Functional Expression of the Human hZIP2 Zinc Transporter*

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Zinc is an essential nutrient for humans, yet we know little about how this metal ion is taken up by mammalian cells. In this report, we describe the characterization of hZip2, a human zinc transporter identified by its similarity to zinc transporters recently characterized in fungi and plants. hZip2 is a member of the ZIP family of eukaryotic metal ion transporters that includes two other human genes, hZIP1 and hZIP3, and genes in mice and rats. To test whether hZip2 is a zinc transporter, we examined 65Zn uptake activity in transfected K562 erythroleukemia cells expressing hZip2 from the CMV promoter. hZip2-expressing cells accumulated more zinc than control cells because of an increased initial zinc uptake rate. This activity was time-, temperature-, and concentration-dependent and saturable with an apparent Km of 3 μM. hZip2 zinc uptake activity was inhibited by several other transition metals, suggesting that this protein may transport other substrates as well. hZip2 activity was not energy-dependent, nor did it require K+ or Na+ gradients. Zinc uptake by hZip2 was stimulated by HCO3− treatment, suggesting a Zn2+-HCO3− cotransport mechanism. Finally, hZip2 was exclusively localized in the plasma membrane. These results indicate that hZip2 is a zinc transporter, and its identification provides one of the first molecular tools to study zinc uptake in mammalian cells.

Zinc is an important nutrient because it is a critical component of numerous metalloenzymes and zinc-dependent transcription factors (1). Zinc is also implicated as a neuromodulator of certain postsynaptic neurons in the brain (2). Because of these important functions, nutritional zinc deficiency can have devastating consequences to human health including growth retardation, immune system dysfunction, and mental disorders. Although severe zinc deficiency is rare, mild deficits may compromise the health of a large portion of the world’s population (3). Genetic factors also influence zinc status in humans. Acute zinc deficiency is observed in individuals with the genetic disorder acrodermatitis enteropathica (AE) (4). AE decreases zinc uptake in the small intestine (5) and other cells of the body (6). In mammals, zinc is absorbed from the diet through the brush border membrane of the epithelial cells lining the small intestine. After absorption, zinc is transported across the basolateral membrane of the epithelial cells into the portal blood where most of the metal ion is loosely bound by albumin. Zinc uptake by mammalian cells has been studied in a variety of tissues and cell types (for review see Ref. 7). These studies have clearly demonstrated that zinc uptake is mediated by transporters located in the plasma membrane of these cells. The specific transporters involved, their biochemical mechanisms of action, their regulation in response to zinc availability, and the specific form of zinc (e.g. Zn2+, zinc-histidine complexes, etc.) they transport remain unresolved.

Molecular insight into zinc uptake in other eukaryotes comes from recent studies of zinc transporters in fungi and plants. The ZRT1 and ZRT2 genes of Saccharomyces cerevisiae encode high and low affinity zinc transporters, respectively (8, 9). Zrt1 and Zrt2 are both similar to the Irt1 transporter of Arabidopsis thaliana (10). Although originally identified as an Fe2+ transporter, Irt1 can transport Zn2+, Mn2+, and Cd2+ as well (11). Four additional and related Arabidopsis proteins, Zip1, Zip2, Zip3, and Zip4, are implicated in zinc transport (12). These proteins are members of a transporter superfamily found in a diverse array of eukaryotic organisms. This family, referred to collectively as the ZIP (ZRT1, IRT1-like protein) family for ZRT, IRT-like protein (13), now contains more than 30 members including 16 in plants (13 in Arabidopsis alone) and 8 in nematodes.

The results obtained from studies of fungal and plant ZIP transporters suggest a remarkably conserved role for the ZIP family in zinc uptake. In this report, we embark on a characterization of the mammalian members of this transporter family with an analysis of a human ZIP protein that we have designated hZip2. Our results indicate that hZip2 is indeed a zinc transporter. This work opens the door to further molecular analysis of zinc uptake in humans and other mammalian species.

EXPERIMENTAL PROCEDURES

DNA and Amino Acid Sequence Analysis—Data base comparisons were performed using BLAST (14), sequence alignments were constructed with CLUSTAL X (15) and SEQVU (Garvan Institute, Sydney), and potential transmembrane domains were identified using TOP-PREDII (16). Standard protocols were used for E. coli cloning procedures and DNA sequencing (17). A full-length hZIP2 cDNA was obtained by PCR screening of a human prostate cDNA library (Genome Systems, Inc.) using primers that were derived from a partial cDNA clone (IMAGE clone ID 504596). The full hZIP2 open reading frame was amplified by PCR using primers with either a NotI (5′-ATATGAA-TGGCGCGGCCATGAGGCAACTACG-3′) or a XhoI (5′-GCTTCA-GAGCTCAGGCCACAGAAGC-3′) site added to their 5′ ends. This fragment was digested with NotI and XhoI and inserted into pBluescript vector (Invitrogen) to generate pCMV-hZIP2. hZip2 was epitope-tagged at its carboxyl terminus with three tandem copies of the hemagglutinin antigen (3×HA) epitope. The 3×HA DNA fragment was generated by PCR using the plasmid pMPY-3×HA (18) as template and the primers

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) AJ186081.

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‡ The abbreviations used are: AE, acrodermatitis enteropathica; CMV, cytomegalovirus; PCR, polymerase chain reaction; HA, hemagglutinin.

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RESULTS

Identification of the hZIP2 Gene—To identify potential zinc transporters in mammals, the amino acid sequences of the fungal and plant ZIPS were compared with mammalian expressed sequence tag sequences in the current data bases. This analysis identified several mammalian cDNAs capable of encoding related proteins. Three human genes (designated hZIP1, hZIP2, and hZIP3), one mouse gene (mZIP1; accession number AA680621), and one rat gene (rZIP1; accession number AI233196) were identified. An apparently full-length cDNA sequence of hZIP1 and a partial sequence of hZIP3 were available (Fig. 1). A plasmid (pZIP2) bearing a full-length cDNA sequence of hZIP2 was isolated from a prostate cDNA library (see Experimental Procedures) and sequenced (GenBank accession number AF186081). The cDNA insert in pZIP2 is 1358 nucleotides in length and codes a 309-amino acid protein (Fig. 1). It appears that the coding region begins at the 5' end, and two in-frame stop codons are present upstream of the putative initiation codon.

The hZIP2 protein product shows significant similarity (greater than 34%) to members of the ZIP family, e.g. the yeast Zrt1 and Arabidopsis Zip2 zinc transporters (Fig. 1). hZIP1 and hZIP3 show an equal degree of similarity to the fungal and plant proteins. The three mammalian proteins are more closely related to one another, sharing greater than 49% similarity, than they are to the fungal and plant proteins. Zrt1, Zip2, and the two full-length mammalian ZIPS are predicted to have eight transmembrane domains and the same topology with both the amino and carboxyl termini located on the extracellular surface of the cell. These features are shared by many of the ZIP family members (13). The available hZIP3 sequence appears to begin after transmembrane domain II.

A number of other features found in hZIP2 are conserved in other ZIPS. A potential metal binding motif containing three histidine residues (... HSHGHL ...) is found in a region between transmembrane domains 3 and 4. This motif is conserved in many of the ZIP family members including Zrt1 (13), but its function is unknown. Three of the transmembrane domains of hZIP2 contain histidine residues that are conserved among most family members (13). The available hZIP3 sequence appears to be related to one another, sharing greater than 49% similarity, than they are to the fungal and plant proteins. Zrt1, Zip2, and the two full-length mammalian ZIPS are predicted to have eight transmembrane domains and the same topology with both the amino and carboxyl termini located on the extracellular surface of the cell. These features are shared by many of the ZIP family members (13). The available hZIP3 sequence appears to begin after transmembrane domain II.

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a similar role for the mammalian ZIP genes. To test the ability of hZip2 to transport zinc, we first attempted to express hZip2 in a yeast zrt1 zrt2 mutant that is defective for zinc uptake. Although this approach was successful for characterizing many of the plant ZIP genes (12), hZip2 expression in yeast failed to complement the yeast mutant, and there was no detectable increase in zinc uptake activity (data not shown).

Lacking an antibody probe against hZip2, we could not assess whether the protein was produced in yeast or localized in the plasma membrane. Therefore, we tested an alternative expression system, human K562 erythroleukemia cells, in which we expected the protein to be expressed well and properly localized. This particular cell line was chosen because of its ability to grow in suspension, a simplifying factor for zinc uptake assays. The hZIP2 open reading frame was cloned into pRC-CMV (generating pCMV-hZIP2) for expression from the CMV promoter. Stable K562 transfectants were generated with pCMV-hZIP2 and the pRC-CMV vector. Northern blot analysis demonstrated that hZIP2 is expressed at a high level, approximately 0.1% of total mRNA, in the pCMV-hZIP2 transfectants (data not shown). No hZIP2 expression was detected in the vector-only control, demonstrating that hZIP2 is not normally expressed at a high level in K562 cells.

$^{65}$Zn accumulation was examined in the stable transfectants expressing hZip2 or the vector-only control, hereafter referred to as K562-hZIP2 and K562-Vec, respectively. At 4 °C, little zinc accumulation was detectable with either cell type (Fig. 2A). At 37 °C, zinc accumulation in K562-Vec cells indicated the presence of endogenous zinc uptake systems. Consistent with an ability of hZIP2 to transport zinc, $^{65}$Zn accumulation was greatly stimulated in K562-hZIP2 cells. To test whether the increased zinc accumulation in K562-hZIP2 cells was due to an increased initial zinc influx rate rather than, for example, increased zinc efflux activity, $^{65}$Zn accumulation was assayed over a much shorter time period (i.e. 0–60 s). K562-hZIP2 cells had an initial zinc uptake rate of 5 pmol Zn/min/10⁶ cells that was approximately 2-fold higher than the endogenous activity (2.3 pmol Zn/min/10⁶ cells) (Fig. 2B).

Endogenous zinc uptake activity measured in K562-Vec cells was concentration-dependent and saturable. When assayed over a range of zinc concentrations, this system showed Michaelis-Menten kinetics with an apparent $K_m$ of 3 μM zinc and a $V_{max}$ of 33 pmol Zn/min/10⁶ cells (Fig. 2C). Zinc accumulation in K562-hZIP2 cells was also concentration-dependent and saturable. The specific contribution of hZIP2 to zinc uptake activity was estimated by subtracting the vector control values from the K562-hZIP2 values (Fig. 2C, dashed line). hZIP2-dependent zinc uptake estimated in this manner had a similar apparent $K_m$ for zinc as the endogenous system (3 μM Zn) and a $V_{max}$ of 58 pmol Zn/min/10⁶ cells. Thus, both the hZIP2-dependent uptake activity and endogenous zinc uptake activity in K562 cells are time-, temperature-, and concentration-dependent. These results indicate that the hZIP2 gene encodes a zinc transporter. Most zinc in serum is bound to albumin. Therefore, an important question regarding the physiological relevance of hZIP2 zinc uptake activity is whether this protein can function in the presence of physiological concentrations of albumin. Adding serum levels of albumin (40 mg/ml) to our uptake assays decreased the uptake rates of both the hZIP2-dependent and endogenous systems by approximately 80%, but did not change the qualitative effects of hZIP2 expression. Thus, hZIP2 can function in the presence of albumin.

hZIP2 Substrate Specificity—To assess whether the endogenous K562 zinc uptake system or hZIP2 is capable of transporting substrates other than zinc, we tested several transition metals for their ability to inhibit zinc uptake activity (Fig. 3A). In these assays, $^{65}$Zn was supplied at 3 μM, and the other metals were added at >6-fold excess (i.e. 20 μM) concentrations. Zinc uptake by the K562 endogenous system was not inhibited by Fe$^{3+}$ or Co$^{2+}$ but was significantly inhibited by Cd$^{2+}$, Cu$^{2+}$, and Mn$^{2+}$ (p < 0.05). Although the mechanism of this inhibition is not known, these data suggest that the inhibitory metals are potential substrates for the endogenous system. In contrast, hZIP2 zinc uptake activity was strongly inhibited by all metals tested. Again, although other mechanisms are possible, hZIP2 may be capable of transporting many different metal ion substrates.

Zinc uptake by both the endogenous activity in K562 cells and hZIP2 was not energy-dependent. Treatment with several different electron transport/oxidative phosphorylation inhibitors (i.e. oligomycin, antimycin A, CN$^-$, N$^3_3$, rotenone, and carbonyl cyanide 3-chlorophenylhydrazone) did not decrease zinc uptake activity (data not shown). Zinc uptake by either system also appeared to be independent of membrane potential. Our standard assay buffer contains a concentration of K$^+$ (150 mM) that is sufficient to depolarize the plasma membrane. However, no effect on uptake activity was observed when cells were incubated in low K$^+$ (1 mM) assay buffer or in the presence of 2 μM valinomycin, a K$^+$ ionophore. Likewise, Na$^+$ was not required for zinc uptake by these systems because it was not included in the standard uptake buffer, and addition of Na$^+$ (6 mM or 150 mM) had no effect on uptake activity. The sulfhydryl-reactive agent N-ethylmaleimide inhibited both hZIP2 and endogenous uptake activities by greater than 50%, suggesting that one or more cysteine residues are important for their function.

Although the endogenous zinc uptake system was pH-independent, hZIP2 zinc uptake was inhibited at pH levels below 7.0 and stimulated at higher pH (p < 0.05) (Fig. 3B). One explanation for this effect is that as pH increases, the concentration of HCO$_3^-$, which is present in the assay solution because of equilibration with atmospheric CO$_2$, also increases. HCO$_3^-$ treatment is known to stimulate zinc uptake activity in cultured fibroblasts and erythrocytes (6, 22, 23). Therefore, we examined the effects of HCO$_3^-$ on the endogenous and hZIP2 activities. HCO$_3^-$ treatment (0.5 mM) had no significant effect...
on the endogenous activity but stimulated hZip2 activity (Fig. 3C). Control experiments indicated that this level of HCO₃⁻ did not change the pH of the solution (data not shown). When assayed at pH 8.0, HCO₃⁻ addition failed to further increase hZip2 uptake activity. This result suggests that effects of high pH are indeed caused by the indirect effects of pH on HCO₃⁻ concentrations.

Subcellular Localization of hZip2—The hypothesized role of hZip2 as a zinc uptake transporter suggests that this protein is found in the plasma membrane. To test this prediction, we epitope-tagged the carboxyl terminus of hZip2 with three tandem copies of the Haemophilus influenzae hemagglutinin antigen (HA) to generate hZIP2–3×HA. This allele was cloned into the pRc-CMV expression vector and transfected into K562 cells. Zinc uptake assays of hZIP2–3×HA transfectants demonstrated that the tagged allele was functional, i.e. an approximately 2-fold increase in uptake activity was observed (data not shown). Localization of the hZIP2–3×HA protein was determined by indirect immunofluorescence using an anti-HA antibody. Little fluorescence was detected in detergent permeabilized or nonpermeabilized cells expressing untagged hZip2 (Fig. 4, A–D). In contrast, permeabilized cells expressing hZIP2–3×HA showed a bright rim of fluorescence at the cell periphery (Fig. 4, E and F) consistent with plasma membrane localization of the epitope-tagged protein. Plasma membrane staining was also observed in nonpermeabilized cells expressing the HA-tagged protein (Fig. 4, G and H). These results indicate that the carboxyl terminus of hZip2 is located on the extracellular face of the plasma membrane as we anticipated from its predicted topology.

DISCUSSION

The human genome contains at least three ZIP family members. Our current hypothesis is that these genes encode zinc uptake transporters. Several lines of evidence from our studies of hZip2 support this hypothesis. First, hZip2 is closely related to several previously characterized zinc transporters from both fungi and plants. Second, hZip2 is localized in the plasma membrane when expressed in K562 cells. Third and most importantly, expression of hZip2 in K562 cells increased the initial rate of zinc uptake in a time-, temperature-, and concentration-dependent fashion. This zinc uptake activity was clearly distinct from the endogenous activity expressed by the cells. These characteristics are indicative of a carrier-mediated transport process dependent on hZip2. Additional support for our hypothesis regarding the human ZIP genes comes from recent studies of zinc uptake in PC-3 prostate carcinoma cells. Costello et al. (24) showed that hZIP1 is expressed in PC-3 cells and that its expression was coordinately regulated with zinc uptake activity in response to testosterone and prolactin treatment. Furthermore, hZIP1 expression was regulated by zinc availability; zinc treatment of PC-3 cells decreased hZIP1 mRNA levels. Similar regulation of zinc transporter expression has been observed for the yeast and plant ZIP genes (8, 12).

Although our results demonstrate the ability of this protein to transport zinc, it may also be capable of transporting other physiologically relevant metal ions such as Fe²⁺, Cu²⁺, and Mn²⁺ and nonphysiological substrates like Cd²⁺ and Co²⁺. This potentially broad substrate range is not unprecedented in the field of metal ion transport. The Dmt1 transporter plays an important role in iron accumulation (25, 26) but is also capable of transporting Zn²⁺, Mn²⁺, Cu²⁺, Co²⁺, Cd²⁺, and Pb²⁺ (27). Ir1, a plant member of the ZIP family, can transport Fe²⁺, Zn²⁺, Mn²⁺, and Cd²⁺ (10, 11). Determining the physiological role of hZip2 in transporting zinc and other metal ions into cells of various tissues may ultimately require an animal model (e.g. knockout mice) for studying mammalian ZIP function.

We can gain some insight into ZIP function in humans by considering the tissues where these proteins are expressed. Repeated attempts to detect hZIP2 mRNA on Northern blots of poly(A)⁺ RNAs derived from different human tissues and cultured cell lines failed to give positive results. It is possible that this gene is normally expressed at low levels and that a more...
sensitive detection method will be required. Alternatively, \textit{hZIP2} may be expressed in only a limited number of tissues and cell types that were not represented on these blots. The available information in the expressed sequence tag data base is consistent with either hypothesis. Only four \textit{hZIP2} expressed sequence tag clones have been isolated and these were only from prostate and uterine cDNA libraries. The observation that these particular tissues express \textit{hZIP2} may be instructive, \textit{e.g.}, cells of the prostate contain the highest zinc level of any soft tissue in the body. Thus, expression of \textit{hZIP2} in these tissues might help meet their particular needs of zinc metabolism. In contrast, \textit{hZIP1} and \textit{hZIP3} have been cloned as expressed sequence tags from a large number of different tissues, indicating that these genes are widely expressed and may play general housekeeping roles.

\textit{hZIP2} was previously identified by subtraction cloning from primary uterine epithelial cells because the gene (designated as “clone 6A1”) was induced when these cells reached contact inhibition (28). Given the clear membership of this gene in the ZIP family, we propose “\textit{hZIP2}” as its designation. 6A1/\textit{hZIP2} expression was not detected in two of four carcinoma cell lines, leading to the proposal that this protein plays a direct role in contact inhibition. We see no growth inhibitory effect of \textit{hZIP2} overexpression in K562 cells (data not shown), suggesting that this hypothesis is incorrect. Rather, an alternative explanation for the observed induction is that at high cell densities, zinc levels in the medium may be decreasing and triggering a transcriptional response to nutrient limitation.

Another unanswered question for \textit{hZip2} is its mechanism of transport. Surprisingly, zinc uptake by \textit{hZip2} was energy-independent. This observation conflicts with results obtained with the yeast zinc transporters that show a strict energy-dependence (8, 9). Therefore, the fungal and human ZIPS may work by different mechanisms. Zinc uptake by \textit{hZip2} was not dependent on the plasma membrane K\textsuperscript{+} or Na\textsuperscript{+} gradients but was stimulated by increased pH and treatment with HCO\textsubscript{3}{-}. We believe that the pH and HCO\textsubscript{3}{-} effects on \textit{hZip2} arise from the same mechanism. At pH 7, the [HCO\textsubscript{3}{-}] is calculated as 0.07 mM, whereas at pH 8, this concentration rises to 0.7 mM. Because 0.5 mM HCO\textsubscript{3}{-} was saturating for the effect (data not shown), adding HCO\textsubscript{3}{-} to assay buffer at pH 8 would not be expected to increase activity. Based on these considerations, we propose that zinc uptake by \textit{hZip2} \textit{in vivo} is mediated by a Zn\textsuperscript{2+}-HCO\textsubscript{3}{-} symport mechanism. Zinc uptake could be driven by the high concentration of HCO\textsubscript{3}{-} in serum (10–20 mM) and, presumably, the concentration gradient of labile zinc across the plasma membrane. Although the total zinc level in a cell is high, very little of that zinc is present in a labile form. Estimates of the labile zinc pool are in the nanomolar range (21). These important mechanistic issues await future study.

A final interesting aspect of the observed stimulation of \textit{hZip2} activity by HCO\textsubscript{3}{-} is its potential relationship to acrodermatitis enteropathica. Vasquez and Grider (6) have shown that zinc uptake in fibroblasts involves two different uptake systems, one stimulated by HCO\textsubscript{3}{-} and a second that is unaffected by this treatment. They also showed that fibroblasts derived from AE patients retained only the system that was unresponsive to HCO\textsubscript{3}{-}. These results suggested that AE interferes specifically with a HCO\textsubscript{3}{-}-responsive zinc uptake activity, and in light of our data, this system may be \textit{hZip2} or a related ZIP transporter. Thus, with the identification of human ZIP transporters, we now have new molecular tools to examine zinc uptake, its genetics, and its regulation in human cells.

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