25675, Q9D856, Q9D909		535	ER 48/PM 26	
BM935173	LZT-Mm8	*Mus musculus*	Fragment		200		
BAB22631	LZT-Mm9	*Mus musculus*	Possible fragment, Q9D1R4		160		KE4 group

Table 1C

Accession number	Family name	Species	Other sequences	Given name	Length	Predicted location	Special features
Q9XTQ7	LZT-Ce1	*C. elegans* chromosome X	(Z99942), CAB17070, E1322656	KE4L	515	ER 44/PM 44	KE4/CATSUP FAM
Q9XUC4	LZT-Ce2	*C. elegans* chromosome IV	Z82285, CAB05297	KE4	404	ER 56/PM 33	KE4/CATSUP FAM
Q17996	LZT-Ce3	*C. elegans* chromosome X	Z50863, CAA90736, T19285		338	ER 48/PM 30	KE4 group
Q9N354	LZT-Ce4	*C. elegans* Y55F3BL.2	AC024828; AAF60809		157		Fragment
Q9XVR4	LZT-Ce5	*C. elegans* chromosome IV	Z81044, CAB02806, E275651		360	ER 22/PM 57	
Q9V3A4	LZT-Dm1	*Drosophila melanogaster*	AAF37226, AF216584, AAF53744, AE003661, AAL13757	CATSUP	449	ER 44/PM 33	CATSUP, kininogen pr00334, KE4 group
Q9VAF0	LZT-Dm2	*Drosophila melanogaster*	AE003771, AAF56969		355	ER 35/PM 52	KE4 group
Q9VUI9	LZT-Dm3	*Drosophila melanogaster*	AAF49687, AE003532		519	ER 44/PM 44	

(continued on next page)

Table 1 (*continued*)

Table 1C Accession number	Family name	Species	Other sequences	Given name	Length	Predicted location	Special features
Q9VSL7	LZT-Dm4	*Drosophila melanogaster*	AAF50401, AE003555		684	ER 44/PM 33	
Q9M647	LZT-At1	*Arabidopsis thaliana*	AF216524, AAF32299, AAF16552, AC012563 Q9SFR8, AAG52008, Q9C9X4	IAR1	569	ER 44/PM 22	IAA-alanine resistance protein, KE4 group
Q29175	LZT-Ss1	*Sus scrofa*	Q9XT01, F14787; CAA23256, AF146397, AAD44801	KE4 PIG			Fragment
Q9PUB8	LZT-Br1	*Brachydanio rerio*	AAF05821, AF196345	KE4-BRARE	352	ER 33/PM 22	

Table 1D Accession number	Family name	Species	Other sequences	Given name	Length	Predicted location	Special features
P40544	LZT-Sc1	*Saccharomyces cerevisiae* chromosome IX	PIR: S49959, CAA86969, Z46881	YIC3 YEAST	346	ER 48/PM 30	KE4 group
Q9UT11	LZT-Sp1	*Schizosaccharomyces pombe*	CAB55170, AL117210, PIR: T39240		453	ER 33/PM 33	KE4 group
AW186578	LZT-Sj1	*S. japonicum*	Fragment		233		
BJ044228	LZT-Xl1	*Xenopus laevii*	BJ081638, BJ047405		155		
CAD15934	LZT-Rs1	*Ralstonia solanaceaerum*			268		
CNS06E09	LZT-Zr1	*Zygosaccharomyces rouxii*			317	ER 44/PM 26	KE4 group
CAD15934	LZT-Rs1	*Ralstonia solanacearum*			268	ER 22/PM 57	
AV996036	LZT-Ci1	*Ciona intestinalis*			162		HNIP MOTIF, KE4 group
BM653518	LZT-Ag1	*Anopheles gambiae*	BH380314		224		

The suggested LZT family name is given in the family name column, where brackets indicate identical, similar or fragments of sequences that have not been included in the overall family alignment. Human sequences are shown in Table 1A, mouse and monkey sequences are shown in Table 1B, other mammals and plant sequences are shown in Table 1C, and remaining fungal and bacteria sequences are shown in Table 1D.

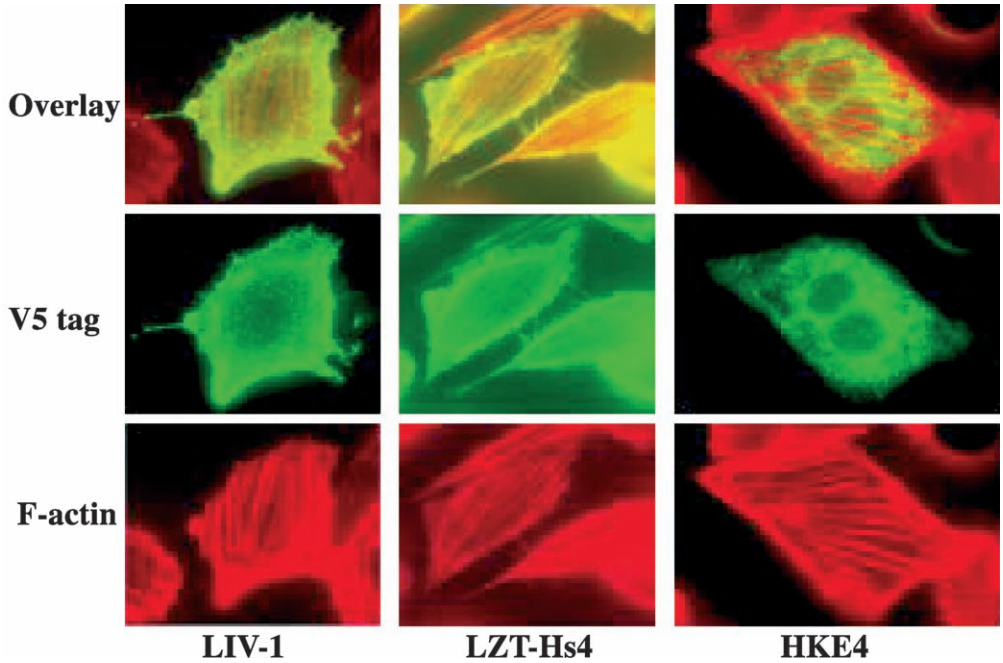

Fig. 4. Fluorescent microscopy of three human LZT family members. Recombinant V5-fusion proteins fluorescently labelled with Alexa-Fluor 488 (green) (middle row) and F-actin filaments stained with Texas-Red phalloidin (bottom row). The overlay on the top row shows plasma membrane staining for LIV-1 and LZT-Hs4 in CHO cells fixed with 4% formaldehyde, whereas HKE4 shows an intracellular location in CHO cells fixed in 4% formaldehyde and 0.4% saponin.

4.1. Histidine-rich repeats

The LZT subfamily sequences contain the same histidine-rich repeat, (HX)*n*, of the ZIP transporters, also on the long cytoplasmic loop, yet the incidence of these repeats is as much as sevenfold more than other ZIP transporters [24] over the whole sequence. In a previous alignment of ZIP sequences [29], two KE4 sequences (human gbD82060 and mouse spQ31125) were included and a region constituting 40 histidine residues in total was observed in the N-terminal region of both sequences. However, these sequences were the human and mouse KE4 sequences (LZT-Hs1 and LZT-Mm1, respectively), which are recognised here as members of the LZT subfamily. Therefore, one characteristic difference of the LZT subfamily from other ZIP transporters is still the increased incidence of histidine-rich repeats [24] throughout the sequence. These repeats are present in the cytosolic loop between TM III and IV, as in ZIP transporters, but also on the extracellular loop between TM II and III and the extracellular N terminus [24]. The presence of these repeats on both sides of the membrane is unprecedented in zinc transporters and although the function of these extra repeats is not known, they would be predicted to have a role in the transport of metal ions by similarity with other such motifs [42].

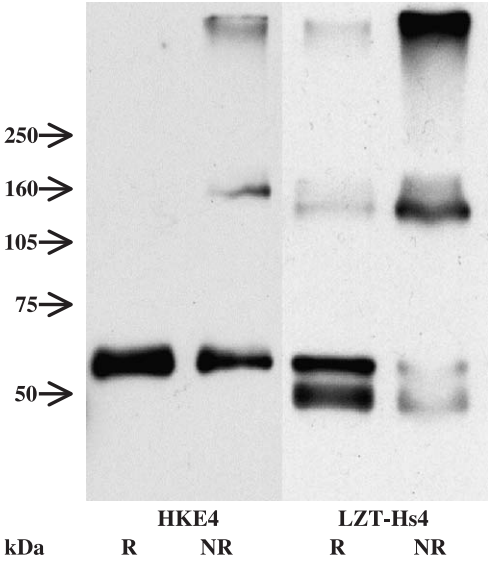

Fig. 5. Western Blot of two human LZT family proteins. CHO cells transiently transfected with HKE4 and LZT-Hs4, with C-terminal V5 tags, were lysed and samples applied to a 10% SDS-PAGE gel. Bands of protein were transferred to nitrocellulose and probed with V5 antibody. Results in reducing (R) and non-reducing (NR) conditions are shown. Both proteins have high molecular weight bands in non-reducing conditions as well as a band that approximates to a trimer.

However, as more family members emerge, it is evident that some sequences are not as histidine-rich. In fact, there is one or more family member from most of the higher species that has few or no histidine residues at all. For example, human LZT-Hs4, mouse LZT-Mm4, and *C. elegans* LZT-Ce3, contain 4, 6 and 0 histidines, in (HX)*n* repeats, respectively. Interestingly, these former two sequences and two others (human LZT-Hs6 and mouse LZT-Mm6) have the initial histidine of the HEXXH motif replaced by a

glutamic acid residue (EEXXH, Fig. 2). We have already mentioned that cells expressing recombinant LZT-Hs4 did not transport extracellular zinc into cells, in contrast to LIV-1. However, this replacement of the conserved histidine with glutamic acid may indicate a preference for transporting ions other than zinc as a QEXXH motif has preference for copper transport [67].

4.2. Zinc-binding site motif of metalloproteases

One feature that is unique amongst the LZT subfamily is the presence of a motif (HEXPHEXG) that fits the consensus sequence for the catalytic zinc-binding site of matrix metalloproteases [50,51] and snake venom metalloproteinases [52] (**HE**XX**H**XXGXX**H**) lacking only the final coordinating histidine. The zinc ligands in this latter motif (marked in bold) consist of three histidine residues and one water molecule, linked to the glutamic acid, E [53,54]. The catalytic zinc ion is essential for the proteolytic activity of matrix metalloproteases [46] and the three histidine residues that coordinate the zinc ion in these molecules are completely conserved. Different subgroups of metalloproteases have been characterised by the distance and identity of the fourth zinc coordinating residue in this motif [55]. For example, the catalytic zinc-binding site of the zincin and PDF groups of metalloproteases (HEXXHXXG), similar to that in LZT sequences, lacks the terminal histidine residue of the matrix metalloprotease motif and, therefore in these proteins, the conserved glycine residue, three residues downstream of the second histidine, generates the proper coordination geometry of the ligands [26,27].

The completely conserved second glutamic acid downstream of the second histidine (marked in bold) in the LZT family motif (HEXPH**E**XGD) is also a good candidate residue to coordinate the zinc ion via a second water molecule [24]. Thermolysin, a zinc-binding metalloprotease, belongs to a group of metalloproteases that use a glutamic acid residue for the fourth ligand to coordinate the zinc ion. In thermolysin, this residue is 20 amino acids downstream from the second histidine in the first motif [54] and present in a small conserved motif (NEXXSD). LIV-1 possesses a similar motif, NDXSD, at residues 706–710. However, there is no evidence for such a motif in any of the other LZT family proteins.

It is also probable, due to the integral TM location of the motif, that the fourth zinc coordinating residue is provided by one of the other TM domains. The prime candidate for this would be TM domain II as it contains a conserved histidine and other residues spaced to be consistent with a position on one side of a helix [24]. Interestingly, two human LZT family members, LZT-Hs4 and LZT-Hs6, both of which have the initial H of the HEXXH motif replaced by a glutamic acid (E) residue (Fig. 2) have the conserved histidine in TM II replaced by a glutamine (Q) residue (Fig. 6). We have already shown that LZT-Hs4 does not transport zinc [50] even though it is located on the plasma membrane, reinforcing the potential importance of these two residues.

The three-dimensional structure of this HEXXH motif has been determined to be a helical tertiary structure [52]. This motif in the LZT family, which is well conserved across all 36 sequences, contains a unique proline residue (Fig. 2). Proline residues can be both inconsistent with the formation of a helical structure [56,57] and also provide increased stability [58–60]. The presence of this motif in a TM domain may therefore be predicted to improve stability [61].

It is noteworthy that TM IV and V of LIV-1 have similarity to the B, D and E chains of haemagglutinin-esterase-fusion glycoprotein of influenza C virus (Hef2) which exists as a helical structure up to the equivalent of the start of the HEXPHEXGD motif (PDB:1flc, residues 96–134). Interestingly, Hef2 helices appear as parallel pairs of helices and TM II of LIV-1 are similar to the second helix of Hef2 which runs parallel to it in the tertiary structure. This suggests that the tertiary structure of TM II and V of LIV-1 may indeed act synergistically. These observations are consistent with the ability of LZT proteins to transport zinc across the plasma membrane through a pore region maintained by interaction of conserved residues in TM II, IV, and V.

4.3. Long N terminus

The LZT subfamily sequences have long N termini, especially when compared to ZIP transporters [29]. The N terminus of LIV-1 itself consists of 317 residues (42%) of the total 749 residues in the full-length sequence. This region in LIV-1 contains 27 histidine residues including the following cluster of 19 histidines, HIHHDHDHHSD-HEHHSDHERHSDHEHHSDHEHHSDHNH, which can be divided into six repeats of the general formula H(H,S)DH (E,D,N)(H,R).

4.4. Conserved TM domains

TM domains I, II, III and VII of the LZT subfamily also contain unique signatures (Fig. 6) not present in any other sequences in the GenBank. The role of TM domain II and its probable interaction with TM IV and V has already been mentioned above. TM IV and V contain the highly conserved consensus sequence for the LZT subfamily with conservation of every third or fourth residue, consistent with their presence on one side of a helix. These adjacent TM domains are well conserved, including three charged residues, with no apparent loop and a conserved mixed charge region directly upstream (Fig. 3). This suggests a possible pore structure involved in the binding and/or transporting of zinc or other ions.

5. The KE4 subfamily of LZT proteins

A number of the LZT subfamily sequences are also KE4 proteins, some of which have been previously aligned with

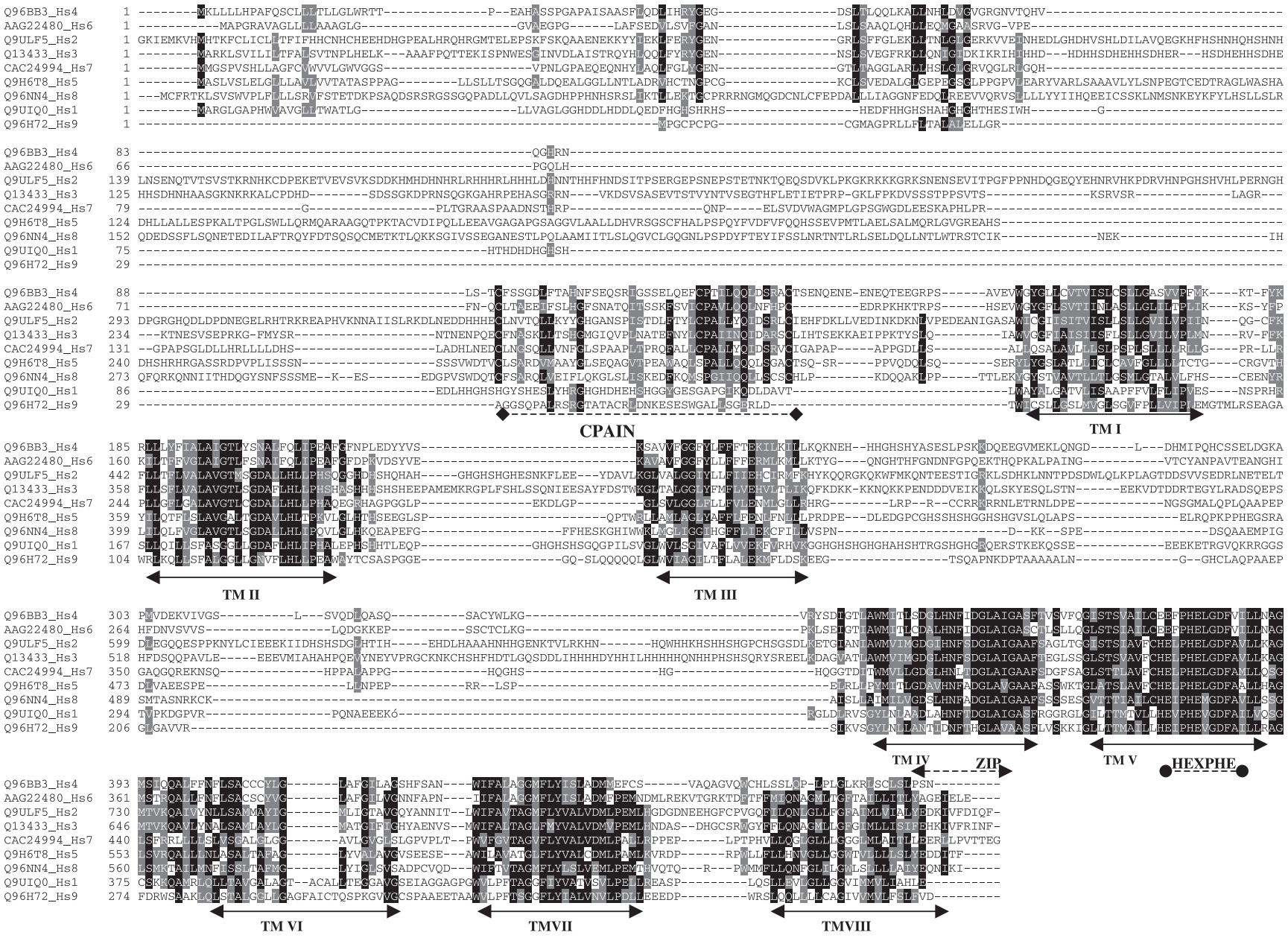

Fig. 6. Alignment of the whole sequence of nine human LZT family members. Residues coloured black and grey correspond to at least 50% identical or complementary residues, respectively. Solid arrows with roman numerals indicate transmembrane domains, dotted arrows indicate conserved motifs, solid square = CPALLY motif, solid circle = HELPHE motif, solid arrows = ZIP consensus. Alignment of sequences was achieved in Clustal V [66].

other ZIP transporters and suggested as both zinc transporters [29,39,40] and members of the LIV-1 subfamily [24]. At present, there are apparently 17 KE4 proteins, which appear from the phylogenetic tree (Fig. 1) to form a separate sub family of LZT proteins. This KE4 subgroup includes CATSUP from *Drosophila* (AAF37226) [41], IAR1 from *Arabidopsis* (AAF32299) [40], mouse KE4, pig KE4 and zebrafish KE4. We have expressed the LZT protein, HKE4 (LZT-Hs1), in CHO cells where it located to intracellular membranes (Fig. 4) and not the plasma membrane as was observed with other LZT proteins. Another KE4 protein, IAR1, a putative transporter with alanine-resistance properties, has been localised to the endoplasmic reticulum and proposed to transport manganese out of this organelle [40]. It has been suggested to be involved in auxin metabolism in plants by inhibiting an auxin conjugate hydroxylase. Interestingly, mouse KE4, was shown to functionally substitute for IAR1 [40]. CATSUP has been suggested to down-regulate tyrosine hydroxylase [41], a rate-limiting enzyme for dopamine production in the brain, in *Drosophila* embryos. This was achieved by performing knockouts in *Drosophila*, which were lethal, and testing mutated partial loss of function alleles.

One interesting feature of the KE4 sequences is the lack of another motif (CPALLY) which is uniquely present in the LZT sequences of human, mouse and monkey origin (Fig. 7), upstream of TM I. This motif, not present in any other sequences in the GenBank, includes cysteine and proline residues, suggesting a conserved tertiary structure. Interestingly, the final conserved cysteine in this motif is mutated to a tyrosine residue (C309Y) in acrodermatitis enteropathica [68]. This may suggest a crucial functional role for this motif but it could also represent disruption of the tertiary structure by a missing cysteine which is usually present in a disulfide bond, as has been observed with other protein mutations [70].

The mouse sequence ermelin has virtually the identical sequence to LIV-1, rather than the endoplasmic reticulum-located KE4 proteins (Table 1, LZT-Mm3), and yet has been localised to the endoplasmic reticulum [39] instead of the plasma membrane as LIV-1 has been. However, the recent appearance of AK028976 (LZT-Mm3) with 765 amino acids suggests that the ermelin sequence is missing most of the N-terminal residues which may account for its endoplasmic reticulum location. In fact, on close inspection of the ermelin DNA sequence (BM721841), another 228 residues that match AK028976 can be obtained from the next reading frame, suggesting a possible sequencing error.

6. Predicted function of LZT subfamily proteins

The exact function of LZT family proteins is unknown, but some function has been suggested in the individual GenBank files. Human LIV-1 (LZT-Hs3) has been implicated in metastatic breast cancer [23], whereas the other proteins in the family group are as yet of unknown function. The oestrogen-regulation of LIV-1 in breast cancer is not without precedent as hZIP1, a human ZIP transporter, is expressed in the malignant prostate cancer cell lines, LN-CaP and PC-3, in a hormone-dependant manner [62]. A role of LIV-1 in breast cancer may be explained by the fact that zinc can induce EGF receptor phosphorylation and MapKinase activation by activation of both the IGF-1 and EGF receptors [63]. This is achieved by stimulation of the tyrosine phosphorylation of these receptors by zinc, possibly by interference with tyrosine dephosphorylation as zinc can inhibit various protein tyrosine phosphatases [64]. These pathways are all of critical importance in the development of a breast cancer phenotype.

One human family member, LZT-Hs6, has been suggested to inhibit cancer cell growth being induced by Bcg and retinoic acid, whereas another, LZT-Hs4, has been implicated in the cell cycle and cell proliferation. The KE4 subfamily has been implicated in ion transport [40] and possible down-regulation of specific hydrolases [41], as mentioned earlier.

Several human LZT family members are expressed in the brain (e.g. LZT-Hs2), where alteration of tyrosine hydroxylase activity may be important for the control of neurodegeneration and growth. Control of zinc is important for the normal functioning of the brain and can be altered in diseases such as epilepsy [65]. Auxin metabolism in plants is also important for the control of growth.

7. Conclusion

The suggestion that the LIV-1 family is intimately involved in zinc homeostasis implies that its function may be critical for cell growth. This is supported by the small

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Q96NN4      LZT-Hs8  310  CFSARQLVEIFLQKGLSLISKEDFKQMSPGIIQQLLSCSC
hZIP4       LZT-Hs5  270  CLSARDVMAAYGLSEQAGVTPEAWAQLSPALLQQQLSGAC
Q96BB3      LZT-Hs4   91  CFSSGDLFTAHNFSEQSRIGSSELQEFCPTILQQLDSRAC
BIGMO-103   LZT-Hs6   74  CLTAEEIFSLHGFSNATQITSSKFSVICPAVLQQLNFHPC
Q9ULF5      LZT-Hs2  341  CLNVTQLLKYYGHGANSPISTDLFTYLCPALLYQIDSRLC
CAC24994    LZT-Hs7  158  CLNGSQLLVNFGLSPAAPLTPRQFALLCPALLYQIDSRVC
LIV-1       LZT-Hs3  259  CFNASKLLTSHGMGIQVPLNATEFNYLCPAIINQIDARSC
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Fig. 7. Alignment of seven LZT human sequences that contain a CPALLY motif. Residues coloured black and grey correspond to 100% identical or complementary residues, respectively. This motif is not present in the KE4 proteins.

amount of suggested function available in the GenBank files. We are therefore encouraged to investigate this exciting new group of proteins, both in normal cells and diseases such as cancer. As zinc is essential for cell growth, membrane proteins capable of transporting zinc into cells will have a crucial role in maintaining the cellular balance between apoptosis and cell growth. Such a function would probably require a family of proteins under tight regulation of expression because faults in the system could easily lead to premature death or cancer.

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