Zinc Transport and Metallothionein Secretion in the Intestinal Human Cell Line Caco-2*

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Caco-2, a human cell line, displays several biochemical and morphological characteristics of differentiated enterocytes. Among these is the ability to transport zinc from the apical to the basal compartment. This process was enhanced following exposure by the apical compartment to increasing concentrations of the metal. High pressure liquid chromatography fractionation of the media obtained from cells labeled with radioactive zinc showed that metallothioneins (MTs), small metal-binding, cysteine-rich proteins, were present in the apical and basal media of controls as well as in cells grown in the presence of high concentrations of zinc. Following exposure to the metal, the levels of Zn-MTs in the apical medium increased, while in the basal compartment the greatest part of zinc appeared in a free form with minor changes in the levels of basal MTs. Metabolic labeling experiments with radioactive cysteine confirmed the apical secretion of MTs. A stable transfectant clone of Caco-2 cells (CL11) was selected for its ability to express constitutively high levels of the mouse metallothionein I protein. This cell line showed an enhanced transport of the metal following exposure to high concentrations of zinc and a constitutive secretion of the mouse metallothionein I protein in the apical compartment. Together, these findings strongly support the hypothesis of a functional role between the biosynthesis and secretion of MTs and the transport of zinc in intestinal cells.

Zinc is an element essential for growth, present in all eukaryotic organisms, where is found as cofactor in many enzymes and proteins (1, 2). Within cells, an appreciable amount of the metal is bound to metallothioneins (MTs), a family of small molecular weight proteins (6000 daltons) with a high content of cysteine residues (3, 4). MTs are present in many tissues, and their synthesis is transcriptionally regulated by a great number of molecules, such as heavy metals (zinc, copper, cadmium), corticosteroids, interleukins, interferon, serum growth factors, and 12-O-tetradecanoylphorbol-13-acetate among others. In humans, eight isoforms have been described, although with different tissue specificity (3–7). Several heavy metals like copper, cadmium, mercury, and zinc are able to bind MTs with different affinity (3). Each MT molecule is able to complex seven atoms of zinc with a binding affinity lower than the other heavy metals (1, 3, 8). Although the MTs accumulate in the cytosol, small amounts are also present in the serum and in the urine of mammals (9). The levels of MT can be regulated by the nutritional status of the animal, for example zinc-depletion (10) or by the exposure to metals in the environment (e.g. cadmium) (11).

In vertebrates, zinc is absorbed in the gut through the apical surface of enterocytes (12, 13). The molecular mechanisms involved in the transepithelial transport of the metal are at the present time still poorly understood. Zinc is thought to be transported by a carrier-mediated saturable process that may be energy-dependent (13). Previous studies with radioactive isotopes have established that the synthesis of MTs is induced within the enterocyte by parenteral or oral administration of zinc (14–16), while in rats and humans the secretion of zinc in the gastrointestinal tract is regulated by the dietary status of the metal (17, 18).

Data in this paper describe the transport of zinc in polarized Caco-2 cells, an in vitro model of enterocyte differentiation (19), and provide evidence of its role in the synthesis and secretion of MTs. Furthermore, zinc transport was found to be affected both by its concentration in the medium as well as by the expression of MT proteins, thus suggesting a cooperative relationship in the regulation of zinc transport in enterocyte cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—All culture reagents were supplied by Sigma. Fetal calf serum was from HyClone (Beiderland, Holland); permeable Transwell filter supports were from Costar Corning (New York, NY). Solid chemicals and liquid reagents were obtained from E. Merck (Darmstadt, Germany), Farmitalia Carlo Erba (Milan, Italy), and Serva Feinbiochemica (Heidelberg, Germany); SDS was purchased from BDH (Poole, United Kingdom). [35S]Cysteine (specific activity >1000 Ci/mmol) and 65Zn (activity between 14.01 and 38.81 mCi/mg) were obtained from...
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NEN Life Science Products; 14C-labeled protein molecular weight markers were from Amersham Pharmacia Biotech; the epithelial volt-ohm-meter was from Millipore Corp. (Bedford, MA); Bio-Rad-Sec 125 HPLC columns (300 × 7.8 mm, 5-μm particle size) were from Bio-Rad. The Roche Molecular Biochemicals (LH2) kit (Mannheim, Germany) was used to measure lactic dehydrogenase activities. Caco-2 cells were a gift of Dr. E. Roudriguez-Boulan (Cornell University Medical College, New York, NY).

Caco-2 Cell Culture—Cells were routinely grown on 100-mm Petri dishes at 37 °C in a mixture of 5% CO2, 95% air in Dulbecco’s modified minimal essential medium high glucose, supplemented with nonessential amino acids (80 μM), penicillin (2 μg/ml), streptomycin (2 μg/ml), 10% NSH, and 10% fetal calf serum containing 4.5 ng/ml zinc, as assayed by atomic flame spectrocopy. At passages between 75 and 90, cells were seeded on polycarbonate Transwell permeable filter supports and grown at confluence between 16 and 21 days. The integrity of the monolayer and the formation of tight junctions were proved by the high values of transepithelial electrical resistance (between 700 and 1000/6×105 ohm) and by the impermeability to radioactive inulin, a marker of paracellular transport. The potential toxic effect of zinc on the integrity of cell membranes was determined by spectroscopically measuring the reduced NAD+ produced by the activity of the cytosolic enzyme lactic dehydrogenase present in the media and in the cell lysates. The presence of microvilli on the apical membranes and the formation of tight junctions, as assessed by electron microscopy, confirmed the morphological differentiation of the cells. All experiments were carried out on cells grown on filter supports at full differentiation and cultured for 20 h in TMH medium containing Dulbecco’s modified minimal essential medium high glucose, deprived of bicarbonate and supplemented with 10 mM TES, 10 mM MOPS, 15 mM NaCl, 2 mM NaH2PO4, pH 7.3 in the absence of serum. Transport and labeling experiments were carried with the same medium without fetal calf serum for a maximum of 20 h.

Zinc Transport Studies—Radioactive 65Zn (14.01–38.81 μCi/mg) was supplied from the apical chamber (control, 5 μM ZnCl2). In metal-exposed cells, ZnCl2 was added to reach final concentrations of 50, 100, and 200 μM. After the 20-h pulse, apical and basal media were collected; the filters were washed twice with TMH medium, pH 7.3, and cells were lysed at 4 °C for 10 min with buffer containing 1% CHAPS. Cell lysates were centrifuged for 10 min at 4 °C in Eppendorf microcentrifuge at maximum speed. Incorporation of 65Zn was evaluated with a Beckman γ-counter.

Protein Labeling and Gel Analysis—Differentiated Caco-2 cells grown on filters were labeled for 20 h with 150 μCi/mmol [35S]L-cysteine (specific activity >1000 Ci/mmol) in complete medium containing 20 μM cold cysteine. Cells were then washed twice in ice-cold phosphate-buffered saline and lysed in 100 μl of 50 mM Tris-HCl, 150 mM NaCl, 5 mM dithiothreitol, 1% Triton X-100, pH 7.4 (20). The apical and basal media and the cell lysates were centrifuged for 10 min in a microcentrifuge at 4 °C, and the supernatant was stored at −20 °C. Incorporation of radioactive amino acid was determined by precipitating the proteins with trichloroacetic acid and counting the radioactivity in a Packard scintillation counter. For electrophoretic analysis, the same amount of radioactive proteins for each sample were acetone-precipitated, resuspended in 20 μl of H2O, reduced, alkylated (21), and analyzed on 20% SDS-polyacrylamide gel electrophoresis (22). After the run, gels were treated with Entensify, dried, and exposed at −80 °C for autoradiography.

HPLC Chromatography—65Zn-Labeled cell lysates or media were loaded on a Bio-Rad-Sec 125 HPLC column (300 × 7.8 mm, 5-μm particle size) equilibrated with 50 mM NaH2PO4, 150 mM NaCl buffer containing 10 mM Na2SO4 (pH 6.8). The column was eluted with the same buffer at a flow rate of 1 ml/min. Fractions of 0.33 ml were analyzed for zinc radioactivity. Purified equine metallothionein was used as standard to calculate the elution profile of Caco-2-derived human MTs.

Construction of the MT Overexpression Plasmid and Transfection Experiments—The plasmid pLTRMT was constructed by digesting the cDNA under the control of the promoter and enhancer from the Friend murine leukemia virus, digested with BglII and EcoRI to remove the CDS RNA and the second intron and the polyadenylation signal of the β-globin gene (24).

Caco-2 cells were cotransfected with the calcium phosphate method using the described pLTRMT plasmid and pRSVHygro (25), a plasmid carrying the resistance gene to hygromycin. Positive clones to the hygromycin resistance were screened for the expression of the mMT1 gene by Northern blot analyses. CL11 clone was chosen for high levels of mMT1 expression.

RESULTS

In Caco-2 Cells, Transport of Zinc Is Dependent on Concentrations of the Metal in the Apical Medium—Experiments of zinc transport were performed on Caco-2 cells grown on permeable filters between 16 and 21 days to reach a fully differentiated status. To test the integrity of cells treated with increasing concentrations of ZnCl2 (50–400 μM) for 20 h, we screened the activity of the cytosolic enzyme lactate dehydrogenase both in the apical and basal media. Cells exposed to ZnCl2 showed no major difference compared with control cells (Table I). A decrease was observed in the lactate dehydrogenase activities present in the apical media of cells exposed to high concentrations of the metal; since in these cells we routinely found higher values of the transepithelial electric resistance (data not shown), zinc positively regulates the tightness of the junctions and/or the stability of the membranes. Taken together, these results suggested us that, under the experimental conditions used, metal exposure did not affect cell integrity. Thereafter, all subsequent experiments were carried out with metal concentrations up to 200 μM for a maximum of 20 h.

65Zn transport was assayed into Caco-2 cells at increasing concentrations of the metal in the apical chamber. In these conditions, the transport from the apical toward the basal chamber was achieved in both cell types (Fig. 1). It is noteworthy that the transport increased with time from 6 to 20 h reaching a peak when the cells were exposed to higher (50–200 μM) concentrations of ZnCl2. The amount of Zn2+ transported in 20 h was calculated to be 0.140 ± 0.02 nmol of Zn2+/cm2 in the basal chamber in the control cells (mean ± S.E., n = 8). In the presence of 50, 100, and 200 μM ZnCl2, transport increased to 1.99 ± 0.02, 7.52 ± 0.01, and 13.08 ± 0.02 nmol/cm2 respectively (Fig. 1, A–C).

Distribution of Zinc in the Cell Lysates and in the Media of Cells Exposed to High Concentrations of the Metal—We next monitored by HPLC chromatography the distribution of zinc (free or in a chelated form) in the apical and basal media as well as in the cell lysates. The Zn2+ distribution was examined in cells grown in the presence of 5 μM ZnCl2 (control cells) (Fig. 2, A–C). Zinc was transported for 20 h in the apical compartment either at 50 (Fig. 2, D–F) or 100 μM ZnCl2 (Fig. 2, G–I). These concentrations of Zn2+ in the apical chamber corresponded to a total of 500, 5000, and 10,000 nmol of total Zn2+, respectively.

As shown in Fig. 2, zinc transport into the basal chamber was similar to that seen in Fig. 1. While in the control cells, 0.12 nmol of ZnCl2/cm2 were transported, in cells exposed to 50 or 100 μM ZnCl2, the nmol of ZnCl2/cm2 were 4.77 and 7.52, respectively.

| Concentration (μM) | Apical (nmol/cm²) | Basal (nmol/cm²) | Lysates (nmol) |
|-------------------|------------------|------------------|---------------|
| Control           | 0.140 ± 0.02     | 0.01 ± 0.02      | 98.7 ± 0.00   |
| 50                | 1.99 ± 0.02      | 0.08 ± 0.00      | 99.04 ± 0.02  |
| 100               | 7.52 ± 0.01      | 0.07 ± 0.00      | 99.04 ± 0.02  |
| 200               | 13.08 ± 0.02     | 0.08 ± 0.00      | 99.04 ± 0.02  |

TABLE I Lactic dehydrogenase activity in control and zinc-exposed Caco-2 cells

Values are the mean of six different experiments and are expressed as percentage of the total enzymatic activity detected in the cell lysates and apical and basal media. Metal-induced cells were exposed to zinc for 20 h.
respectively, equivalent to 40,157 and 2482 pmol of Zn\(^{2+}\) (Fig. 2, B, E, and H, and Table II). In the cell lysates as well as in the apical and basal media, Zn\(^{2+}\) was distributed in several peaks. Purified human and rabbit MT markers separated by HPLC chromatography eluted with a peak with retention time at 10 and 21 min (Fig. 2). Unbound zinc had a retention time between 11.3 and 21 min (Fig. 2K). This elution time does not exclude the possibility that part of the metal found in these fractions can also be bound in the apical and basal media to small molecules, like amino acids (i.e. cysteine and histidine) or glutathione.

Analyses of the distribution of \(^{65}\)Zn in the cellular lysates of the control cells with the same technique showed that part of the metal co-eluted with the MT fractions, while the rest appeared associated with proteins of different molecular weights, having retention times between 5 and 9 min (Fig. 2C). The fraction of radioactive zinc associated with MTs increased from 51.9 pmol of Zn\(^{2+}\) in control cells to 215.6 and 420.1 pmol of Zn\(^{2+}\) in cells exposed to either 50 or 100 \(\mu\)M ZnCl\(_2\) (Fig. 2, C, F, and I, and Table II, retention times 9–11).

Interestingly, in both the apical and basal media one of the major peaks of zinc had the same retention times as the intracellular MTs and the purified MT markers (Fig. 2, retention times 9–11; compare A, B, D, E, G, and H with J), thus indicating the presence of secreted MTs. In the absence of metal exposure, zinc was present mainly as Zn-MT complexes in both the basal and the apical compartments of control cells (Fig. 2, A and B). Following exposure to increased concentrations of Zn\(^{2+}\) in the apical chamber, two changes in Zn\(^{2+}\) distribution were noted. First, there was a marked increase of the unbound form, which increased from 6.4 pmol in control cells to 1120.6 and 1955.5 pmol in cells grown in the presence of 50 and 100 \(\mu\)M ZnCl\(_2\), respectively (Table II and Fig. 2, retention times 13–21; compare B with E, H, and K). Second, the levels of the Zn-MT complexes in the apical media were higher than in the corresponding basal compartments and increased consistently from 184.9 pmol in control cells to 709 and 910.2 pmol in cells exposed to 50 and 100 \(\mu\)M ZnCl\(_2\), respectively (Table II and Fig. 2, retention times 9–11; compare A, D, and G with B, E, and H).

We also found high levels of unbound zinc in the apical medium of metal-exposed cells (Fig. 2, retention times 11.3–21; D and G). Although the apical compartment was the site of loading of the metal at the beginning of the experiment, we cannot rule out the hypothesis that an aliquot was derived from a process of secretion of the cells, as suggested by pulse-chase experiments and the ability of Caco-2 cells to transport the metal from the basal to the apical compartment.\(^2\)

**Synthesis and Secretion of Metallothioneins in Zinc-exposed Cells**—One approach to confirm the data of the secretion of MTs in the extracellular compartments obtained in the previous experiment consisted in labeling Caco-2 cells for 20 h with \(^{35}\)S-cysteine in presence or absence of 200 \(\mu\)M ZnCl\(_2\) in the apical chamber. The radioactivity present in both cell extracts and media were then analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3).

We found that induced Caco-2 cells synthesized MTs (Fig. 3, compare lanes 1 and 2). Exposure to 200 \(\mu\)M ZnCl\(_2\) induced the accumulation in the apical medium of MTs (Fig. 3, compare lanes 3 and 4), in agreement with the experiment of labeling with radioactive zinc described in Fig. 2. No effect was observed on the levels of MTs in the basal medium (Fig. 3, compare lanes 5 and 6).

We also found the presence of discrete amounts of other cysteine-rich proteins in the apical and basal media (Fig. 3, lanes 3–6). However, these secreted proteins showed higher molecular weights compared with MTs and did not increase their biosynthesis in response to metal excess.

The **Overexpression of the mMTI Protein Positively Regulates the Transport of Zinc in Cells Grown in the Presence of High Concentrations of the Metal**—The concurrent association of biosynthesis and secretion of MTs with the transport of zinc suggests an active role for these proteins in the mobilization of pools of the metal between the cell and the apical and basal compartments. We therefore analyzed the effect of the constitutive expression of MTs on the transport of zinc and on the distribution of MTs in the apical and basal media.

Caco-2 cells were cotransfected permanently with a plasmid conferring resistance to the drug hygromycycin and another plasmid expressing the mouse mMTI gene under the control of the long terminal repeat promoter of the Friend murine leukemia virus. Several clones were isolated, and one of them, CL11, was chosen for the ability to express constitutively high levels of the mMTIa isoform. Fig. 4 shows the gel fractionation of proteins obtained from the metabolic labeling of the cells for 20 h with \(^{35}\)S-cysteine in presence or absence of 200 \(\mu\)M ZnCl\(_2\) supplemented from the apical compartment. CL11 cells did synthesize the mMTI constitutively (Fig. 4, lane 3); this protein migrated slightly more slowly than human MTs, as demonstrated by similar experiments performed on other murine cell lines (data not shown); following exposure to zinc, CL11 cells accumulated both mMTI and human MTs (Fig. 4, compare lane 4 with lanes 2 and 3). Analysis of the apical media from CL11 cells showed that the overexpression of the mMTI isoform allowed its constitutive secretion (Fig. 4, lane 7) and that the exposure of cells to zinc further stimulated the secretion of MTs (Fig. 4, compare lane 8 with lanes 6 and 7). In the basal medium, no major differences in the levels of MTs were observed in metal-exposed cells or controls of normal and transfected cells (data not shown).

We then compared the transport of Zn\(^{2+}\) in the apical compartment in Caco-2 versus CL11 cells, over a period of 6 h at concentrations of 5 \(\mu\)M ZnCl\(_2\) (control cells) and 50 or 200 \(\mu\)M ZnCl\(_2\), respectively (Fig. 5). In the control cells, there was no difference in the transport of \(^{65}\)Zn. At the higher Zn\(^{2+}\) concentrations in the apical chamber, CL11 cells showed a greater

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\(^2\) O. Molletto, X. Alvarez-Hernandez, and A. Leone, unpublished results.
transport of Zn$^{2+}$ into the basal chamber. Therefore, the higher intracellular levels of MTs in the transfected cells increased the mobilization of zinc from the apical toward the basal compartment, but only after exposure of the cells for several hours to zinc, suggesting that the enhancement of the transport of the metal requires the participation of different biochemical components (i.e. transporters, MT, and others), whose activity and/or biosynthesis should be, at least in part, zinc-dependent.

**DISCUSSION**

In mammals the absorption of zinc occurs almost exclusively in the small intestine through saturable and nonsaturable mechanisms (13, 26). The molecular intermediates responsible for such processes have not been fully identified. The recently cloned divalent metal transporter (DMT1) (27) appears to be able to mediate the uptake of iron as well as other ions, including zinc (14). Within the cells, a family of zinc transporters with different intracellular localization have been isolated: ZnT1, ZnT2, ZnT3, and ZnT4 (28–31). None of these transporters appear to be present exclusively in the intestine, and all display different tissue specificity. For example, ZnT3 expression is limited to the nervous system and the testis (30). Therefore, the control of zinc homeostasis appears to be a complex mecha-

**TABLE II**

| Values are expressed in pmoles, calculated according to the amount of radioactive and cold zinc detected in the fraction of the HPLC chromatography experiment described in Fig. 2. | **Apical** | **Basal** | **Lysates** |
| --- | --- | --- | --- |
| Control | TRT 245.8 | TRT 40 | TRT 129.9 |
| | RT 9–11 184.9 | RT 9–11 8.5 | RT 9–11 51.9 |
| | RT 11.3–21 50.3 | RT 11.3–21 6.4 | RT 11.3–21 33.9 |
| 50 μM ZnCl$_2$ | TRT 2564 | TRT 1577 | TRT 740 |
| | RT 9–11 709 | RT 9–11 345.9 | RT 9–11 215.6 |
| | RT 11.3–21 1756.4 | RT 11.3–21 1120.6 |
| 100 μM ZnCl$_2$ | TRT 5456 | TRT 2482 | TRT 1501 |
| | RT 9–11 910.2 | RT 9–11 251.1 | RT 9–11 420.1 |
| | RT 11.3–21 4295.4 | RT 11.3–21 1955.5 |
anism, mediated in different tissues by the presence and/or the modulation of the activity of various intermediates (i.e. synthesis and activity of transporters, synthesis of zinc-binding proteins like MTs, regulation of the levels of glutathione, and others), most probably according to the need of the metal and/or to the exposure to different concentrations.

We found that Caco-2 cells, a well characterized model of in vitro differentiation of human enterocytes (19), were constitutively able to transport zinc from the apical toward the basal compartment and to secrete MTs. The process of secretion of MTs appeared polarized toward the apical compartment and regulated by the concentrations of zinc in the medium; i.e. following exposure to the metal, an enhancement of both the transport of zinc toward the basal chamber and the secretion of MTs toward the apical compartment was observed. In the basal medium, HPLC chromatography showed that the greatest part of zinc was found as free metal, while a minor aliquot was bound to MTs.

This is the first observation of the secretion of MTs in polarized cells. Little information is available about the mechanisms controlling the passage of leaderless secretory (LLS) proteins through membranes, and either translocation (of the plasma...
membrane or of intracellular membranes) or pinching off from the plasma membranes of vesicles enriched in a given LLS have been proposed (32, 33). In prokaryotes and lower eukaryotes, pathways of secretion of LLS proteins use dedicated ATP-binding cassette membrane transporters (33). In high eukaryotes, direct evidence for the participation of the ATP-binding cassette transporters in the secretion of LLS proteins is still not available, although it has been shown that drugs such as glibenclamide that block ATP-binding cassette activity also inhibit the secretion of LLS proteins (34).

We do not know at the moment if MTs are secreted in a metal-free or in a metal-chelated form. In the first case, we hypothesize the existence of two distinct mechanisms, one allowing the translocation of the apo-MT across the cellular membrane and the other regulating the efflux of zinc. Reconstitution of the metal-MT would than occur by protein folding in the media, where free zinc ions would be present. According to the properties of rapid exchange of the metal from the β and α domains of MTs and to other MTs (35, 36), it is conceivable in the media, where free zinc ions would be present. According to the properties of rapid exchange of the metal from the β and α domains of MTs and to other MTs (35, 36), it is conceivable that the free and the MT-chelated zinc pools present in the apical and basal medium could be interchanged, at least in some part, and that several environmental factors (such as pH, presence of metal-binding proteins like albumin, or reducing agents) could modulate this effect. The secretion of MTs appears independent from the type of metal bound; we found that Caco-2 and Madin-Darby canine kidney cells, a kidney-derived dog polarized cell line, were able to accumulate MTs in the apical medium following not only zinc, but also cadmium and copper exposure (data not shown). Other laboratories reported the presence of Zn-MTs, as well as Cu- and Cd-MTs, in the blood and urine of rodents and humans (9–11), with the levels of MT dependent upon the nutritional or environmental exposure to metals. Plasma Zn-MT levels, for example, appeared to be influenced by the nutritional intake of the metal and were higher in normal zinc-fed rats than in zinc-deprived animals (10, 11). Finally, we observed that the secretion of MTs was regulated not only by the extracellular concentrations of zinc, but also by the intracellular levels of the same proteins, as shown by the constitutive secretion of the mMTI protein in the stable transfected cell line CL11.

Different hypotheses have been raised on the role of MTs in the control of zinc metabolism in the gut; some authors have postulated that these proteins could sequester the metal and render it unavailable for transfer to the circulating plasma (37, 38), while others favor a more active function in the general mechanism of transport of the metal (18). Our experiments show that in Caco-2 cells MTs were present in the medium and in the cells, both in basal conditions and following exposure to high concentrations of the metal. MTs bound significant levels of zinc in the apical compartment, and their presence in it increased in metal-exposed cells. In permanently transfected CL11 cells, the constitutive overexpression of the mouse mMTI protein increased the transport of the metal, but only in cells grown in presence of high concentrations of zinc. Thus, MTs per se are not able to mobilize the metal, but they contribute to its transport, with a need for other yet not characterized metal-dependent biochemical mechanisms. These mechanisms might include, for example, an increase in the kinetics of transporters or the stimulation of the activities of metal chaperones. Interestingly, evidence of metal-mediated trafficking of proteins has already been reported; both the Menkes (ATP7A) and the Wilson (ATP7B) proteins, two copper-binding P-type ATPases that regulate the efflux of the metal, mobilize toward an endosomal compartment after increase of the extracellular concentrations of copper (39, 40).

Similar conclusions for a role of MT to act as a zinc pool have been suggested by Davis et al. (41). In MT transgenic mice containing a high number of copies of the mMT-I gene in their genome, the elevated levels of the protein were not associated with greater intestinal zinc accumulation, while in MT knock out mice zinc treatment increased the intestinal zinc concentration significantly compared with the zinc-treated animals. In the latter case, the absence of MT would explain the elevated levels of zinc found in the serum and in the intestine, possibly due to an inefficient mucosa-to-lumen flux.

Taken together, our results demonstrate that the exposure of the apical membrane of Caco-2 cells to high levels of zinc achieves at the same time three different, important effects on the cellular metabolism of the metal. First, Zn$^{2+}$ activates the transport machinery. Second, Zn$^{2+}$ enhances the accumulation of MTs, which contributes in zinc-exposed cells to an increase in the transport of the metal, as shown by the experiments of the overexpression of the mMTI protein. Third, Zn$^{2+}$ increases the levels of secreted MTs, especially into the apical compartment. In vivo, these tightly regulated molecular mechanisms would coordinately link two important aspects of the metabolism of zinc in intestinal cells: the increased absorption in the presence of high levels of metal in the diet and the removal of the excess of zinc as a MT-chelated form in the lumen of intestine (Fig. 6).

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