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Review

Eukaryotic zinc transporters and their regulation

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Abstract

The last ten years have witnessed major advances in our understanding of zinc transporters and their regulation in eukaryotic organisms. Two families of transporters, the ZIP (*Z*rt-, *I*rt-like *P*rotein) and CDF (*C*ation *D*iffusion *F*acilitator) families, have been found to play a number of important roles in zinc transport. These are ancient gene families that span all phylogenetic levels. The characterized members of each group have been implicated in the transport of metal ions, frequently zinc, across lipid bilayer membranes. This remarkable conservation of function suggests that other, as yet uncharacterized members of the family, will also be involved in metal ion transport. Many of the ZIP family transporters are involved in cellular zinc uptake and at least one member, the Zrt3 transporter of *S. cerevisiae*, transports stored zinc out of an intracellular compartment during adaptation to zinc deficiency. In contrast, CDF family members mediate zinc efflux out of cells or facilitate zinc transport into intracellular compartments for detoxification and/or storage. The activity of many of these transporters is regulated in response to zinc through transcriptional and post-transcriptional mechanisms to maintain zinc homeostasis at both the cellular and organismal levels.

Introduction

Zinc is an essential nutrient for all organisms on earth. This metal ion plays critical roles in a wide variety of biochemical processes. For example, zinc is a cofactor required for the function of over 300 different enzymes including representatives from all six major functional enzyme classes (Vallee & Auld 1990). Zinc is also an important structural cofactor for many proteins including the ubiquitous zinc finger DNA binding proteins (Rhodes & Klug 1993). Because of these essential roles, organisms must maintain adequate intracellular zinc concentrations to support cell growth even when extracellular or dietary levels are low. To accomplish this feat, cells have evolved with efficient uptake systems to allow accumulation of zinc even when it is scarce. These uptake systems use integral membrane transport proteins to move zinc across the lipid bilayer of the plasma membrane.

Once inside a eukaryotic cell, a portion of the zinc must be transported into intracellular organelles to serve as a cofactor for various zinc-dependent enzymes and processes present in those compartments. For example, the mitochondrial isozyme of alcohol dehydrogenase is a zinc-dependent enzyme (Sytkowski 1977). Therefore, transporter proteins must be present in organelle membranes to facilitate this flux of zinc. Zinc can also be stored in certain intracellular compartments when supplies are high and used later if zinc deficiency ensues. Again, zinc transporters are required to facilitate this transport in and out of organelles.

Although zinc is essential, excess zinc can be toxic to cells (for example, see Koh *et al.* 1996). The mechanism of zinc toxicity is not known but the metal may bind to inappropriate intracellular ligands or compete with other metal ions for enzyme active sites, transporter proteins, etc. Therefore, while maintaining adequate levels of zinc for growth, cells must also con-

trol intracellular levels when exposed to excessive zinc concentrations. Several mechanisms exist to detoxify excess zinc including the binding of the metal to cytoplasmic macromolecules. Metallothionein proteins may play such a detoxification role (Hamer 1986). Zinc transporters can also aid in detoxification by facilitating intracellular sequestration within organelles, or efflux of zinc across the plasma membrane. Finally, in multicellular organisms, cellular zinc efflux systems are required for the distribution of dietary zinc to other tissues. For example, in the enterocyte of the mammalian intestine, zinc transporters must take up zinc from the intestinal lumen and then efflux that zinc across the basal lateral membrane into the portal blood.

From this discussion, it should be clear that zinc transporters play a variety of essential roles in zinc metabolism. This review considers our current knowledge of zinc transporters in eukaryotic organisms. Because the activity of these transporters, and hence zinc homeostasis, is often controlled by zinc status, the mechanisms of regulation will also be described. Zinc transporter activity is known to be regulated at both transcriptional and post-translational levels.

Families of zinc transporters in eukaryotes

Many types of transporters have been implicated in zinc transport processes. In bacteria, transporters belonging to the ABC family have been shown to function in zinc uptake. For example, the ZnuABC proteins of *E. coli* are a major source of zinc accumulation for these cells (Patzer & Hantke 1998). Zinc efflux transporter proteins belonging to the family of P-type ATPases have also been identified. The ZntA transporter of *E. coli* is one such protein. ZntA plays an important role in zinc detoxification by pumping the metal ion out of the cell when intracellular zinc levels get too high (Rensing *et al.* 1997).

In eukaryotes, neither ABC transporters nor P-type ATPases have yet been found to play physiological roles in zinc transport. Rather, two other families of transporters have been implicated in this process. The ZIP family plays prominent roles in zinc uptake, transporting zinc from outside the cell into the cytoplasm. ZIP transporters have also been found to mobilize stored zinc by transporting the metal from within an intracellular compartment into the cytoplasm. A second group of transporters, the CDF family, transports zinc in the direction opposite to that of the ZIP proteins, promoting zinc efflux or compartmentalization by pumping zinc from the cytoplasm out of the cell or into the lumen of an organelle. The next two sections will provide an overview of these two families of transporters. Specific examples of the roles they play in eukaryotic zinc transport and their regulation will be considered in later sections of this review. A key point to be made is that all of the characterized members of these families play roles in metal ion transport with zinc often being the substrate. This observation suggests that many other members function in zinc transport. The roles of the many characterized proteins implicated in zinc transport have been assessed based on mutant phenotypes (e.g., sensitivity to zinc excess or deficiency), gene or protein regulation in response to zinc, and direct assays of zinc transport using $^{65}Zn^{2+}$ as a tracer.

The ZIP family of metal ion transporters

The ZIP transporters, so-called for being '*Z*rt-, *I*rt-like *P*roteins' are named after two of the first members of the family to be identified, Zrt1 of *Saccharomyces cerevisiae* and Irt1 of *Arabidopsis thaliana* (Eide *et al.* 1996; Zhao & Eide 1996a). Zrt1 is a zinc uptake transporter in yeast and Irt1 is an iron transporter in plants. At the time of this writing, 86 ZIP members are found in the non-redundant protein sequence database at the National Center for Biotechnology Information (NCBI) (Figure 1). This list includes proteins from eubacteria, archeae, fungi, protozoa, insects, plants, and mammals. Recent reviews described the ZIP family as containing only 20–30 members (Eng *et al.* 1998; Guerinot 2000). The 3-fold increase in the number of member genes reported here is largely due to our use of more sensitive database analysis tools. Previous comparisons were performed using BLAST (Basic Local Alignment Search Tool) (Altschul *et al.* 1990) while our analysis used PSI-BLAST (Position-Specific Iterated BLAST), a more sensitive method of database comparisons that relies on a position-specific scoring matrix (Altschul *et al.* 1997). While previous BLAST analyses suggested that the family was limited to eukaryotes, members of the ZIP family have now been found in archeae and eubacteria as well. Thus, contrary to our previous view (Eng *et al.* 1998), we now conclude that the ZIP family has a very ancient origin.

The ZIP family can be split into several subfamilies based on a higher degree of sequence conservation within these groups. Guerinot previously divided the

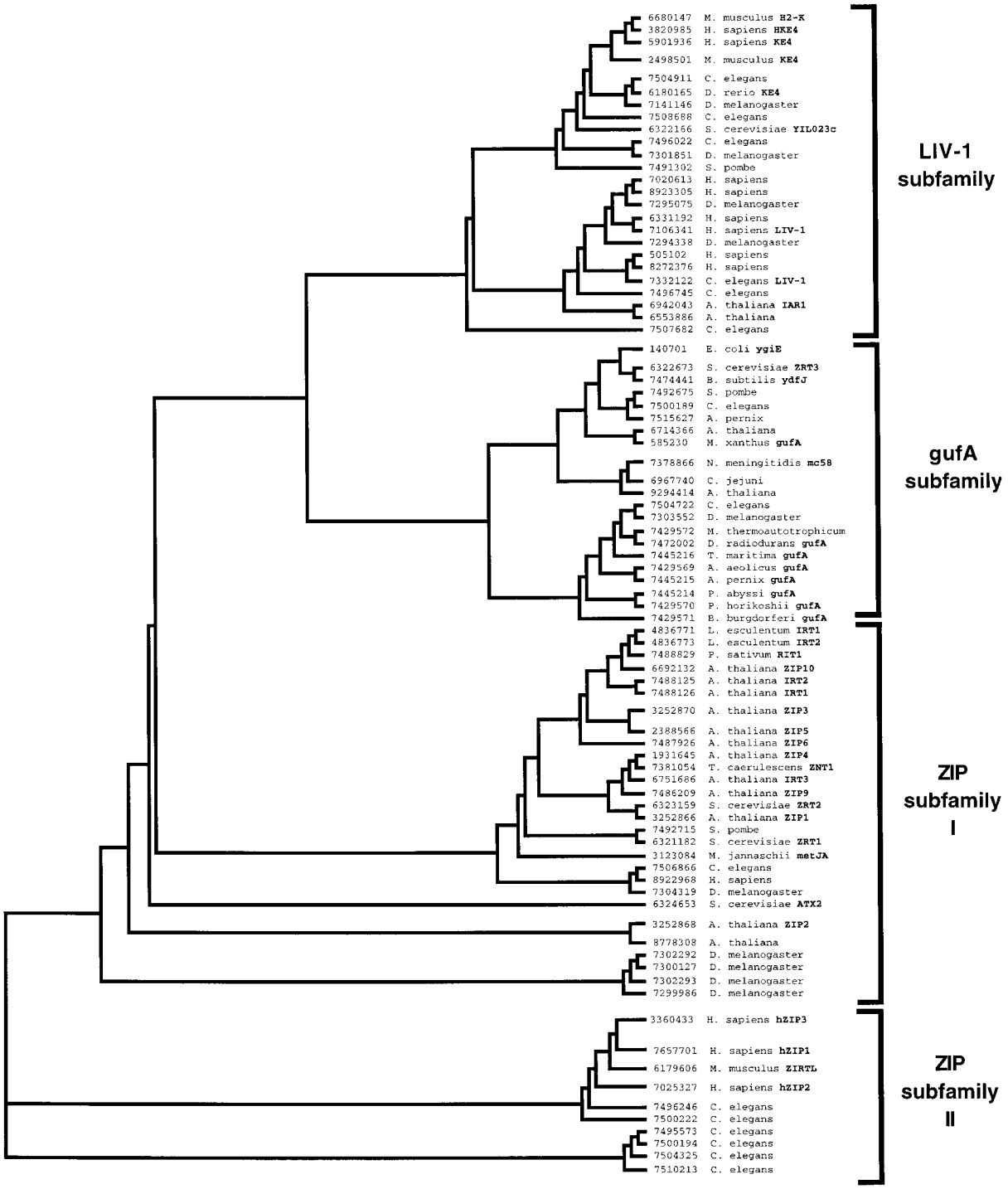

Fig. 1. The ZIP family of metal ion transporters. A dendrogram is shown describing the sequence relationships of ZIP members identified in the NCBI nonredundant protein database (12/00). Related sequences were identified using a PSI-BLAST-generated Position-Specific Scoring Matrix and the resulting dendrogram is a neighbor-joined tree generated using CLUSTALX (Thompson *et al.* 1997). The corresponding accession numbers, source organism, and gene name (*bold*), if any, are given. The different subfamilies within the ZIP family are indicated with *brackets*.

known eukaryotic ZIP proteins into subfamilies I and II (Guerinot 2000) and we have retained that classification here. Subfamily I consists largely of fungal and plant members whereas subfamily II is a smaller group of mammalian and nematode proteins. As described below, many members of ZIP subfamilies I and II are involved in zinc uptake. Our PSI-BLAST analysis identified two additional subfamilies. One new group is designated the gufA subfamily after the gufA protein of *Myxococcus xanthus*. GufA is a protein of unknown function first identified by genomic sequencing (McGowan *et al.* 1993). Several related genes, also of unknown function, have been identified in other prokaryotes and have been named gufA as well. Among the gufA subfamily are proteins from eukaryotes. This includes Zrt3, which is now known to be a zinc transporter in *S. cerevisiae*. The presence of Zrt3 in the gufA cluster of proteins clearly links this subfamily to roles in metal ion transport. The fourth subfamily, the LIV-1 subfamily, is restricted to eukaryotes. LIV-1, the gene after which this subfamily is named, encodes a protein of unknown function that is expressed at higher levels in certain metastatic breast cancer tissues (Manning *et al.* 1995). No members of the LIV-1 subfamily have been functionally characterized. However, their relationship to other ZIP proteins suggests a role in metal ion transport. This role was first proposed by Eng *et al.* (1998). This hypothesis was also proposed by Taylor (2000) based on the high abundance of histidines in various regions of the LIV-1 proteins, a common feature of both ZIP and CDF families. We find here that the sequence similarity between LIV-1 and other ZIP proteins is much greater than was recognized in previous studies.

Most ZIP proteins are predicted to have eight transmembrane domains but some members may have as few as five. Because many of the protein sequences have been determined by computer-assisted editing of probable introns from genomic sequences, the variability in the number of transmembrane domains may be due in large part to errors in this conceptual splicing process. All of the functionally characterized members of the family are predicted to have eight transmembrane domains so this seems the most accurate domain structure for most of the family. The majority of ZIP proteins share a similar predicted topology where the amino and carboxy termini are extracytoplasmic (Figure 2A). Elements of this topology have been confirmed for some members of the family, e.g., the amino terminus of Zrt1 and the carboxy terminus of hZip2 have been shown to be on the extracellular surface of the plasma membrane (Gaither & Eide 2000; Gitan *et al.* 1998). Another feature shared by many of the ZIP proteins is a long loop region located between transmembrane domains III and IV. This region is referred to as the 'variable region' because both its length and sequence shows little conservation among the family members (Guerinot 2000). One feature of the variable region that is shared by several of the ZIP proteins is the presence of many histidine residues. For example, in Zrt1, this sequence is . . . HDHTHDE. . . and in Irt1, the sequence is . . . HGHGHGH. . . . While the function of this motif is unknown, we recognize it as a potential metal binding domain and its conservation in many of the ZIP proteins suggests a role in metal ion transport or its regulation. Finally, the variable region is predicted to be cytoplasmic and this location has been confirmed for Zrt1 (Gitan & Eide 2000).

The greatest degree of conservation among ZIP proteins is found in transmembrane domains IV-VIII (Figure 3). Transmembrane domains IV and V are particularly amphipathic and contain conserved histidine residues frequently with adjacent polar or charged amino acids. Some examples of these motifs are shown in Figure 3. Given their amphipathic nature and sequence conservation, transmembrane domains IV and V are predicted to line an aqueous cavity in the transporter through which the cationic substrate passes (Eng *et al.* 1998). Consistent with this model, mutation of the conserved histidines or adjacent polar/charged residues in transmembrane domains IV and V of Irt1 eliminated its transport function (Rogers *et al.* 2000). Finally, residues important in determining the substrate specificity of Irt1 were mapped to the loop region between domains II and III (Rogers *et al.* 2000). This region is predicted to lie on the outer surface of the membrane and could be the site of initial substrate binding during the transport process.

The mechanism of transport used by the ZIP proteins is still unclear. Zinc uptake by human hZip2 zinc transporter was found to be energy independent (Gaither & Eide 2000). This observation is in conflict with studies of the yeast zinc transporters Zrt1 and Zrt2 which showed strict energy dependence (Zhao & Eide 1996a). Therefore, fungal and human ZIPs may work by different mechanisms. Zinc uptake by hZip2 was not dependent on K^+ or Na^+ gradients but was stimulated by HCO_3^- (Gaither & Eide 2000). It was suggested that hZip2 functions *in vivo* by a Zn^{2+}-HCO_3^- symport mechanism. Alternatively, zinc uptake by these proteins may simply be driven by the concen-

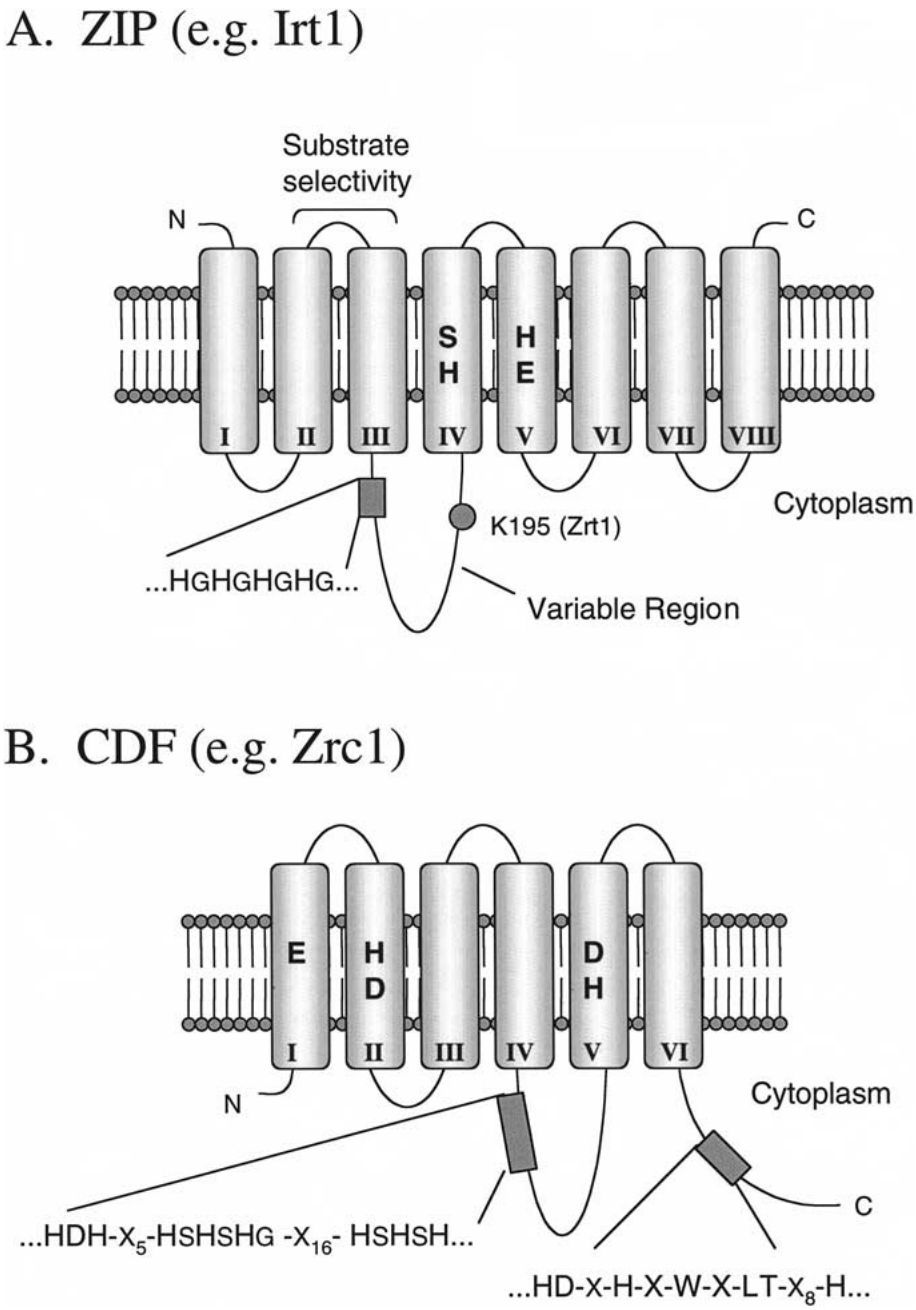

Fig. 2. Predicted membrane topology of ZIP and CDF proteins. A. Irt1, like most other ZIP transporters is predicted to have eight transmembrane domains (I–VIII). The conserved and functionally important residues within domains VI and V are indicated as is the variable region, the ubiquitinated K195 in Zrt1, and the extracellular loop region that affects Irt1 substrate specificity. B. Zrc1, like most other CDF transporters, is predicted to have six transmembrane domains (I–VI). Conserved polar or charged residues within transmembrane domains I, II, and V are indicated. The locations of histidine-rich regions of Irt1 and Zrc1 that are potentially involved in metal binding are also indicated.

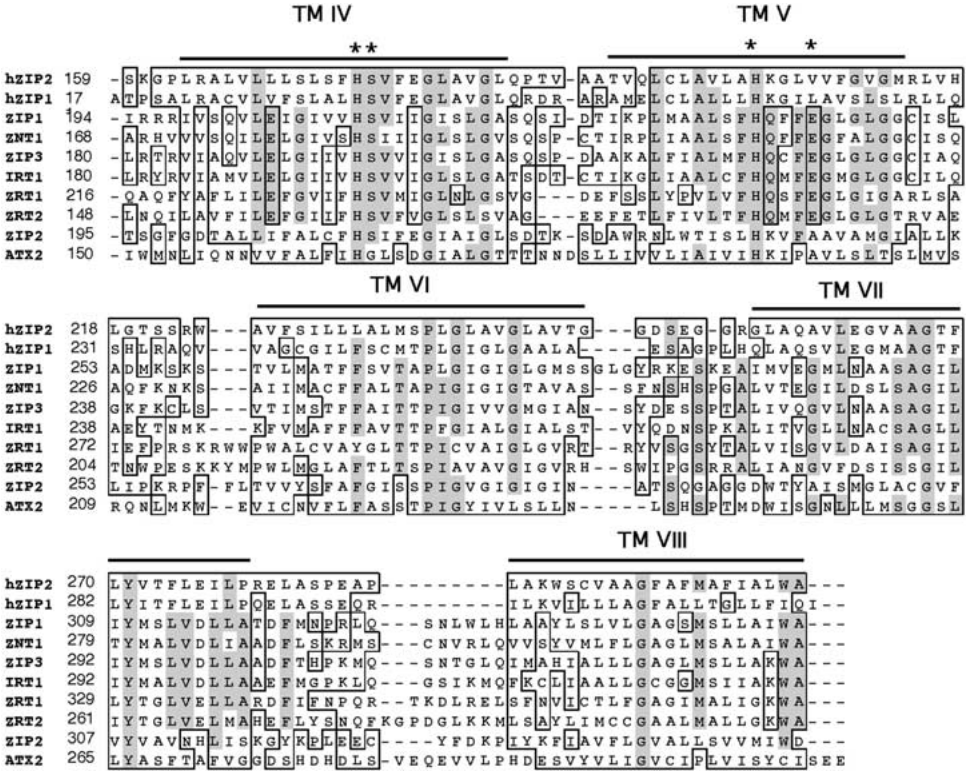

Fig. 3. Sequence conservation in transmembrane domains IV-VIII of characterized ZIP family members. The amino acid sequences of several ZIP transporters are shown and the approximate locations of transmembrane domains (TM) are indicated by a *line* over the corresponding sequences. Similar amino acids ($\geq$ 85% of the sequences shown) are *boxed* and residues that are identical in $\geq$ 65% of the sequences are *shaded*. The conserved histidines and nearby polar or charged residues in the amphipathic TM IV and V that are required for function of Irt1 (Rogers *et al.* 2000) are indicated with *asterisks*.

tration gradient of the metal ion substrate. Although the total level of zinc in a cell is high (e.g., 200 μM) (Palmiter & Findley 1995), very little of that zinc is present in a 'free' or labile form. Estimates of the labile pool of zinc in cells are in the nanomolar range (Suhy & O'Halloran 1995). Therefore, a concentration gradient of labile zinc across the plasma membrane may be an important driving force for Zn^{2+} uptake. The negative-inside membrane potential found in cells may also be a driving force for uptake of this cation (Stein 1990; Zhang & Allen 1995).

The CDF family of metal ion transporters

Like the ZIPs, members of the CDF family of proteins are found in organisms at all phylogenetic levels. The name CDF stands for 'cation diffusion facilitator' and is based on the early recognition that these proteins commonly play roles in metal ion transport (Nies & Silver 1995). As discussed in later sections of this review, many members of this family have been implicated specifically in the transport of zinc from the cytoplasm out of the cell or into organellar compartments. The CDF family was recently reviewed by Paulsen and Saier (1997). This article described a family of thirteen members. Thanks to more sequence data and better database analysis tools, that number has now grown to 101 (Figure 4).

Also like the ZIPs, the CDF family can be divided into different sub-groups based on clusters of proteins with greater sequence similarities. Based on this clustering, we have divided the CDF family into three subfamilies, I, II, and III. CDF subfamily I contains mostly prokaryotic members from both eubacterial and archeael sources while subfamilies II and III contain approximately equal representation of eukaryotic and prokaryotic members. The CDF proteins listed in Figure 4 range in size from 199–1677 amino acids with most members in the 300–550 residue range. Again, some of the variability, especially for the smaller sequences, may be due to errors in the conceptual assignment of splice junctions. The member

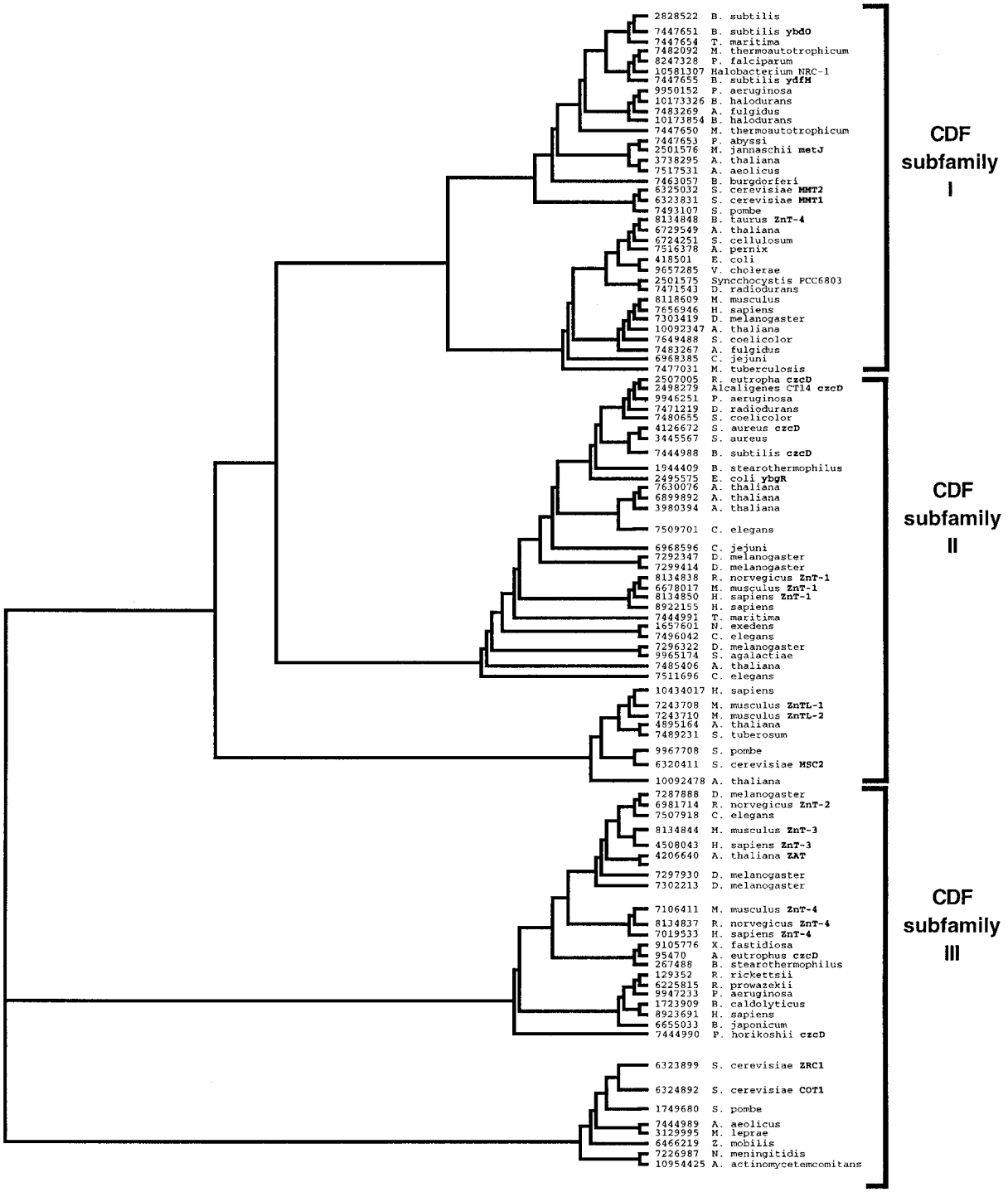

Fig. 4. The CDF family of metal ion transporters. A dendrogram is shown describing the sequence relationships of CDF members identified in the NCBI nonredundant protein database (12/00). Related sequences were identified using a PSI-BLAST-generated Position-Specific Scoring Matrix and the resulting dendrogram is a neighbor-joined tree generated using CLUSTALX (Thompson *et al.* 1997). The corresponding accession numbers, source organism, and gene name (*bold*), if any, are given. The different subfamilies within the CDF family are indicated with *brackets*.

proteins from bacteria average about 300 amino acids while the eukaryotic members are usually larger averaging approximately 450 amino acids. Most members of the CDF family are predicted to have six transmembrane domains. Their predicted membrane topology is similar to that shown for one such protein, Zrc1 from *S. cerevisiae*, depicted in Figure 2B. Notable exceptions to this rule are the Msc2 protein of *S. cerevisiae* and a closely related protein from *Schizosaccharomyces pombe* (accession number 9967708) which are predicted to have 12 transmembrane domains. Comparing the amino acid sequence of these novel proteins to other members of the CDF family suggests that the amino-terminal six transmembrane domains may have been fused onto an archetypical six-domain CDF member some time during their evolution.

Most of the CDF proteins share a similar predicted topology where both the amino and carboxy termini are cytoplasmic (Figure 2B). Some elements of this topology have been confirmed for the czcD protein from *Ralstonia* sp. Strain CH34 (Anton *et al.* 1999). Another feature common among CDF proteins is a long loop region located between transmembrane domains IV and V. This loop frequently contains a histidine-rich motif, $(HX)_n$ where n ranges from 3–6 and X is often S or G, that could function as a potential metal binding domain (Figure 5A). In addition, a second potential metal-binding motif found in the cytoplasmic C-terminal tail has the sequence . . . H-D/E-X-H-X-W-X-L-T$-X_8$-H. . . (Figure 5B). The function of these domains has not been determined but their sequence conservation and potential for metal binding is intriguing.

The greatest degree of conservation among CDF proteins is found within transmembrane domains I, II, and V (Figure 5C, D). These three transmembrane domains are also highly amphipathic suggesting an important role in substrate transport. This hypothesis is supported by the observation that certain polar or charged amino acids within these domains are among the most highly conserved. To date, the importance of these residues to the metal ion transport function of these proteins has not been investigated. The mechanism of transport used by the CDF proteins has not been closely examined. Analysis of a mammalian CDF, the ZnT-1 protein, suggested that its zinc transport activity was not dependent on ATP (Palmiter & Findley 1995). However, given the negative-inside membrane potential of the plasma membrane and the likelihood that this efflux is also occurring against a zinc concentration gradient (see above), secondary active transport by some mechanism seems probable.

Zinc transport and its regulation in yeast

Much of our understanding of zinc transport and its regulation comes from studies of the yeast *S. cerevisiae*. Many elements of the following discussion are summarized in Figure 6. Zinc uptake in *S. cerevisiae* is time-, temperature-, concentration-dependent and saturable (Fuhrmann & Rothstein 1968; Mowll & Gadd 1983; White & Gadd 1987). Kinetic studies of zinc uptake by cells grown with different amounts of zinc in the medium suggested the presence of at least two uptake systems. One system has a high affinity for zinc with an apparent K_m of 1 μM Zn^{2+} and is active in zinc-limited cells (Zhao & Eide 1996a). The second system has a lower affinity for zinc (apparent K_m of 10 μM Zn^{2+}) and its activity is detectable in zinc-replete cells (Zhao & Eide 1996b). These apparent K_m values are overestimates of the true K_m values because they do not consider the chelation properties of the uptake assay media. Equilibrium calculations suggest that the actual K_m values are approximately 10 and 100 nM for the high and low affinity uptake systems, respectively. Both high and low affinity uptake systems are specific for zinc and probably do not contribute to the accumulation of other metals (Zhao & Eide 1996b).

The *ZRT1* and *ZRT2* genes encode the transporter proteins of the high and low affinity systems, respectively (Zhao & Eide 1996a, b). Zrt1 and Zrt2 are closely related to each other sharing 44% amino acid sequence identity and 67% similarity. Both are members of the ZIP family of metal ion transporters. A *zrt1 zrt2* mutant is viable (Zhao & Eide 1996b) suggesting that additional, as yet uncharacterized zinc uptake systems are also present in this yeast. These other systems are unlikely to be major sources of zinc under any but the most zinc-replete conditions given that *zrt1 zrt2* mutant cells require 105-fold more zinc to grow than wild type cells (Zhao & Eide 1996b). Zinc uptake through other metal transporters or channels could contribute to these uncharacterized zinc accumulation mechanisms. Alternatively, zinc uptake in the *zrt1 zrt2* mutant could be occurring by fluid-phase endocytosis and subsequent mobilization of the accumulated zinc from the vacuole (see below).

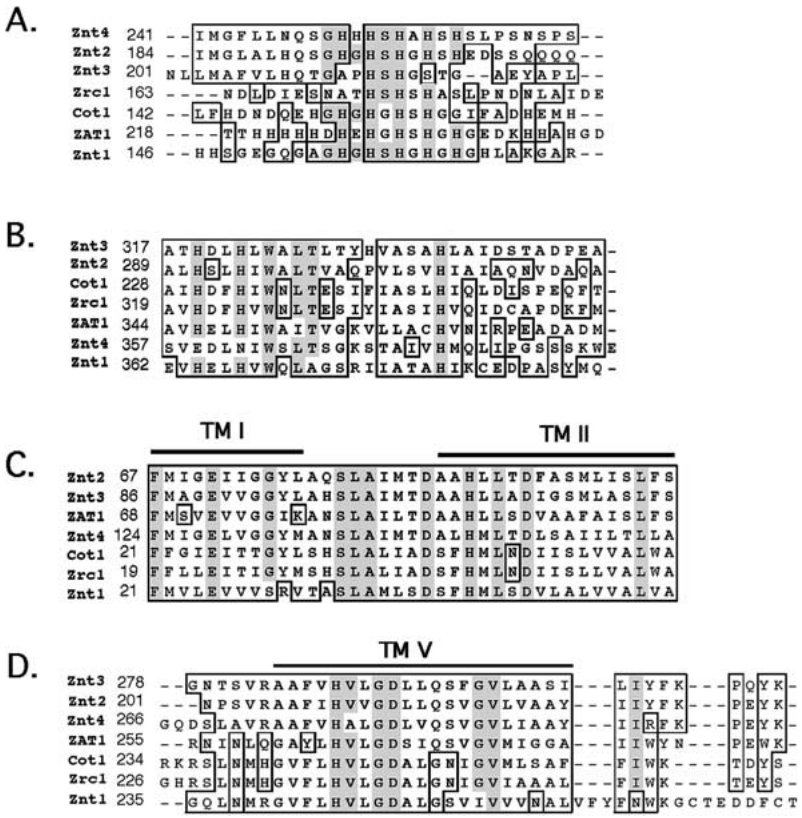

Fig. 5. Sequence conservation in various domains of representative CDF family members. The amino acid sequences of several CDF transporters are shown and the approximate locations of transmembrane domains (TM) are indicated by a *line* over the corresponding sequence. Similar amino acids ($\geq$ 85% of the sequences shown) are *boxed* and residues that are identical in $\geq$ 65% of the sequences are *shaded*. A. Histidine-rich sequences in the predicted cytoplasmic loop between TM IV and V; B. A potential metal binding domain in the carboxy-terminal tail regions; C. Transmembrane domains I and II; D. Transmembrane domain V.

Transcriptional control of zinc uptake in yeast

Zinc uptake in *S. cerevisiae* is controlled at the transcriptional level in response to intracellular zinc levels. The high affinity system is induced more than 30-fold in zinc-limited cells resulting from increased transcription of the *ZRT1* gene (Zhao & Eide 1996a). The low affinity system is also regulated through the control of *ZRT2* transcription (Zhao & Eide 1997). Regulation of these genes in response to zinc is mediated by the product of the *ZAP1* gene (Zhao & Eide 1997). *ZAP1* encodes a transcriptional activator with seven carboxy-terminal C_2H_2 zinc finger domains and two amino terminal activation domains (Figure 7). Zap1 was also found to regulate its own transcription through a positive autoregulatory mechanism. This type of regulatory circuitry would allow for an amplified response to changes in zinc levels and Zap1 activity under progressively zinc-limiting conditions.

Zap1 binds to zinc-responsive elements (ZREs) in the promoters of the *ZRT1, ZRT*2, and *ZAP1* genes (Zhao *et al.* 1998). A ZRE consensus sequence, 5′-ACCYYNAAGGT-3′, was identified and found to be both necessary and sufficient for zinc-responsive transcriptional regulation. Despite each gene having one or more consensus ZRE in their promoters, there is differential zinc responsiveness among the *ZRT1, ZRT*2, and *ZAP1* genes. Significantly more zinc is required to repress Zap1-dependent expression of the *ZRT2* promoter than is required to repress either the *ZRT1* or *ZAP1* promoters. These data suggest that Zap1 activity on the *ZRT2* promoter may be altered by accessory factors (e.g., other transcription factors) that modulate Zap1's response to zinc on this promoter. If zinc controls the affinity of Zap1 for its ZRE binding sites, one possible model is that other proteins bind to the *ZRT2* promoter and help stabilize binding of Zap1 to

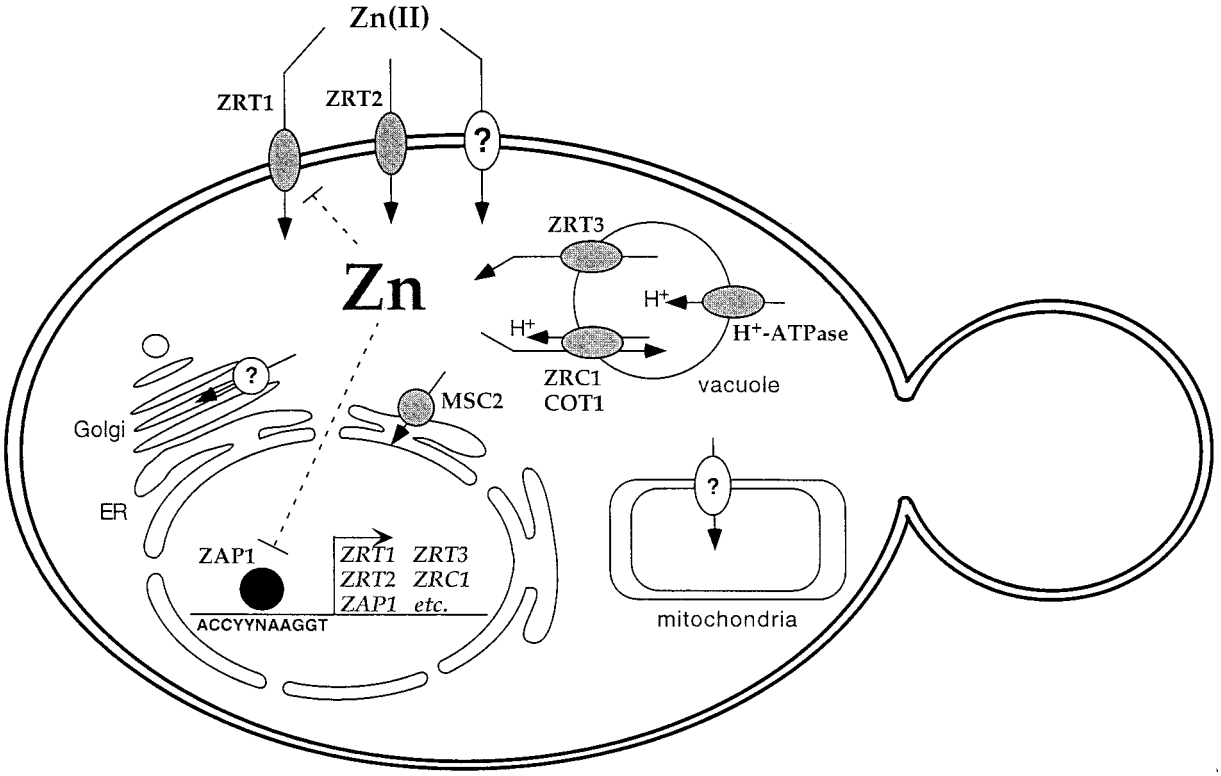

Fig. 6. Model of zinc transport and its regulation in *S. cerevisiae*. Proteins involved in zinc transport are shown in *gray* and the Zap1 regulatory protein is depicted in *black*. *Solid arrows* indicate transport steps and *dashed lines* indicate regulatory interactions. Transporters that are anticipated to exist but have not been identified are indicated by the *question marks*.

the ZREs, thus increasing the affinity of Zap1 for these sites.

The differential sensitivity of the *ZRT1, ZRT2*, and *ZAP1* promoters to zinc is also consistent with the different functions of these proteins and suggests the following scenario: basal (i.e., Zap1-independent) expression of the Zrt2 low affinity transporter is sufficient to supply zinc to cells under zinc-replete conditions (Zhao & Eide 1997). As cells enter the initial phases of zinc limitation, their first response is to increase the activity of the Zrt2 transporter. As zinc limitation becomes more severe, the Zrt1 transporter is induced to provide high affinity uptake activity for zinc acquisition. Finally, increased expression of the *ZAP1* gene, allowing for maximum expression of its target genes, would only be needed under conditions of extreme zinc-limitation.

Another intriguing question that remains to be answered is precisely how zinc regulates Zap1 activity. Recent studies that dissect the functional domains of Zap1 have assisted greatly in answering this question. Two activation domains, designated AD I and AD II, were mapped within the protein and both function *in vivo* (Bird *et al.* 2000b) (Figure 7). The complete DNA binding domain was mapped to the carboxy-terminal five zinc fingers (Bird *et al.* 2000a; Zhao *et al.* 1998). Mutations that disrupt the formation of each of these fingers were tested for their ability to complement a *zap1* mutant *in vivo* and for DNA binding affinity *in vitro* (Bird *et al.* 2000a). Each finger was found to be required for high affinity DNA binding. Consistent with this hypothesis, purified Zap1 protein was found to have a stoichiometry of five zinc atoms per monomer of protein (Bird *et al.* 2000a). Mutation of zinc fingers 1 and 2 in AD II had no effect on this stoichiometry suggesting these fingers bind zinc with low affinity. The function of fingers 1 and 2 remains unclear.

The portion of the Zap1 protein required for zinc responsiveness co-localizes to the DNA binding domain and the five C-terminal zinc fingers (Bird *et al.* 2000b). For example, fusion of the Zap1 DNA binding domain (amino acids 687–880) to the Gal4 activation domain resulted in a functional protein *in vivo*.

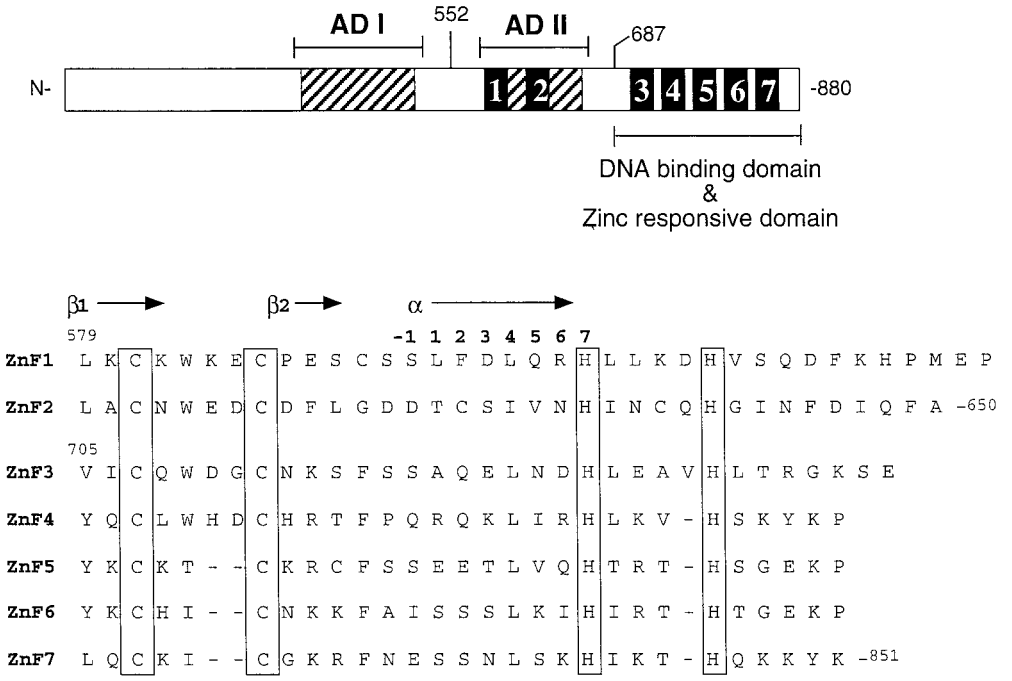

Fig. 7. Potential domain structure of Zap1. The Zap1 activation domains AD I and AD II (*hatched boxes*) and zinc fingers (numbered 1–7, *filled boxes*) are shown. The location of the DNA binding and zinc-responsive domains are also indicated and the sequences of the Zap1 zinc fingers are shown below. The conserved Zn^{2+} ligands are *boxed* and the locations of the presumed $\beta 1$, $\beta 2$ and α-helix structures are indicated. The amino acids relative to the start site of the α-helix are *numbered* -1 to 7.

This fusion was zinc regulated to a similar degree as the wild type Zap1 protein. The co-localization of the DNA binding domain and zinc-responsive domain suggested that DNA binding activity of Zap1 may be controlled by zinc binding to this region of the protein. However, the ability of Zap1 to confer zinc-responsive gene expression on a heterologous DNA binding domain, i.e. the DNA binding domain from the Gal4 activator (Bird *et al.* 2000b), suggested that zinc impairs activation domain function. While others are possible, we propose the following models of how Zap1 activity is regulated by zinc. First, we hypothesize that Zap1 is the direct zinc sensor and contains one or more low affinity regulatory zinc binding sites in the DNA binding domain in addition to the five high affinity C_2H_2 zinc fingers. Binding of zinc to these regulatory sites may stabilize a conformation (e.g. a multimeric complex) that sterically impairs both the DNA binding interface and the accessibility of the activation domain to general transcription factors. Alternatively, binding of zinc to the regulatory sites might recruit another protein that represses Zap1 function. In this model, either Zap1 or the accessory protein may contain the regulatory zinc binding site(s). Future studies will determine which, if any, of these models is correct.

Post-translational control of zinc uptake in yeast

A second mechanism in *S. cerevisiae* regulates zinc transporter activity at a post-translational level. In zinc-limited cells, Zrt1 is a stable plasma membrane protein. Exposure to high levels of extracellular zinc triggers a rapid loss of Zrt1 uptake activity and protein. This inactivation occurs through zinc-induced endocytosis of the protein and its subsequent degradation in the vacuole (Gitan *et al.* 1998). Our molecular understanding of these events are summarized in Figure 8. Mutations that inhibit the internalization step of endocytosis also inhibited zinc-induced Zrt1 inactivation and the major vacuolar proteases were required to degrade Zrt1 in response to zinc. Furthermore, immunofluorescence microscopy showed that Zrt1 is in the plasma membrane of zinc-limited cells and is transferred to the vacuole via an endosome-like compartment upon exposure to zinc. Zrt1 inactivation is a relatively specific response to zinc; Cd^{2+} and Co^{2+} trigger the response but less effectively than zinc. Excess zinc does not alter the stability of several other

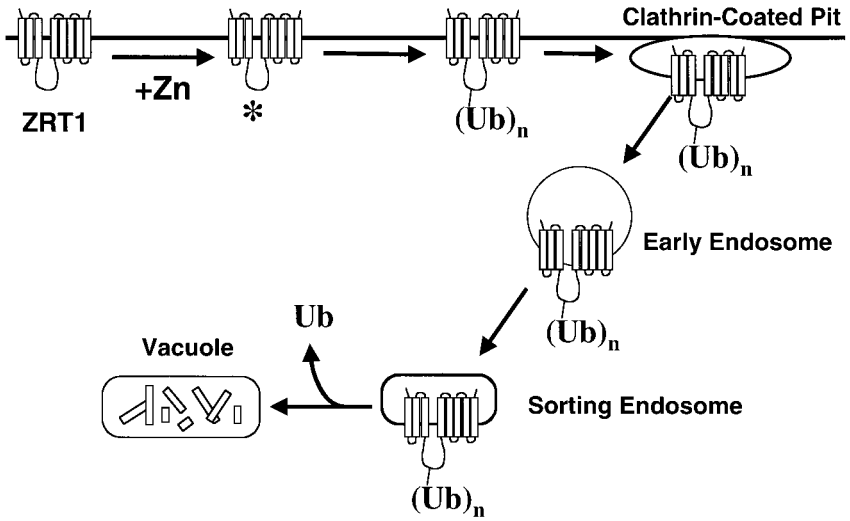

Fig. 8. A model of zinc-responsive post-translational inactivation of Zrt1. High zinc causes the ubiquitination of Zrt1 which results in the protein's migration into clathrin-coated pits and subsequent endocytosis. Zrt1 then passes through the endocytic pathway to the vacuole where it is degraded by vacuolar proteases. The model proposes that zinc alters the conformation of Zrt1 (indicated by the *asterisk*) to make it a better substrate for ubiquitination.

plasma membrane proteins. Therefore, zinc-induced Zrt1 inactivation is a specific regulatory mechanism to shut off zinc uptake activity in cells exposed to high extracellular zinc levels. This system thereby prevents the overaccumulation of this potentially toxic metal.

The mechanism of zinc-induced endocytosis raises a number of exciting new questions. First, while it is clear that zinc induces endocytosis of Zrt1, it is unknown if this response is induced by a mechanism that senses intracellular or extracellular metal ion levels. Second, it is unclear if the signal being monitored is Zn^{2+} ions *per se*, the activity of a zinc-dependent or zinc-inhibited enzyme, or a more indirect consequence of high metal accumulation. The observation that Co^{2+} and Cd^{2+} also induce endocytosis of Zrt1 (Gitan *et al.* 1998) is potentially instructive. Both Co^{2+} and Cd^{2+} have similar coordination chemistries to Zn^{2+} and will bind to protein ligands in a similar fashion. Therefore, the simplest hypothesis is that Zn^{2+} ions trigger endocytosis directly and that Co^{2+} and Cd^{2+} mimic that signal. The lower activity of Co^{2+} and Cd^{2+} in triggering the response may be due to a greater specificity of the sensing mechanism for Zn^{2+} or different uptake efficiencies for different metal ions. A third unanswered question is how the zinc signal is transmitted to Zrt1. This could occur through the metal binding directly to the transporter or through an indirect signal transduction pathway. Recent studies demonstrate that Zrt1 is ubiquitinated prior to endocytosis suggesting that this modification serves as a signal for recruitment of the protein into clathrin-coated pits (Gitan & Eide 2000). A similar role for ubiquitin has also been found for other *S. cerevisiae* and some mammalian plasma membrane proteins (Hicke 1997). Zinc-induced ubiquitination of Zrt1 occurs on a lysine residue (K195) located in the variable region of the protein (Figure 2A). Therefore, the principle question is currently how does zinc control ubiquitination of Zrt1?

The post-translational regulatory system is clearly separate from the transcriptional control system given that inactivation of Zrt1 activity occurs normally in a *zap1* deletion mutant (Gitan *et al.* 1998). However, these two systems undoubtedly work together to maintain the homeostatic control of intracellular zinc levels. It is interesting to note that the transcriptional control system exerts its greatest effect on ZRT1 expression when cell-associated zinc levels vary between 0.01 and 0.07 nmol Zn/10^6 cells (i.e., $\sim$0.5 - 4 $\times$ 10^7 atoms zinc/cell) (Gitan *et al.* 1998). Approximately 90% repression of a *ZRT1* promoter-*lacZ* fusion was observed when cell-associated zinc levels rose to 0.07 nmol/10^6 cells (Zhao *et al.* 1998). In contrast, the post-translational response is triggered only at cell-associated zinc levels of greater than 0.07 nmol Zn/10^6 cells. Thus, we envision a two-tiered regulatory system in which the transcriptional control can respond to moderate changes in zinc avail-

ability and the post-translational control responds to more extreme zinc excess. A likely scenario in which the post-translational control would be important for maintaining zinc homeostasis is when zinc-limited cells are suddenly exposed to high levels of zinc. The rapid down-regulation of zinc uptake by Zrt1 endocytosis helps to prevent overaccumulation of zinc. This fast response would not be possible solely through the transcriptional control of a stable plasma membrane protein. During inactivation of zinc uptake activity, other systems may be induced to facilitate storage of the excess zinc or mediate its efflux from the cell.

Intracellular zinc transport in yeast

Once zinc is taken up across the plasma membrane, some of the metal must be transported into organelles such as the mitochondria and compartments of the secretory system to serve as a cofactor of zinc-dependent proteins found within those compartments. Furthermore, the vacuole has been implicated in the storage and detoxification of zinc (Ramsay & Gadd 1997). Very little is known about the specific transporters involved in intracellular zinc trafficking. Three potential intracellular zinc transporters have been identified in *S. cerevisiae*. These transporters are three members of the CDF family, Zrc1, Cot1, and Msc2. *ZRC1* was isolated as a determinant of zinc resistance, i.e. overexpression of *ZRC1* results in increased ability of these cells to tolerate high zinc levels (Kamizono *et al.* 1989). A *zrc1* mutation was later found to increase sensitivity to lipid hydroperoxides and decrease glutathione levels by approximately 40% (Kobayashi *et al.* 1996). The relationship between these phenotypes and zinc, if any, is unknown. The *COT1* gene was isolated in a similar fashion to *ZRC1*, i.e., as a suppressor of cobalt toxicity, but was later found to confer zinc resistance as well (Conklin *et al.* 1994; Conklin *et al.* 1992). Disruption of either *ZRC1* or *COT1* resulted in greater sensitivity to excess zinc further supporting the role of these genes in zinc compartmentalization.

Zrc1 and Cot1 are closely related proteins (60% identity) with approximately 400 amino acids and 6 potential transmembrane domains. The physiological roles of these transporters remain unclear. Neither *ZRC1* nor *COT1* are essential genes, and a *zrc1 cot1* mutant is also viable. Thus, these two genes do not together provide a function essential for growth. Neither protein appears to catalyze zinc efflux from the cell. While Cot1 was originally proposed to be a mitochondrial protein (Conklin *et al.* 1992), the subcellular location of both Zrc1 and Cot1 has recently been identified as the vacuole (Li & Kaplan 1998). This localization was determined with overexpressed proteins and so must be viewed with caution. However, with this caveat aside, these results suggest that these transporters are responsible for zinc sequestration into the vacuole (Li & Kaplan 1998). Because zinc transport into the vacuole has been attributed to a Zn^{2+}/H^{+} antiport system (Bode *et al.* 1995; Nishimura *et al.* 1998), this leads to the conclusion that this is the transport mechanism used by Zrc1 and Cot1. This mechanism would then provide a simple explanation for the zinc sensitivity observed in vacuolar H^{+}-ATPase mutants (Eide *et al.* 1993; Ramsay & Gadd 1997); i.e., mutants defective for vacuolar acidification lack the H^{+} gradient necessary to drive zinc sequestration.

More recently, we have identified a gene in yeast, *ZRT3*, that also plays a role in vacuolar zinc transport (MacDiarmid *et al.* 2000). Although distantly related to Zrt1 and Zrt2, Zrt3 is a potential transport protein that is a member of the ZIP family. Like the *ZRT1* and *ZRT2* genes, *ZRT3* is a ZAP1 target gene and is upregulated in zinc-limited cells. Our analysis of Zrc1, Cot1, and Zrt3 has generated the following scenario of zinc storage in yeast. Zinc-replete wild type cells generate a vacuolar zinc storage pool through the action of the Zrc1 and Cot1 transporters. This pool of stored zinc is in a labile form that can be mobilized when cells are deprived of extracellular zinc. Mobilization of the vacuolar store is the role of Zrt3 whose expression is induced under zinc-limiting conditions. Several aspects of this model have already been confirmed (MacDiarmid *et al.* 2000).

Finally, a third member of the CDF family has recently been implicated in zinc transport in *S. cerevisiae*. This transporter is encoded by the *MSC2* gene. While Msc2 is a member of the CDF family, it differs from most other members by having twelve rather than six transmembrane domains and two rather than one histidine-rich region. Paradoxically, *MSC2* was first identified by a transposon insertion allele that caused an increased frequency of meiotic sister chromatid (MSC) recombination events (Thompson & Stahl 1999). This effect was found to be allele-specific and did not occur when the *MSC2* gene was deleted. The connection between *MSC2* and recombination is still a mystery but a subsequent analysis has suggested a role of Msc2 in zinc transport (Li & Kaplan 2000). An *msc2* deletion mutation caused decreased viability

on respired carbon sources and an abnormal cellular morphology when cells were grown at an elevated temperature. Both of these phenotypes were suppressible by zinc supplementation suggesting some defect in zinc metabolism in this strain. The *msc2* mutant also had alterations in intracellular zinc content and an apparent increase in the regulatory zinc pool sensed by Zap1. The Msc2 protein was localized to the nuclear envelope. An attractive hypothesis is that Msc2 mediates zinc transport into the intermembrane space of this compartment. This intriguing model awaits further testing.

Zinc transport and its regulation in plants

Our understanding of zinc transport and its regulation in plants is increasing rapidly with the identification of both ZIP and CDF family genes in many plant species. The number of ZIP family members in plants is remarkable. The *Arabidopsis* genome alone contains 18 such genes representing members from three of the four subclasses of ZIP proteins; only ZIP subfamily II does not contain any plant members. The high number of potential metal ion transport proteins in plants and animals (see below) no doubt stems from the greater diversity of tissue-specific roles to be played by these proteins in multicellular organisms. Irt1 (Iron-regulated transporter 1) was the first ZIP protein to be identified in any organism (Eide *et al.* 1996). The *IRT1* gene was cloned because its expression in a yeast mutant defective for iron uptake suppressed the growth defect of this strain in low iron media. Biochemical studies confirmed that Irt1 expression increased iron uptake in this yeast strain (Eide *et al.* 1996) and later studies demonstrated that Irt1 can also transport Zn^{2+}, Mn^{2+}, and Cd^{2+} (Korshunova *et al.* 1999). The function of Irt1 in plants, however, appears to be largely iron accumulation. Irt1 is expressed solely in roots and only in roots of iron-limited plants. Therefore, if Irt1 participates in the accumulation of metals other than iron, e.g., zinc, it is likely to do so only under iron-limiting conditions. This prediction is consistent with the observation that iron-limited plants accumulate higher levels of other metal ions such as zinc, manganese, and cadmium (Cohen *et al.* 1998; Welch *et al.* 1993).

Four other *Arabidopsis* ZIP transporters, Zip1-4, may play roles in zinc transport (Grotz *et al.* 1998). Expressing Zip1, Zip2, or Zip3 in *S. cerevisiae* confers increased zinc uptake each with distinct biochemical properties. These results indicate that these proteins are zinc transporters. Zip4 expressed in yeast failed to increase zinc uptake perhaps due to poor expression or mislocalization of the protein in the yeast cell. *ZIP1* is expressed predominantly in roots while *ZIP3* and *ZIP4* mRNA could be detected in both roots and shoots. Furthermore, *ZIP1, ZIP3*, and *ZIP4* mRNA are induced under zinc-limiting conditions. These results further suggest a role for these proteins in zinc transport. The tissue-specific expression and subcellular localization of these proteins is not known so their precise roles can not yet be assessed. The zinc-responsive regulation of mRNA levels in response to zinc availability demonstrates that some mechanism of regulation exists in plants. This regulation may occur through a transcriptional control mechanism, like that found in yeast, or alternatively by a mechanism that regulates mRNA stability.

Incorrect regulation of zinc transporter expression would likely have a great impact on zinc accumulation in plants. This premise may in part explain the physiology of a unusual group of plants, the metal ion hyperaccumulators. Metal ion hyperaccumulators are plants that take up large quantities of metal ions from the soil. They are of great research interest because of their potential to remove metal pollutants from surface soils in a process called phytoremediation (Raskin 1995). Among the best known hyperaccumulators is *Thlaspi caerulescens*, a member of the Brassicaceae family that also includes *Arabidopsis*. Certain ecotypes of *T. caerulescens* are capable of accumulating up to 30,000 ppm Zn in their shoots without apparent toxic consequences (Brown *et al.* 1995). By comparison, non-hyperaccumulators normally accumulate only 0.1% of that level. Thus, hyperaccumulators must have remarkable ability to accumulate and detoxify metal ions. Biochemical analysis of Zn^{2+} uptake by *T. caerulescens* found that the V_{max} was elevated almost 5-fold compared to a non-hyperaccumulating ecotype, *T. arvense*, with no difference in K_m (Lasat *et al.* 2000). These results suggested that expression of zinc uptake transporters is higher in *T. caerulescens*. A ZIP family member was recently cloned from *T. caerulescens* and *T. arvense* and called *ZNT1* (Pence *et al.* 2000). Znt1 is expressed at a low level and regulated by zinc status in *T. arvense*. In striking contrast, this gene is expressed at a much higher level in *T. caerulescens* and is unaffected by zinc availability. Znt1 expression can explain the increased zinc accumulation in this and perhaps other metal hyperaccumulating plant species.

The genomes of plant species also contain many members of the CDF family; *Arabidopsis* alone encodes ten CDF member genes. These proteins are likely to function in subcellular zinc compartmentalization, as was the case for Zrc1 and Cot1 in yeast, as well as in zinc efflux. To date, only one plant CDF member has been studied, the Zat protein of *Arabidopsis* (van der Zaal *et al.* 1999). *ZAT* mRNA expression is not zinc regulated but the protein does appear to be a zinc transporter. Transgenic plants overexpressing the *ZAT* gene show increased zinc resistance. Zn content in roots of these transgenic plants was also found to increase suggesting that Zat transports zinc into an intracellular compartment, e.g., the vacuole, of root cells. As is the case with any multicellular organism, zinc transporters are required for both cellular zinc uptake as well as efflux to allow the utilization of the metal. In plants, for example, a zinc efflux transporter is required to pass zinc from the root tissue into the xylem for distribution to aerial portions of the plant. CDF proteins such as Zat probably perform this function as well.

Zinc transporters and their regulation in mammals

Several zinc transporters from both the ZIP and CDF families are found in mammalian organisms and many of these have been implicated in zinc transport. To date, twelve ZIP genes have been identified in humans and three have been found in the mouse (Figure 1). Functional data are only available for two of the human genes, *hZIP1* and *hZIP2*, and none of the mouse genes have been characterized. hZip1 and hZip2 appear to play roles in zinc uptake across the plasma membrane. *hZIP2* mRNA expression has only been detected in prostate and uterine tissue indicating restricted tissue-specificity. Functional assays indicated that the hZip2 protein is a functional zinc transporter (Gaither & Eide 2000). When *hZIP2* was overexpressed in K562 erythroleukemia cells grown in culture, these cells accumulated more zinc than control cells due to an increased zinc uptake activity. Moreover, hZip2 protein was localized to the plasma membrane of these cells. These results indicated that *hZIP2* may serve in zinc uptake in the few tissues where it is expressed.

Zinc uptake mediated by hZip2 was biochemically distinct from the endogenous activity of the K562 cell line in a number of respects. For example, zinc uptake mediated by hZip2 was stimulated by HCO_3^- treatment whereas the endogenous system was not affected. Furthermore, several other metal ions [e.g., Co^{2+}, Fe^{2+}, and Mn^{2+}] severely inhibited zinc uptake by hZip2 but the endogenous activity was far less sensitive. We have recently determined that another ZIP transporter, hZip1, is the endogenous zinc uptake system in K562 cells (Gaither & Eide 2001). First, K562 cells express *hZIP1* mRNA and the functional hZip1 protein is localized to the plasma membrane of these cells. Second, overexpression of *hZIP1* mRNA by approximately 2-fold increased zinc uptake activity by 2-fold as well. This increased uptake activity in hZip1 overexpressing cells was biochemically indistinguishable from the endogenous system. Finally, antisense oligonucleotides targeted to inhibit *hZIP1* expression also inhibited the endogenous zinc uptake activity. These results strongly suggest that hZip1 is the endogenous transporter in K562 cells. The antisense *hZIP1* oligonucleotide treatment reduced zinc uptake to 10–20% of control levels suggesting that hZip1 is the major pathway of zinc uptake in these cells.

In marked contrast to the *hZIP2* gene, *hZIP1* is expressed in a wide variety of different cell types. Thus, our results suggest that hZip1 may be the primary component of zinc uptake in many human tissues. This conclusion was supported by a recent study in which a correlation was found between *hZIP1* expression levels and zinc uptake in human malignant cell lines derived from the prostate. Prostate cell lines LNCap and PC-3 possess high levels of zinc uptake activity that is stimulated by prolactin and testosterone. Costello *et al.* (1999) found that *hZIP1* is expressed in LNCap and PC-3 cells and this expression is increased by prolactin and testosterone treatment. Expression of hZIP1 was also repressed by adding zinc to the medium suggesting some regulation of zinc uptake occurs in response to cellular zinc status. A closely related ortholog of hZip1 from the mouse was recently reported (Lioumi *et al.* 1999). This protein was named Zirtl for 'zinc-iron regulated transporter-like' protein. Like *hZIP1*, the *ZIRTL* gene is expressed in a wide variety of tissues. Zirtl fused to GFP was reported to localize to intracellular organelles. However, these studies were preformed without confirmation that the tagged protein retained wild type function. Therefore, it is possible that localization of the GFP-tagged Zirtl protein does not reflect the normal location of the native protein.

One paradox that arises from our studies of hZip1 and hZip2 is that these transporters have a surprisingly low affinity for their substrate. Both transporters have K_m values of approximately 3 μM for free Zn^{2+} ion. Similar K_m values have been reported for zinc transporters in a large number of mammalian cell types (Reyes 1996). The paradox arises when we consider the free Zn^{2+} concentration in mammalian serum. While the total zinc concentration of serum is approximately 20 μM, very little metal is present in an unbound form (Magneson *et al.* 1987). In serum, $\sim$75% Zn^{2+} is bound to albumin and 20% is bound to α2-macroglobulin. Much of the remaining zinc is complexed with amino acids such as histidine and cysteine. Because of the high chelation capacity of serum, the free Zn^{2+} concentration in serum is calculated to be in the low nM range. Given this extremely low concentration of substrate, it was initially unclear how these transporters could contribute to zinc accumulation by mammalian cells under physiological conditions. The solution to this paradox comes from considering the capacity of these transporters relative to the zinc requirements of the cell. Our studies demonstrated that the capacity (i.e., V_{max}) for uptake is so high relative to the cellular demand for zinc that sufficient levels can be obtained despite the apparent low affinity of the transporters.

The potential role of the DCT1/DMT1/Nramp2 Fe^{2+} transporter in zinc uptake should be included in this discussion. DCT1/DMT1/Nramp2 is a member of the Nramp family of transporters and is unrelated to either ZIP or CDF proteins. Gunshin *et al.* (1997) provided evidence that DCT1/DMT1/Nramp2 was capable of Zn^{2+} uptake; *Xenopus* oocytes expressing DCT1/DMT1/Nramp2 displayed cation influx currents indicative of Zn^{2+} movement across the membrane. However, more recent results have indicated that the currents recorded in these oocytes result from Zn^{2+}-induced proton fluxes rather than transport of the metal ion (Sacher *et al.* 2001).

Several members of the CDF family have been implicated in zinc transport processes in mammals. The isolation and characterization of these transporters has been extensively reviewed by McMahon & Cousins (1998a) and will be considered only briefly here. Mammalian CDF family members are involved in the efflux of zinc from the cell or the transport of zinc into intracellular organelles. The analysis in Figure 4 lists seven CDF genes in humans, six in the mouse genome plus a small number of others from the rat and other mammals. Four of the mammalian genes, *ZnT-1, ZnT-2, ZnT-3*, and *ZnT-4*, have been functionally characterized to some extent so that their roles in zinc metabolism seem clear. ZnT-1 is a zinc efflux transporter in the plasma membrane of mammalian cells (Palmiter & Findley 1995). Given this localization, ZnT-1 may play a role in the cellular detoxification of zinc by exporting unneeded metal ion out of the cell. This role is consistent with the observation that cells that overexpress this transporter show higher zinc resistance than control cells. ZnT-1 may also play a role in the dietary absorption of zinc in the intestine and the recovery of zinc from urine in the renal tubules of the kidney. In the intestine, ZnT-1 is expressed in the enterocytes of the duodenum and the jejunem, i.e. the primary sites of zinc absorption (McMahon & Cousins 1998b). ZnT-1 protein is found localized to the basolateral membrane of enterocytes suggesting a role in transporting zinc out of the enterocyte and into the blood stream. ZnT-1 is also found on the basolateral surface of renal tubule cells (McMahon & Cousins 1998a), a position that would be expected of a protein involved in transporting zinc absorbed from urine back into the circulation. It has been well established that the loss of zinc in urine is very low due to efficient renal reabsorption (Victery *et al.* 1981).

ZnT-2 may play a role in intracellular zinc sequestration and storage similar to that proposed for Zrc1 and Cot1 in yeast. This protein is located in the membrane of an acidic endosomal/lysosomal compartment that accumulates zinc when cells are grown under high zinc conditions (Palmiter *et al.* 1996a). This compartment has been recently identified as the late endosome (Kobayashi *et al.* 1999). ZnT-3 also transports zinc into an intracellular compartment where the metal may play a role in neuronal signaling. *ZnT-3* mRNA has been detected only in the brain and testis and is most abundant in the neurons of the hippocampus and the cerebral cortex (Palmiter *et al.* 1996b). ZnT-3 protein is localized to membranes of synaptic vesicles in these neurons, suggesting that the protein transports zinc into this compartment. Consistent with this hypothesis, a subset of glutamatergic neurons contain histochemically reactive zinc in their synaptic vesicles. The localization of ZnT-3 protein was coincident with these zinc-containing vesicles (Palmiter *et al.* 1996b). Moreover, a *ZnT-3* null mouse line generated by targeted gene disruption failed to accumulate zinc in these vesicles (Cole *et al.* 1999). Thus, ZnT-3 is required for transport of zinc into synaptic vesicles in some types of neurons where it may play a neuromodulatory role (Fredrickson *et al.* 2000).

The fourth characterized mammalian CDF protein is ZnT-4. ZnT-4 is expressed in the mammary gland and is responsible for zinc transport into milk (Huang & Gitschier 1997). In fact, mutations in the *ZnT-4* gene are responsible for the *lethal milk* (*lm*) mutant mouse. The *lm* gene is so-named because pups of any genotype suckled on *lm/lm* dams die before weaning. Death is due to zinc deficiency from an insufficient supply of zinc in the milk (Piletz & Ganschow 1978). ZnT-4 has also been found to be expressed in the intestinal enterocytes where it is localized in endosomal vesicles concentrated at the basolateral membrane (Murgia *et al.* 1999). Thus, like ZnT-1, the function of ZnT-4 in the intestine may be to facilitate transport of zinc into the portal blood.

Our knowledge of how mammalian zinc transporters are regulated is still rudimentary. One point that is becoming increasingly clear is that zinc efflux in many cell types is regulated by zinc. One of the first indications of this effect came from studies of transient forebrain ischemia in gerbils (Tsuda *et al.* 1997). Differential display analysis demonstrated that ZnT-1 mRNA is up-regulated during ischemia, a condition which is known to cause zinc influx into neurons (Koh *et al.* 1996). Consistent with the *ZnT-1* regulation being the result of zinc influx, cultured neurons transiently increased *ZnT-1* mRNA when exposed to zinc. Thus, transcriptional control of *ZnT-1* may contribute to zinc detoxification by promoting efflux. *ZnT-1* is expressed in many cell types so this may be a general mechanism of cellular zinc homeostasis. The transcriptional control of *ZnT-1* may also play a role in zinc absorption. *ZnT-1* mRNA levels were increased in enterocytes following an oral dose of zinc (McMahon & Cousins 1998b). Given the location of the ZnT-1 protein on the basolateral membrane of these cells, a likely hypothesis is that up-regulation of ZnT-1 promotes zinc absorption by facilitating transport into the portal blood. A recent study by Andrews and colleagues (Langmade *et al.* 2000) has shown that increased expression of *ZnT-1* by zinc is mediated by the zinc-responsive MTF-1 transcription factor. This intriguing protein is considered elsewhere in this issue (Andrews, this issue). Cousins and coworkers (Liuzzi, *et al.* 2001) have shown that *ZnT-2* mRNA is up-regulated in response to increased dietary zinc levels suggesting that this gene is also a target of MTF-1 regulation.

Regulation of zinc uptake transporters in mammals is far less well understood. Some biochemical evidence suggests that regulation of these transporters in response to zinc status does occur. For example, zinc uptake in brush border membrane vesicles was found to increase in zinc-deficient rats (Menard & Cousins 1983). Similarly, cultured endothelial cells grown under low zinc conditions had a higher rate of zinc uptake than zinc-replete cells (McClung & Bobilya 1999). These data suggest that zinc deficiency can increase the expression or activity of zinc uptake transporters in some cell types. The mechanism of this regulation is unknown but a potential clue comes from the study of *hZIP1* expression in cultured malignant prostate cell (Costello *et al.* 1999). *hZIP1* mRNA levels were reduced in zinc-treated cells suggesting that a transcriptional control mechanism similar to that described in yeast may be present in mammalian cells. Should it exist, such a mechanism would play a critical role in mammalian zinc homeostasis.

Concluding remarks

Research over the past ten years has produced major advances in our knowledge of zinc transporters and their regulation in eukaryotic organisms. The identification of the ZIP and CDF families of metal ion transporters represents a major step forward. Study of these transporters has identified their various roles in zinc uptake, efflux, compartmentalization, storage, and detoxification. Moreover, the regulatory mechanisms that control the activity of these transporters in response to zinc status are becoming increasingly clear. These include both transcriptional and post-transcriptional mechanisms of regulation and they play important roles in zinc homeostasis and metabolism. Despite this progress, however, we are still very far from a complete picture of these processes in any organism. This is even true of yeast where the current model is the most complete among eukaryotes. Perhaps the greatest challenge that lies ahead will be determining the different roles of the ZIP and CDF family members found in plants and animals. The high number of such proteins in mice, for example, suggests that they play diverse functions in metal ion transport. Functional analyses of these transporters will identify their substrates and determine their biochemical mechanisms of action. Localization studies will determine the tissue- and cell-specific expression patterns of these proteins and define their subcellular locations. These data will tell us much about the potential roles these proteins play in zinc transport. Genetic studies, e.g., targeted gene disruption in mice,

will assist in determining transporter function through the phenotypic analysis of the resulting mutants. Finally, the mechanisms that regulate the activity of these transporters need to be analyzed to place these proteins into the context of cellular and organismal zinc physiology and homeostasis. The mechanisms of zinc sensing used in these regulatory circuits will be an exciting new area of research. Clearly, the next ten years of research into zinc transporters and their regulation promises to be as exciting as the last decade.

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