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

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Running title: Zinc transport and MT secretion in Caco-2 cells

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SUMMARY

Caco-2 cells, a human cell line, displays several biochemical and morphological characteristics of differentiated enterocytes. Among these they were able to transport zinc from the apical to the basal compartment. This process was enhanced following exposure from the apical compartment to increasing concentrations of the metal. HPLC fractionation of the media obtained from cells labelled 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 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 (mMTI).This cell line showed an enhanced transport of the metal following exposure to high concentrations of zinc and a constitutive secretion of the mMTI 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.

Keywords:: metallothionein / zinc / metal-transport / protein secretion / intestinal cells

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INTRODUCTION

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, (6,000 daltons) with a high content of cysteine residues (3, 4). MTs are present in many tissues, and their synthesis is transcriptionally regulated by a wide numbers of molecules, such as heavy metals (zinc, copper, cadmium), corticosteroids, interleukins, interferon, serum growth factors, and TPA among others. In human eight isoforms have been described, even though 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 7 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 describes the transport of zinc in polarized Caco-2 cells, an *in vitro* model of enterocyte differentiation (19), and provide evidences of its role in the synthesis and secretion of MTs. Furthermore, zinc transport was found to be affected both from its concentration in the medium as well as from the expression of MT proteins, thus suggesting a cooperative relationship in the regulation of zinc transport in enterocyte cells.

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EXPERIMENTAL PROCEDURES

Materials - All culture reagents were supplied by Sigma Chemicals (St. Louis, MO, USA). Fetal Calf Serum was from Hyclone, Beiderland, Holland; permeable Transwell filter supports were from Costar Corning, New York, NY, USA. Solid chemicals and liquid reagents were obtained from E. Merck, Darmstadt, Germany, Farmitalia Carlo Erba Milan, Italy, Serva Feinbiochemica Heidelberg, Germany; SDS was purchased from BDH Poole, UK. [^{35}S]cysteine (specific activity > 1000 Ci / mmol) and [^{65}Zn] (activity between 14.01 and 38.81 mCi / mg) were obtained from Du Pont-New England Nuclear, Boston, MA, USA; ^{14}C-molecular weight markers were from Amersham Life Sciences, Bucks, England; epithelial volt-ohmmeter was from Millipore, Bedford, Ma, USA; Biorad-Sec 125 HPLC columns (300 x 7.8 mm, 5 μm particle size) were from Biorad (Hercules, CA, USA). The Boehringer (LDH) kit, Mannheim, Germany, was used to assay lactic dehydrogenase activities. Caco-2 cells were a kind gift of Dr. E. Roudriguez-Boulan, Cornell University Medical College, New York, NY, U.S.A.

CaCo-2 cells culture - Cells were routinely grown on 100 mm Petri dishes at 37° C in a mixture 5% CO_2- 95% air in Dulbecco Modified Minimal Essential Medium high glucose (DMEM), supplemented with non essential aminoacids, Penicillin 60 u/ml, Glutamine 2 mM, Streptomycin 100 u/ml and 20% fetal calf serum containing 4.5 ng / ml zinc, as assayed by atomic flame spectroscopy. 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 (TEER) (between 700 and 1000 W / well) 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.

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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 on cells grown on filter supports at full differentiation and cultured for 20 h in TMH Medium containing DMEM, deprived of bicarbonate and supplemented with 10 mM TES, 10 mM MOPS, 15 mM HEPES, 2 mM NaH_2PO_4, pH 7.3 in absence of serum. Transport and labelling experiments were carried with the same medium without FCS for a maximum of 20 h .

Zinc Transport Studies -- Radioactive ^{65}Zn (14.01-38.81 mCi / mg) was supplemented from the apical chamber (control, 5 µM $ZnCl_2$). In metal-exposed cells, $ZnCl_2$ 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 10 min at 4° C in Eppendorf microfuge at maximum speed. Incorporation of ^{65}Zn was evaluated with a Beckman gamma counter.

Protein Labelling and Gel Analyses - Differentiated Caco-2 cells grown on filters were labelled for 20 h with 150 µCi / ml ^{35}S-cysteine (specific activity > 1000 Ci / mmole) in complete medium containing 20 µM cold cysteine. Cells were then washed twice in ice cold phosphate buffered saline (PBS) and lysed in 100 µl 50 mM Tris-HCl, 150 mM NaCl, 5 mM DTT, 1% Triton X-100 pH 7.4 (20). The apical and basal media and the cell lysates were centrifuged for 10 min in a microfuge at 4° C and the supernatant was stored at -20° C. Incorporation of the labeled aminoacid was determined by precipitating the proteins with trichloroacetic acid and counting the radioactivity in a Packard scintillation counter. For electrophoretic analysis, the same amounts of radioactive proteins for each sample were acetone-precipitated, resuspended in 20 µl H_2O, reduced, alkylated (21) and analyzed on 20% SDS-PAGE (22). After the run, gels were treated with Entensify, dried and exposed at -80° C for autoradiography.

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HPLC Chromatography - ^{65}Zn labelled cell lysates or media were loaded on a Biorad-Sec 125 HPLC column (300 x 7.8 mm, 5 µm particle size) equilibrated with NaH_2PO_4 50 mM, NaCl 150 mM buffer containing 10 mM NaN_3 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 analysed for zinc radioactivity. Purified equine metallothionein was used as standard to calculate 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 murine MTI gene (23) with the BglII restriction enzyme, at the site corresponding to the 5' untranslated region of the mRNA in the first exon, and with the EcoRI restriction enzyme, at the site corresponding to the end of the entire 3' flanking region of the gene. This DNA fragment was inserted in the expression plasmid pFLTR containing the human CD8 cDNA under the control of the promoter and enhancer from the Friend murine leukaemia virus, digested with BglI and EcoRI to remove the CD8 cDNA and the second intron and the polyadenylation signal of the -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 mMTI gene by Northern blot analyses. CL11 clone was chosen for high levels of mMTI expression.

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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 $ZnCl_2$ (50 - 400 µM) for 20 h, we screened the activity of the cytosolic enzyme lactate dehydrogenase (LDH) both in the apical and basal media. Cells exposed to $ZnCl_2$ showed no major difference compared to control cells (Table I). A decrease was observed in the LDH 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 (TEER) (data not shown), it results that zinc postively 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 cells integrity. Thereafter, all subsequent experiments were carried out with metal concentrations up to 200 µM for a maximum of 20 h.

^{65}Zn 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 h to 20 h reaching a peak when the cells were exposed to higher (50 to 200 µM) concentrations of $ZnCl_2$. The amount of Zn^{2+} transported in 20 h was calculated to 0.140 ± 0.02 nmoles of Zn^{2+} / cm^2 in the basal chamber in the control cells (mean ± SE, n=8). In the presence of 50, 100 and 200 µM $ZnCl_2$ transport increased to 4.38 ± 1.23, 7.52 ± 1.15 and 13.08 ± 2.50 nmoles transported into the basal chamber (mean ± SE, n=8), respectively (Fig. 1).

***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 Zn^{2+} distribution was examined in cells, grown in presence of 5µM $ZnCl_2$ (control cells) (Fig.

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2, panels A, B, C) or cells exposed for 20 h in the apical compartment either to 50 (Fig. 2, panels D, E, F), or 100 μM $ZnCl_2$ (Fig. 2, panels G, H, I). These concentrations of Zn^{2+} in the apical chamber corresponded to a total of 500, 5000 and 10000 pmoles of total Zn^{2+}, 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 nmoles of $ZnCl_2$ / cm^2 were transported, in cells exposed to 50 or 100 μM $ZnCl_2$, the nmoles of $ZnCl_2$ / cm^2 were 4.77 and 7.52, respectively, equivalent to 40, 1577 and 2482 pmoles of Zn^{2+} (Fig. 2, panels B, E, 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 min (Fig. 2, panel J). Unbound zinc had a retention time between 11.3 and 21 min (Fig. 2, panel K).This elution time do 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 aminoacids (i.e. cysteine, 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 MTs fractions, while the rest appeared associated with proteins of different molecular weights, having retention times between 5 and 9 min (Fig. 2, panel C). The fraction of radioactive zinc associated with MTs increased from 51.9 pmoles of Zn^{2+} in control cells to 215.6 and 420.1 pmoles of Zn^{2+} in cells exposed to either 50 or 100 μM $ZnCl_2$ (Fig. 2, panels C, F, 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 panels A, B, D, E, G, H to panel J), thus indicating that 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, panels A, 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 pmoles in control cells to 1120.6 and 1955.5 pmoles in cells grown in presence of 50 and 100 μM $ZnCl_2$, respectively (Table II and Fig. 2, Retention Times 13-21, compare panel B to panels E, H

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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 pmoles in control cells to 709 and 910.2 pmoles in cells exposed to 50 and 100 μM $ZnCl_2$, respectively (Table II and Fig. 2, Retention Times 9-11, compare panels A, D, G to panels B, E, H).

We also found high levels of unbound zinc in the apical medium of metal-exposed cells (Fig. 2, Retention Times 11.3-21, panels D, G). Even though the apical 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 (Moltedo, Alvarez-Hernandez and Leone, unpublished results).

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 μM $ZnCl_2$ in the apical chamber. The radioactivity present in both cell extracts and media were than analysed by SDS PAGE (Fig. 3).

We found that induced Caco-2 cells synthesised MTs (Fig. 3, compare lanes 1 and 2). Exposure to 200 μM $ZnCl_2$ induced the accumulation in the apical medium of MTs (Fig. 3, compare lanes 3 and 4), in agreement with the experiment of labelling 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 to MTs and did not increase their biosynthesis in response to metal excess.

The overexpression of the mouse MTI protein positively regulates the transport of zinc in cells grown in presence of high concentrations of the metal.

The concurrent association of biosynthesis and secretion of MTs with the transport of zinc

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suggests an active role for these proteins in the mobilisation of pools of the metal between the cell and the apical and basal compartments. We therefore analysed 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 the resistance to the drug hygromycin 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 mouse MTIa isoform. Fig. 4 shows the gel fractionation of proteins obtained from the metabolic labelling of the cells for 20 h with ^{35}S-cysteine in the presence or absence of 200 μM $ZnCl_2$ supplemented from the apical compartment. CL11 cells did synthesise the mouse MTI constitutively (Fig. 4, lane 3); this protein migrated slightly slower 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 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 hours at concentrations of 5 μM $ZnCl_2$ (control cells) and 50 or 200 μM $ZnCl_2$, 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 transport of Zn^{2+} into the basal chamber. Therefore, the higher intracellular levels of MTs in the transfected cells increased the mobilisation 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, others), whose activity and/or biosynthesis should be - at least in part - zinc-

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dependent .

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DISCUSSION

In mammals the absorption of zinc occurs almost exclusively in the small intestine through saturable and non saturable 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 localisation 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 mechanism, 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, 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 characterised model of *in vitro* differentiation of human enterocytes (19) were constitutivably able to transport zinc from the apical toward the basal compartment and to secrete MTs. The process of secretion of MTs appeared polarised 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 polarised 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 (ABC) membrane transporters (33). In high

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eukaryotes direct evidence for the participation of the ABC transporters in the secretion of LLS proteins is still not available, although it has been shown that drugs such as glibenclamide which blocks ABC 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 should hypothesise the existence of two distinct mechanisms, one allowing the translocation of the apoMT across the cellular membrane, 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 and 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 MDCK cells, a kidney-derived dog polarised 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 postulated that these proteins could sequester the metal and render it unavailable for transfer to the circulating plasma (37, 38), while others favoured 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

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following exposure to high concentrations of the metal. MTs bound significative 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 mobilise the metal, but contribute to its transport, with the need of other yet not characterised 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 have already been reported: both the Menkes (ATP7A) and the Wilson (ATP7B) proteins, two copper-binding P-type ATPases which regulates the efflux of the metal, mobilise 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 to 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 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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LEGENDS TO FIGURES

Fig.1 Dose and time dependence of the transport of zinc in the basal chamber of Caco-2 cells.

Differentiated Caco-2 cells cultured for between 18 and 21 days after seeding on polycarbonate permeable filter supports were grown for 20 h in absence of serum and than incubated for 6, 12 and 20 h with either trace doses of ^{65}Zn (control, 5 μM $ZnCl_2$), or 50, 100 and 200 μM $ZnCl_2$ supplemented from the apical chamber in TMH medium. At the end of the pulse, filters were washed twice with TMH medium pH 7.3 and the amounts of ^{65}Zn in the basal medium were determined with a Beckman gamma counter. Abscissa, Time of induction. Ordinate, nmoles of Zn^{2+} / cm^2. Values are the mean ± S.E. (n =8) (control, 200 μM) and (n =3) (50 and 100 μM).

Fig.2. Separation by HPLC chromatography of ^{65}Zn labelled cell lysates and media from control and metal-exposed Caco-2 cells.

Differentiated Caco-2 cells on polycarbonate permeable filter supports were labelled with trace amounts of ^{65}Zn (activity 14.01 mCi / mg) supplemented from the apical chamber in TMH medium for 20 h in absence (control, 5 μM $ZnCl_2$) or presence of $ZnCl_2$ to reach final concentrations of 50 or 100 μM. At the end of the pulse, cells lysates (C, F, I) apical (A, D, G) and basal (B, E, H) media were separated by HPLC chromatograpy on a Biorad-Sec 125 column. The amount of ^{65}Zn radioactivity was determined using a gamma scintillation counter and the total content of zinc in each fraction was normalized according to the amount of cold zinc added. Abscissa, retention times in minutes. Ordinate, pmoles of total Zn^{2+}. On the right side of the figure the migration of equine metallothionein (J) and ^{65}Zn marker (K) are shown. Ordinate J, absorbance at A280. Ordinate K, cpm x 10^{-3}.

Fig.3. Effect of zinc exposure on protein biosynthesis in Caco-2 cells. Differentiated Caco-2 cells on polycarbonate permeable filter supports were grown for 20 h in absence of serum and then incubated for 20 h with 150 μCi / ml ^{35}S-Cysteine (specific activity > 1000 Ci / mmole) in TMH medium containing 20 μM cold cysteine, in presence (+) or absence (-) of 200

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μM $ZnCl_2$ in the apical chamber. Incorporation of the radioactive aminoacid was evaluated after TCA precipitation and equivalent amounts of labelled lysates (Lanes 1, 2), apical (Lanes 3, 4) and basal media (Lanes 5, 6) were concentrated by acetone precipitation, reduced, alkylated and separated on 20% SDS-PAGE. Migration of the ^{14}C-labelled protein molecular weight markers and MTs are indicated on the left and on the right side, respectively.

Fig. 4. Synthesis and secretion of MT in Caco-2 and CL11 cells.

Differentiated Caco-2 (Lanes 1, 2, 5, 6) and CL11 cells (Lanes 3, 4, 7, 8) on polycarbonate permeable filter supports were grown for 20 h in absence of serum and then incubated for 20 h with 150 μCi / ml ^{35}S-Cysteine (specific activity > 1000 Ci / mmole) in TMH medium containing 20 μM cold cysteine, in presence (+) or absence (-) of 200 μM $ZnCl_2$ in the apical chamber. Incorporation of the radioactive amminoacid was evaluated after TCA precipitation and equivalent amounts of labelled lysates (Lanes 1-4) and apical media (Lanes 5-8) were concentrated by acetone precipitation, reduced, alkylated and separated on 20% SDS-PAGE. Only the lower part of the original gel is shown. Migrations of the ^{14}C-labelled protein molecular weight markers and human and murine MTs are indicated on the left and on the right side, respectively.

Fig. 5. Transport of zinc in Caco-2 and CL11 cells.

Differentiated Caco-2 or CL11 cells between 18 and 21 days after seeding on polycarbonate permeable filter supports were grown in TMH medium for 20 h in absence of serum and than incubated for 6 h with either trace doses of ^{65}Zn (control, 5 μM $ZnCl_2$), or presence of $ZnCl_2$ to reach final concentrations of 50 or 200 μM in the apical chamber. At the end of the pulse, filters were washed twice with TMH medium pH 7.3 and the amounts of ^{65}Zn in the medium were determined with a Beckman gamma counter. Abscissa, Time of induction. Ordinate, nMoles of Zn^{2+} / cm^2. Values are the mean ± S.E. (n =3) .

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Fig. 6. Model of the transport of zinc and metallothionein secretion in intestinal cells. A model of intestinal cells grown in presence of control (-) or elevated (+) levels of $ZnCl_2$ in the apical chamber is shown. Basal transport of zinc and secretion of MTs in control cells are indicated with broken arrows. Increased transport of zinc and secretion of MTs in cells grown in excess metal are indicated with thick arrows. Circles indicate Metallothionein proteins.

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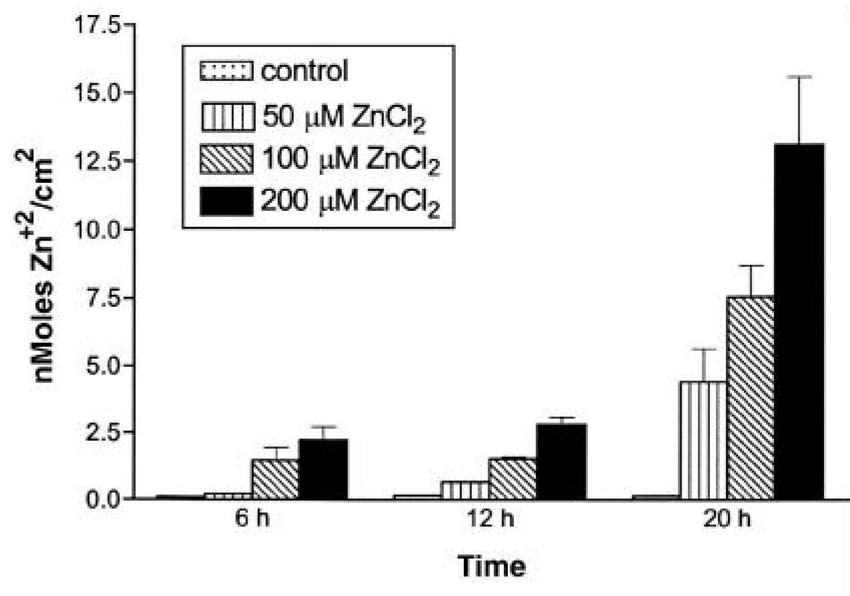

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APICAL BASAL LYSATES

Control

50

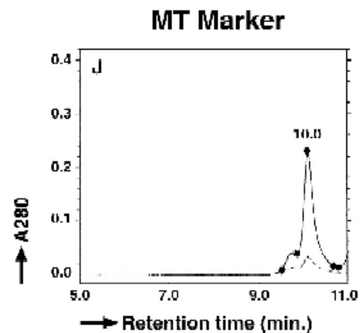
100

A B C D E F G H I

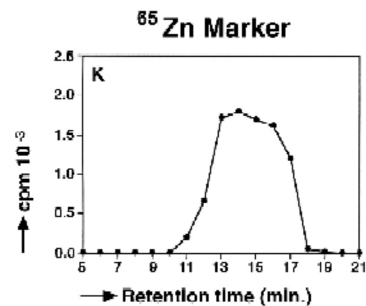
pMoles of Zinc

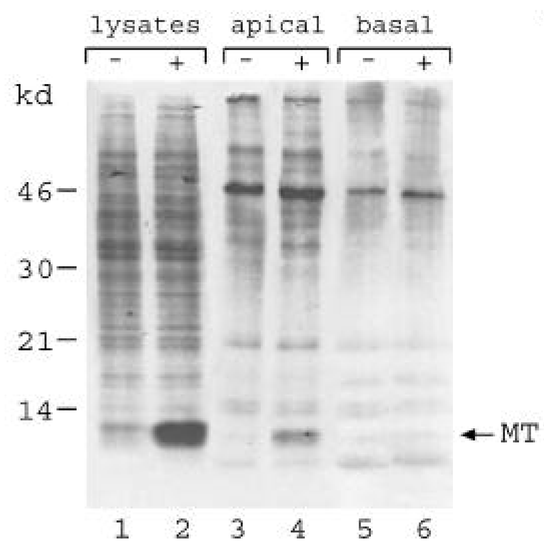
Retention time (min.)

MT Marker



^{65}Zn Marker





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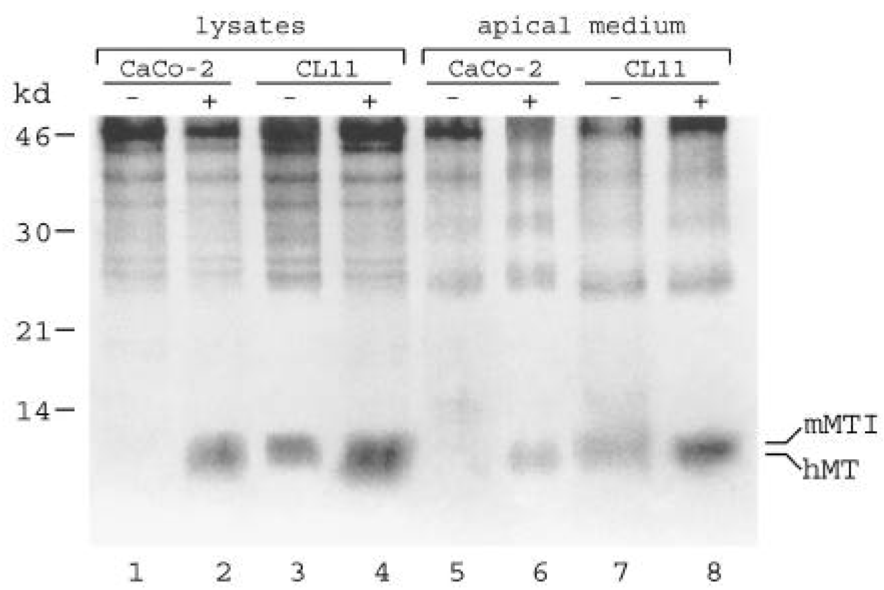

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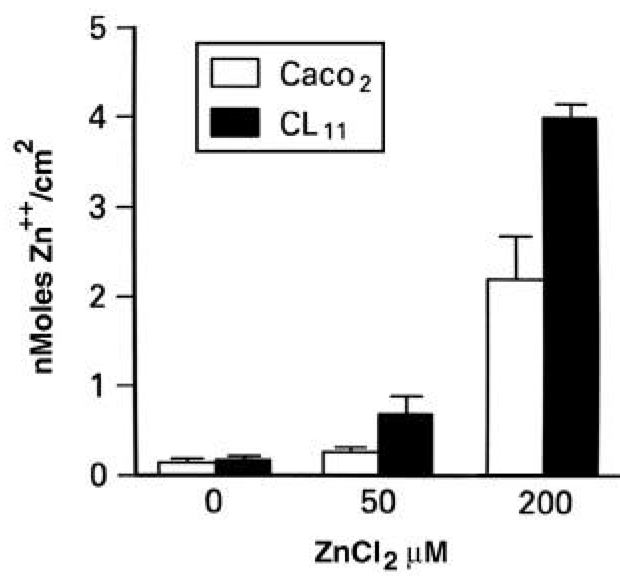

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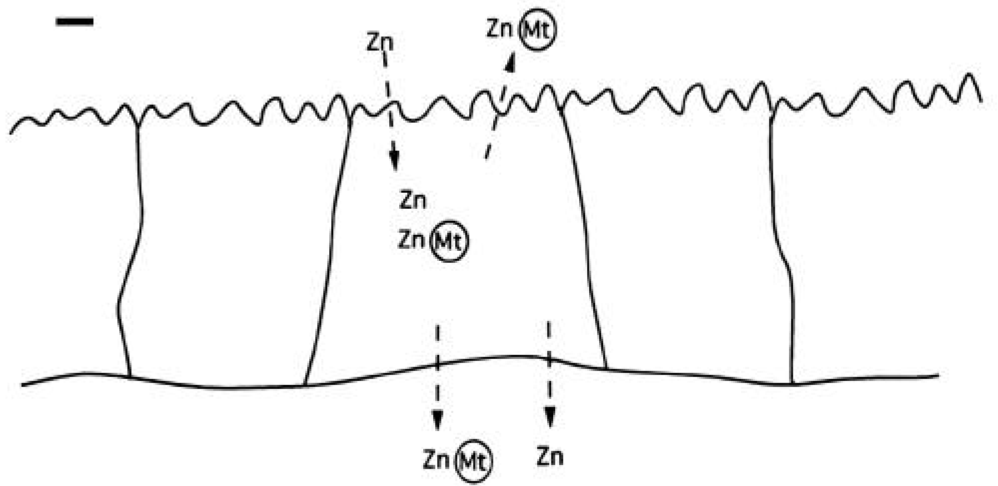

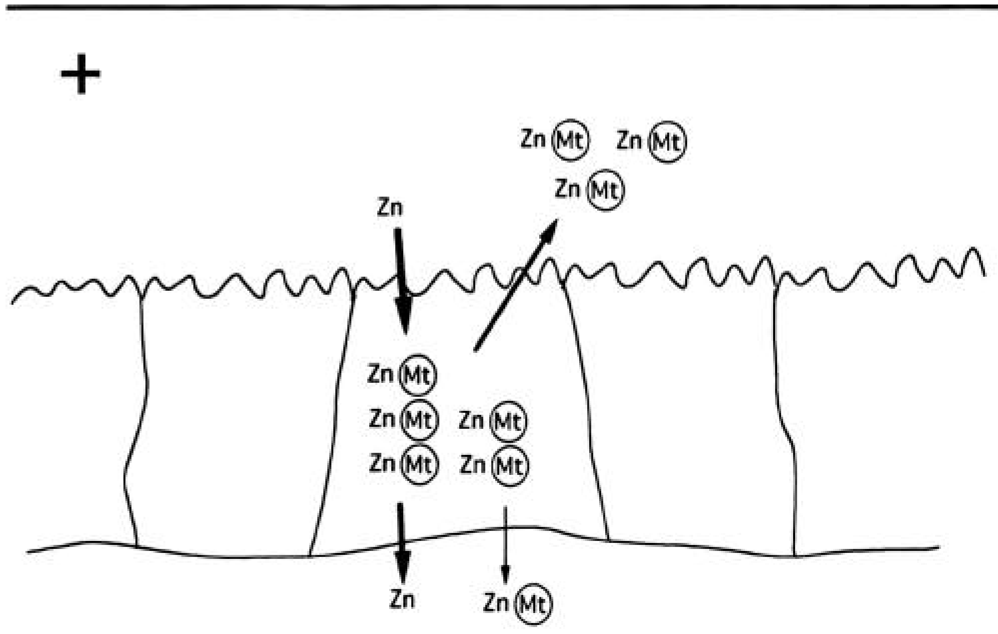

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TABLE I

Lactic Dehydrogenase Activity in control and zinc-exposed CaCo-2 cells *

	Control	**100 µM $ZnCl_2$**	**200 µM $ZnCl_2$**	**400 µM $ZnCl_2$**
Apical media	1.99 ± 0.72	1.43 ± 0.05	1.18 ± 0.02	0.91 ± 0.04
Basal media	0.02± 0.02	0.08 ± 0.00	0.07± 0.01	0.06 ± 0.04
Lysates	97.9 ± 0.70	98.04± 0.07	98.7 ± 0.00	99.04 ± 0.02

* Values are the mean of six different experiments and are expressed as percent of the total enzymatic activity detected in the cell lysates, apical and basal media. Metal-induced cells were exposed to zinc for 20h.

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TABLE II

Zinc content of the fractions obtained by HPLC chromatography of the lysates, the apical anc basal media of control and metal-exposed Caco-2 cells*

	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**		

*Values are expressed in pMoles, calculated according to the amount of radioactive and cold zinc detected fraction of the HPLC chromatography experiment described in Fig. 2.

Total Retention Time of the fractions 5 to 21 min., TRT; Retention Times of the fractions 9 to 11 min., R Retention Times of the fractions 11.3-21, RT 11.3-21.

Retention Times 9-11 correspond to the migration of the MT marker. Retention Times 11.3-21 correspon migration of the ^{65}Zn marker.

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Zinc transport and metallothionein secretion in the intestinal human cell line CaCo-2

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