Cloning, expression, and vesicular localization of zinc transporter Dri 27/ZnT4 in intestinal tissue and cells

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Murgia, Chiara, Isabella Vespignani, Joanna Cerase, Fabio Nobili, and Giuditta Perozzi. Cloning, expression, and vesicular localization of zinc transporter Dri 27/ZnT4 in intestinal tissue and cells. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G1231–G1239, 1999.—We have identified the Dri 27 cDNA on the basis of its upregulated expression during rat intestinal development. It encodes a hydrophobic protein of 430 amino acids that shares significant homology with members of the mammalian zinc transporter family ZnT. The murine homologue of Dri 27 (named ZnT4) was recently associated with the mouse mutation “lethal milk.” The primary sequence of Dri 27/ZnT4 displays features characteristic of polytopic membrane proteins. In this paper, we show that Dri 27/ZnT4 is localized in the membrane of intracellular vesicles, the majority of which concentrate in the basal cytoplasmic region of polarized enterocytes. A Dri 27/ZnT4 myc-tagged construct, transiently transfected in intestinal Caco-2 cells, partially colocalizes with the β-subunits of the clathrin adaptor complexes AP-1 and AP-2 in a subpopulation of endosomal vesicles. By subcloning distinct portions of the protein in frame with glutathione-S-transferase, we also provide experimental evidence of their function as zinc-binding and protein-protein-interaction domains.

small intestine; zinc transport; endosomal trafficking

The essential role of intestinal epithelial cells is the regulated absorption of nutrients and their transfer to the circulation to meet body needs. Because of the wide variation in dietary nutrient composition, a key function of enterocytes is the selective absorption of those substances whose excessive intake could be harmful. The polarized cells that constitute the absorptive intestinal mucosa are constantly renewed by sequential cell division, migration, and exfoliation that occur continuously in the adult epithelium along the crypt-villus axis (28). Such processes are strictly associated with cell differentiation and increasing expression of genes involved in nutrient absorption and transport (15).

Metal ions are essential nutrients necessary for a multitude of biochemical processes, either as catalytic components for enzymatic reactions or as structural elements of metalloproteins (13). Adequate intake of these trace elements is therefore essential for normal functioning of cells and tissues, although in high concentrations they can display toxic effects, either by triggering uncontrolled redox reactions leading to the production of reactive oxygen species or by interfering with metal-dependent cellular processes. Intracellular homeostasis for the different transition metals is achieved through the coordinate regulation of specific proteins involved in their uptake, efflux, and intracellular compartmentalization. Understanding these complex regulatory mechanisms requires the identification and molecular characterization of the specific transporters involved in such processes.

Each metal ion displays a specific site of absorption along the adult gastrointestinal tract (27). The absorption of zinc occurs almost exclusively in the small intestine and displays both saturable and nonsaturable components (reviewed in Refs. 6, 38, and 41). A specific transporter responsible for the intestinal uptake of zinc has not yet been identified at the molecular level, although biochemical evidence has been provided in specific cell types (10). The recently cloned iron transporter DCT 1 was also shown to mediate the uptake, with lower affinity, of other ions, including zinc (17). The only characterized mammalian zinc transporters belong to the recently described ZnT protein family. Although none of them appears to be responsible for zinc uptake, the ZnT proteins are highly homologous and differ from each other in their tissue specificity and intracellular localization. ZnT1 and ZnT2 were cloned by functional complementation of the zinc sensitivity of a mutated BHK cell line (30, 32). ZnT1 appears to be localized to the plasma membrane, where it presumably mediates zinc efflux; ZnT2 is involved in vesicular sequestration of this ion. ZnT3 was identified by homology with ZnT2; its expression is restricted to the nervous system and testes (31), and the protein is responsible for zinc transport into synaptic vesicles (8, 44). Very little is known about the zinc transport mechanism mediated by ZnT4, the most recently isolated member of this family. This protein was shown to confer zinc resistance to yeast mutants, and a truncated form of the protein was found to represent the molecular basis of the recessive mouse mutation “lethal milk” (lm) (18). The milk produced by homozygous lm/lm females contains insufficient zinc to support the needs of the growing pups (1).

In a search for genes upregulated during intestinal epithelial differentiation, using a subtractive hybridization approach, we have isolated two novel cDNA clones that were named Dri 27 and Dri 42 (Dri stands for differentially expressed in rat intestine) (2, 4). In this study, we report the isolation of the full-length Dri 27 cDNA clone, its expression in the intestinal tissue, and the functional characterization of the encoded protein as the rat homologue of the recently described mouse ZnT4. Moreover, we provide the first experimental evidence for direct zinc binding to the histidine-rich...
domain of the Dri 27/ZnT4 transporter and for its localization to the membranes of intracellular vesicles in intestinal cells.

**MATERIALS AND METHODS**

Tissue preparation and RNA extraction. The portion of small intestine between the pylorus and the ileocecal valve was dissected from Sprague-Dawley rats that were anesthetized by intraperitoneal injection of 20 mg/100 g body wt of Farnotal (Farmitalia-Carlo Erba, Milan, Italy). The dissected tissue was quickly rinsed in cold Hank's buffered saline (Flow Laboratories, Irvine, UK) and immediately frozen in liquid nitrogen. Total RNA was extracted from frozen pulverized tissues as described by Chirgwin et al. (7).

cDNA libraries. The rat intestinal cDNA library used in this study was previously described (4). The rat brain cDNA library was purchased from Stratagene (La Jolla, CA). Both libraries were cloned into the EcoRI site of the phage vector λZAP II. Library screening and in vivo excision of insert-containing pBluescript SK− from plaque-purified, recombinant lambda phages were performed according to the manufacturer's directions.

DNA sequence determination. The DNA sequence was determined on double-stranded templates either by the Sanger's method of dideoxy chain termination, as modified by the use of Sequenase (US Biochemicals, Cleveland, OH) and 33S-labeled dATP (40), or by automatic sequencing (Genenco sequencing service, M-Medical, Florence, Italy).

cDNA constructs and expression of fusion proteins. The cDNA constructs used in this study were obtained either by restriction digestion or from fragments generated by PCR, using Dri 27-specific primers. All constructs were verified by sequencing of the fusion junctions. Taq polymerase (Perkin-Elmer, Norwalk, CT) was used for amplification as recommended by the manufacturer. PCR primers had 20 nt of identity with the template, 6 nt containing a restriction site, and were all synthesized by Genenco (oligonucleotide synthesis service, M-Medical). Constructs glutathione-S-transferase (GST)-His and GST-Leu, containing the Dri 27 putative metal-binding domain (residues 231–283) and the leucine zipper-containing segment (residues 29–135), were obtained by subcloning amplified Dri 27 fragments into the vector pGEX-2T (Amersham Pharmacia Biotech, Uppsala, Sweden), in frame with the carboxyl terminus of bacterial GST. GST-HD-Zip1 was a kind gift of Ida Ruberti (Centro di Studio per gli Acidi Nucleici, Consiglio Nazionale delle Ricerche, Rome, Italy) with the gift of Silvia Biocca, University of Rome Tor Vergata, Rome, Italy). The vector was co-transfected with the pBluescript SK− digested by intraperitoneal injection of 20 mg/100 g body wt of Farmotal (Farmitalia-Carlo Erba, Milan, Italy). The disected tissue was quickly rinsed in cold Hank's buffered saline (Flow Laboratories, Irvine, UK) and immediately frozen in liquid nitrogen. Total RNA was extracted from frozen pulverized tissues as described by Chirgwin et al. (7).

Cell culture, transfection, metabolic labeling, and antibodies. Human colon adenocarcinoma Caco-2 cells (a kind gift of A. Zweibaum, Institut National de la Sante et de la Recherche Medicale, Villejuif, France) were used between passages 80 and 100 and cultured as previously described (37). Both Caco-2 and Cos-1 cells were transiently transfected using polyethyleneimine (PEI; Aldrich Chemical, Milwaukee, WI) as transfection agent (5). Briefly, plasmids were incubated in 1.5 mM PEI for 12 min at room temperature in 150 mM NaCl, and the mixture was diluted with serum-free medium and added to mid-log phase cells. After 1 h at 37°C, cells were washed and fed with fresh medium. Immunofluorescence and metabolic labeling were performed 24–48 h after transfection. For metabolic labeling, cells were incubated for 3 h with 100 µCi/ml of [35S]methionine plus cysteine (NEN Life Science Products, Boston, MA) in methionine-cysteine-free medium. The polyclonal antibody KS was raised in rabbit against a synthetic 14-amino-acid peptide spanning residues 370–383 of the Dri 27 sequence (Fig. 2). Peptide synthesis, coupling to ovalbumin, and immunization were performed by Nogen (Sequans, Strasbourg, France). The immune serum was further purified by protein A-Sepharose affinity chromatography according to standard procedures. The purified serum was used at a 1:50 dilution for both immunofluorescence and immunoprecipitation. The anti-c-myc monoclonal antibody (clone 9E10) was purchased from Sigma and used at 1:100 dilution for immunofluorescence experiments and at 1:500 dilution for immunoprecipitation. For double immunofluorescence experiments, the following primary antibodies were employed: polyclonal anti-c-myc (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-transferrin receptor (Zymed, San Francisco, CA), monoclonal anti-β1- and β2-adaptins (Sigma). Secondary antibodies were goat anti-rabbit IgG (Cappel, Organon Teknika, Durham, NC) and donkey anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). The secondary antibodies were labeled with either tetramethylrhodamine isothiocyanate or FITC and were both used at a 1:200 dilution.

Immunofluorescence. For subcellular localization, cells were seeded on glass coverslips in 24-well tissue culture plates and grown to 50% confluency. Twenty-four hours after transfection, cells were fixed with 2% paraformaldehyde in PBS+ (1× PBS containing 1 mM MgCl2 and 0.1 mM CaCl2) for 30 min at room temperature and treated by conventional immunofluorescent techniques. Primary and secondary antibodies were diluted in 1× PBS+, 2% BSA, and 0.05% Triton X-100. For tissue immunofluorescence, portions of proximal duodenum, jejunum, and distal ileum from 21-day-old rats were dissected, and longitudinal cryosections were prepared as described in Ref. 3. Sections were fixed and treated as described for cells.

Biochemical methods. To test the ability of Dri 27 to bind metal ions, chromatography columns were packed with chelating Sepharose fast flow (Pharmacia Biotech) and the gel was immobilized with metal ions according to the instructions from the manufacturer. Purified GST or GST-His (both 150 µg) were applied to the column in loading buffer (50 mM phosphate buffer, pH 8.0, and 300 mM NaCl), washed with the same buffer, and eluted with increasing concentrations of imidazole. To examine possible protein-protein interactions, metabolically labeled cells were extracted in NP-40 lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, and 0.025% SDS) containing a cocktail of proteinase inhibitors for 30 min on ice. GST-Leu and the control fusion GST-HD-Zip1,
still bound to glutathione-agarose beads, were incubated overnight at 4°C with cell lysate. The protein complexes were washed with lysis buffer, boiled in denaturing sample buffer, and fractionated by SDS-PAGE.

RESULTS

Isolation and molecular analysis of a full-length Dri 27 cDNA clone. The nucleotide sequence of the Dri 27 cDNA fragment originally isolated by subtractive hybridization had shown no homology with previously known sequences in the GenBank/EMBL database. Northern blot hybridizations using this cloned fragment as probe identified a single mRNA of ~5 kb (2). The cloned sequence appeared to represent the 3’ end of this transcript, as it did not contain an ORF and terminated with a consensus poly(A) addition site. We were unable to isolate a full-length clone after repeated screening of a rat intestinal cDNA library, synthesized in our laboratory (4), and because the Dri 27 mRNA is very abundant also in the brain (Fig. 3), we screened a rat brain library using as hybridization probe the largest intestinal clone (1.7 kb). Screening of 120,000 plaques yielded 6 positives, one of which contained a 5.4-kb insert. The complete nucleotide sequence of this clone was determined and shown to represent the full-length cDNA. The main features of the Dri 27 cDNA are 267 bp of untranslated region (UTR) at the 5’ end, with a very high GC content (80%) and a long 3’ UTR of 3.9 kb, ending with a poly(A) stretch. The ATG codon at position 268 initiates an ORF of 1.3 kb, encoding a protein of 430 amino acids. This sequence was submitted to the EMBL, GenBank, and DDBJ nucleotide sequence databases, where it is listed under the accession number Y16774.

Dri 27 protein is homologous to eukaryotic metal ion transporters. Homology searches within the GenBank/EMBL protein sequence database revealed that the primary sequence of the Dri 27 protein shares significant homology to a widely conserved protein family of metal ion transporters (9, 18, 20, 30, 32, 42). The murine homologue of Dri 27, named ZnT4, is the most recently cloned member of this family (18). Alignment of the primary sequences of the four rat ZnT proteins and the two yeast transporters is shown in Fig. 1A. At the level of primary sequence, the six proteins can be aligned according to three distinct blocks of higher homology, which are evolutionarily conserved. The overall length, as well as the spacing between the conserved blocks, differs in each sequence. It is interesting to note that the similarity among these proteins is very strong at the structural level, as all of them display six alternating hydrophobic/hydrophilic domains, indicative of polytopic membrane proteins (Fig. 1B). Such striking structural conservation had previously emerged for the members of a wider family of heavy metal ion transporters, which includes the ZnTs (CDF family), whose structure is evolutionarily conserved from bacteria to mammals (33). Between the putative transmembrane segments IV and V, a cluster of alternating histidine residues is present in all the eukaryotic proteins (aligned at Fig. 1A, bottom), which have been proposed to bind metal ions through the imidazole ring.

The rat Dri 27/ZnT4 protein has a predicted molecular mass of 47.7 kDa. The characteristic features of this protein, its amino acid sequence, and the predicted membrane insertion topology are summarized in Fig. 2. In Dri 27/ZnT4, the hydrophilic segments facing the noncytoplasmic side of the membrane are very short (between 7 and 16 residues), whereas the histidine-rich domain is present within a cytoplasmic loop of 39 residues. Of the four members of the ZnT family, Dri 27/ZnT4 is the only one that contains a potential protein-protein interaction domain, represented by a leucine zipper of four consecutive L-(x)₆ repeats in the primary sequence of the C-terminal domain.

![Fig. 1. Comparison of primary sequences and hydrophathy profiles of mammalian and yeast transporters. A: primary sequences of rat zinc transporters ZnT1, ZnT2, and ZnT3, yeast zinc transporter 1 (ZRC 1), and yeast cobalt transporter 1 (COT 1) are aligned in order of decreasing homology to primary sequence of rat Dri 27/ZnT4. Numbers at end of each sequence indicate length of each protein in amino acid residues. Numbers within blocks indicate percent similarity with respect to corresponding block in Dri 27/ZnT4 sequence. Vertical bars within first block of homology mark the position of histidine-rich domain in each protein, whose amino acid residues are aligned at bottom. Numbers to left of aligned sequences at bottom indicate position of first residue of histidine-rich domain within primary sequence of each protein. B: hydrophathy profiles of proteins aligned in A, calculated according to the Kyte and Doolittle algorithm (24). Hydrophobic residues lie above horizontal line, hydrophilic residues below. Asterisks mark putative transmembrane segments.](http://ajpgi.physiology.org/)
amino-terminal hydrophilic segment. Leucine zippers are commonly found in transcription factors (reviewed in Ref. 19), but they have been recently described also in transporter proteins (Ref. 21 and references therein). The primary sequence contains also three potential sites for N-glycosylation, but none of them appears to be modified posttranslationally when the Dri 27 cDNA is translated in vitro in the presence of pancreatic microsomal membranes (data not shown).

Dri 27/ZnT4 expression. The Northern blot hybridizations in Fig. 3A show that the Dri 27 gene is transcribed to different extents in all the adult rat tissues examined. The strongest expression is observed in brain and testes, followed by small intestine, medulla, lung, kidney, and the proximal and distal portions of the gastrointestinal tract (stomach and colon). Other tissues display lower levels of transcription of the Dri 27 gene.

To investigate whether transcription of this gene might be affected by nutritional zinc deprivation, we compared the steady-state levels of Dri 27/ZnT4 mRNA in zinc-deprived rats with respect to those in the corresponding tissues of animals fed control diets. Total RNA was extracted from tissues expressing the highest levels of this transcript, namely the small intestine, brain, and testes. The results in Fig. 3B show that transcription of the gene remains unchanged in these tissues in response to zinc deprivation.

Dri 27/ZnT4 is localized in the membrane of intracellular vesicles. A construct containing the entire ORF of the Dri 27/ZnT4 cDNA was tagged at the carboxy terminus with a 10-amino acid peptide epitope from c-myc, and the resulting construct was transiently transfected into cells of the human adenocarcinoma cell line Caco-2 and detected using an anti-myc monoclonal antibody that immunoprecipitates a single band of the predicted molecular weight from transfected cells (Fig. 4A). Figure 4B shows immunostaining of Dri 27/ZnT4 in an overexpressing Caco-2 cell 48 h after transfection. The protein is clearly associated with intracellular vesicles of heterogeneous size, mostly concentrated in the perinuclear region and sparsely distributed throughout the entire cytoplasm. No staining is detected on the plasma membrane. To further investigate the nature of such vesicles, we performed double-labeling experiments using antibodies specifically directed against proteins whose association with specific intracellular compartments is well characterized. The results in Fig.
show that a subpopulation of Dri 27/ZnT4-containing vesicles also contain transferrin receptor (TfR), which recycles between the endosomal compartment and the plasma membrane (14). A monoclonal antibody recognizing both β1- and β2-adaptins, subunits of the two clathrin-adaptor complexes AP-1 and AP-2, respectively, also shows partially overlapping localization with the transfected protein (Fig. 5, bottom). The anti-β-adaptin antibody does not allow distinction between AP-1 and AP-2, which mediate clathrin assembly on coated vesicles budding from the trans-Golgi network or from the plasma membrane (26). As expected, a smaller extent of overlap is observed with TfR than with the β-adaptins, because the transfected protein and TfR are simultaneously present as specific cargo proteins in only a subset of endosomal vesicles, whereas AP-1 and AP-2 associate with all clathrin-coated vesicles that traffic along the same route. Although these experiments do not allow more precise determination of the endpoint of Dri 27/ZnT4 vesicle trafficking, they provide a strong indication of their association with the endosomal compartment.

Immunolocalization of Dri 27/ZnT4 in rat small intestine. The epithelial cells that layer the small intestinal mucosa display morphological and functional polarization of cellular components. To investigate whether Dri 27/ZnT4-containing vesicles show a polarized distribution in differentiated enterocytes, we next stained sections of small intestinal epithelium with a specifically raised antipeptide antibody. The antibody preparation was first tested by immunoprecipitation (Fig. 4A). Both the antipeptide antibody and the commercial anti-myc antibody select a single immunoprecipitated band from the extracts of Cos cells transfected with the myc-tagged Dri 27/ZnT4 cDNA. The antipeptide antibody KS failed to immunoprecipitate the endogenous protein from either simian Cos cells (Fig. 4A) or human Caco-2 cells (data not shown), thus demonstrating species specificity towards the rat epitope. Immunostaining of the protein by indirect immunofluorescence on cryosections of rat small intestine, using the KS antibody, is shown in Fig. 6. Along the crypt-villus axis, the distribution of the protein (Fig. 6A) parallels the synthesis of its mRNA, visualized by in situ hybridization (Fig. 6C). The protein is absent in the proliferative cells of the crypts; its expression starts at the crypt-villus junction and is maintained in the differentiating enterocytes along the villus. Specific staining is concen-
trated mainly at the basal side of epithelial cells, although weaker staining is observed also throughout the apical cytoplasm. Diffuse fluorescence is detected in the microvillar membrane, the significance of which is unclear at present because it is also observed in sections stained with the preimmune serum (Fig. 6D). A phase-contrast photograph of the same field in Fig. 6A is shown in Fig. 6B as a control for the integrity of the tissue.

Histidine-rich domain of Dri 27/ZnT4 binds zinc as well as other transition metal ions in vitro. To gain further insight into the role that the Dri 27/ZnT4 protein might play in zinc cellular metabolism, we have sought to characterize the function of two presumably important regions of the protein, namely the histidine-rich domain and the leucine zipper. We have independently subcloned each of these regions in frame with the bacterial protein GST. The resulting fusion proteins (GST-His and GST-Leu) were purified from overexpressing bacteria by affinity chromatography on glutathione-agarose beads and used in in vitro binding experiments. Because the zinc-binding capacity of the histidine-rich domains in ZnT proteins had never been demonstrated experimentally, we first tested the ability of GST-His, which contains the eight histidine residues in the cytoplasmic loop (Fig. 2), to specifically bind different transition metal ions (Zn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, and Co$^{2+}$), immobilized on a Sepharose matrix. Metal ion binding was displaced by increasing concentrations of the histidine analog imidazole, and the protein content was measured in each fraction. Figure 7A shows that, in the absence of imidazole, GST-His is retained on the columns containing any of the four transition metals tested. Step elution with increasing imidazole concentrations has shown that, whereas bound GST is released at low imidazole concentrations, GST-His is stably bound to the metal and elutes as a sharp peak when 250 µM imidazole is added to the column. The optical density units at 280-nm wavelength of the fractions shows no difference, among the metal ions tested, in the imidazole concentration required to displace binding in vitro. When GST-His was applied to a Sepharose column containing...
magnesium ions, the protein was entirely recovered in the flowthrough, thus demonstrating binding specificity toward transition metal ions (data not shown). SDS-PAGE of the fractions from the zinc-Sepharose columns is shown in Fig. 7B.

Amino-terminal segment of Dri 27/ ZnT4 mediates specific protein-protein interactions. To test the ability of Dri 27/ZnT4 to participate in protein-protein interactions, we used the fusion protein GST-Leu, which contains 120 amino acids in the amino-terminal segment of the rat zinc transporter, including the leucine-zipper motif (Fig. 2). Total proteins were extracted from metabolically labeled Caco-2 or Cos cells. The extract was incubated with either the purified recombinant GST-Leu protein or with a control GST fusion containing a heterologous leucine zipper from Arabidopsis thaliana [GST-HD-Zip1 (39)], under conditions allowing specific protein-protein interactions (described in MATERIALS AND METHODS). Because both GST fusion proteins were still bound to glutathione-agarose beads, after incubation the isolated complexes were pulled down by centrifugation and fractionated on SDS-PAGE gels. The results in Fig. 8 show that two prominent bands with molecular masses of ~80 and ~100 kDa, respectively, were specifically selected by GST-Leu from the protein extracts of either Caco-2 (lanes 1 and 2) or Cos cells (lanes 3–6). The presence of a band of comparable size to the entire Dri 27/ZnT4 protein in the complexes selected by GST-Leu does not allow us to rule out entirely the possibility of homodimerization. However, we believe that this case is rather unlikely because the intensity of this band is not increased in Cos cells overexpressing Dri 27/ZnT4 by transfection (lanes 5 and 6). Parallel immunoprecipitation of myc-tagged Dri 27/ZnT4 from transfected and nontransfected Cos cells is shown in lanes 7 and 8 to account for the amount of protein expressed by transfected cells. The binding specificity of GST-Leu toward the two polypeptide bands shown in Fig. 8 was reproducible also when using GST alone in place of GST-HD-Zip1 as negative control (data not shown). We have observed that the relative intensity of the two specific bands varies in independent experiments; therefore, we cannot exclude the possibility that the 80-kDa band might represent a degradation product of the 100-kDa polypeptide. In conclusion, our results show a role of the amino-terminal portion of Dri 27/ZnT4, which contains the leucine-zipper motif, in heterodimeric interactions with at least one specific partner.

DISCUSSION

We have reported in this paper the isolation and functional analysis of the rat protein Dri 27, a zinc transporter whose mouse homologue, ZnT4, was recently cloned and identified as the molecular basis of the lm mouse mutation (18). Homozygous lm/lm females produce zinc-deficient milk, thus causing the death of their pups before weaning (1, 36). Although this is the most severe phenotype of the mutants, homozygous lm/lm mice fostered by wild-type mothers can survive throughout adulthood displaying a minor pleiotropic phenotype that is still poorly understood (12). The initial studies on the expression of ZnT4 were carried out in mammary tissue and cell lines, and the cellular mechanisms by which the mutant protein interferes with zinc metabolism are still an open question.

Our laboratory has been primarily concerned with the isolation and molecular characterization of genes that are upregulated during intestinal epithelial differentiation (2–4). Because the absorptive intestinal mucosa is the primary site of zinc uptake and we originally isolated the Dri 27 cDNA from this tissue on the basis of its upregulation during intestinal epithelial differentiation, we have sought to understand the role that its gene product might play in differentiated enterocytes through a detailed analysis of its intracellular localization and of its distinct functional domains.

We have shown that Dri 27/ZnT4 is expressed almost ubiquitously in rat tissues and that transcription of the corresponding gene is not affected by zinc deprivation. Within the ZnT family, ubiquitous expression was reported only for ZnT1, whereas both ZnT2 and ZnT3 display tissue-restricted expression (31). Because ZnT4 and ZnT2 expression profiles overlap in the gut and both proteins appear to be localized in endosomal vesicle membranes (Ref. 23 and this study), it would be interesting to investigate whether in this tissue they might perform similar functions. The two proteins are in fact predicted to display the same topological configuration within the membrane bilayer, their zinc binding domains are both cytoplasmically oriented, and ZnT2-coated vesicles have been shown to accumulate zinc (30). A possible, even partial redundancy between the functions of the two proteins might explain the lack of a major intestinal phenotype in lm mice.

The results presented in this paper show for the first time the vesicular localization of Dri 27/ZnT4 and the partial overlap of such vesicles with markers of vesicular trafficking. Immunofluorescence on cryosections of rat small intestinal mucosa reveals that the majority of the Dri 27/ZnT4-containing vesicles are clustered in the basal side of polarized enterocytes in vivo, although
staining is observed also throughout the apical cytoplasm of enterocytes. We cannot rule out at present that translocation of Dri 27/ZnT4-containing vesicles to different membrane compartments might occur in vivo in response to specific stimuli that are absent in the tissue-culture medium. A regulatory mechanism of this kind has been described for the glucose transporter GLUT4, which has a vesicular localization in adipose cells and muscle cells, but it is rapidly translocated to the plasma membrane in response to insulin (Ref. 34 and references therein). In the case of ZnT4, the first likely candidate for a regulatory response of this kind is zinc, as other metal ions have been already reported to induce trafficking of their own transporters. The human Menkes protein, which plays a key role in copper transport, is localized on intracellular vesicles that show a reversible, copper-dependent redistribution from the Golgi apparatus to the plasma membrane (25, 35). Several mammalian diseases related to metal ion transport and homeostasis have been recently associated with defects in intracellular trafficking, and increasing experimental evidence points to cargo selection as a key regulatory mechanism for proper sorting of metal ion-loaded intracellular vesicles. The regulatory mechanism for proper sorting of metal ion-loaded intracellular vesicles. The Acrodermatitis enteropathica mutation (AE), resulting in defective intestinal uptake of zinc in humans, was recently associated with the absence from fibroblasts of AE patients of TIP47, a protein involved in cargo selection for mannose-6-phosphate receptor trafficking (11, 16). Moreover, the mouse mutation mocha, which results in lack of expression of the β-subunit of the AP-3 complex, simultaneously affects endosomal transport, zinc concentration in synaptic vesicles, as well as the expression levels of ZnT3 (22). In light of these studies, future work should be aimed at elucidating the regulatory mechanisms underlying vesicular compartmentalization of zinc and the expression of specific zinc transporters.

The alternating histidine residues in the ZnT proteins have been postulated to represent metal ion-binding domains, but this function had never been shown experimentally. The data presented in this paper provide therefore the first experimental evidence that zinc and other metal ions specifically bind in vitro to the histidine residues in the putative metal ion-binding domain that characterizes the ZnT family of transporters. Moreover, with this experiment we have demonstrated that the binding specificity toward a specific ion is not always conferred by the length or the composition of the His-rich sequence per se but rather likely by the structural features of the protein in which it resides.

A unique feature of Dri 27/ZnT4 is the presence of a leucine zipper motif in the amino-terminal cytoplasmic region, which is absent in the other ZnT proteins. The presence of a leucine zipper in a transporter protein is not unusual (Ref. 21 and references therein), although in this latter class of proteins the function of this sequence motif has not been investigated thoroughly. By expressing the leucine zipper-containing fragment of Dri 27/ZnT4 as a GST fusion protein, we have demonstrated specific binding to two polypeptide bands of apparent molecular masses of 80 kDa and 100 kDa in the extracts of metabolically labeled Caco-2 and COS cells. The identity of these protein bands is presently under study, and it would be extremely interesting to elucidate whether their interaction with Dri 27/ZnT4 could either lead to the formation of a multisubunit transporter complex or play a role in functional regulation or intracellular compartmentalization of the transporter protein.

Having determined the colocalization of Dri 27/ZnT4 with β-adaptins, we have investigated whether the 100-kDa band might correspond to one of the subunits of equivalent molecular mass in the AP complexes (reviewed in Ref. 26). However, the results of immunoblotting experiments using antibodies against the adaptins α, β1, β2, and γ did not show identity of any of these subunits of AP-1 and AP-2 with the Dri 27/ZnT4 interacting polypeptides. Future work should be aimed at elucidating the regulatory mechanism of these interacting proteins, as well as at identifying which of the amino acid residues in the amino-terminal segment of the zinc transporter are responsible for such interaction.

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