Intracellular distribution of labile Zn(II) and zinc transporter expression in kidney and MDCK cells

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Ranaldi, Giulia, Giuditta Perozzi, Al Truong- Tran, Peter Zalewski, and Chiara Murgia. Intracellular distribution of labile Zn(II) and zinc transporter expression in kidney and MDCK cells. Am J Physiol Renal Physiol 283:F1365–F1375, 2002. First published August 13, 2002; 10.1152/ajprenal.00094.2002.—Kidneys play a key role in zinc balance. The portion of Zn(II) that enters the glomerular filtrate is efficiently reabsorbed along the nephron through a mechanism yet to be identified. We used the Zn(II)-specific fluorophore Zinquin to visualize intracellular Zn(II) accumulated in the kidney epithelium and compared it with the intracellular localization of the vesicular zinc transporter ZnT4 both in vivo and in vitro. The Madin-Darby canine kidney (MDCK) cell line, stably overexpressing rat ZnT4, was used as a tissue culture model of the kidney epithelium. Zinquin labeling of MDCK cells revealed rapid internalization of Zn(II) and compartmentalization in intracellular bodies interspersed throughout the cytoplasm. In polarized kidney cells, ZnT4 protein was localized on the membrane of intracellular vesicles concentrated around the nucleus, mostly on the basal side. Results of double stainings demonstrated that ZnT4-containing vesicles do not overlap with Zn(II) bodies. Zinquin fluorescence, confirmed by automated microscopy in rat kidney, indicated that consistent with its physiological role, the central glomerulus was weakly stained, whereas the epithelium that lines convoluted tubules was strongly labeled. Double staining of rat kidney with Zinquin and anti-ZnT4 antibodies confirmed the in vitro observations, as Zinquin fluorescence appeared to be distinct from ZnT4 immunofluorescence. To gain further insight into which of the known zinc transporters might be involved in Zn(II) metabolism in the kidney, we have also characterized by RT-PCR the expression of other proteins involved in Zn(II) transport. All of the mRNAs examined [ZnT1, -T2, -T4, and human Zrt, Irt-like protein 1 (hZIP1)], with the exception of hZIP2, were present in adult rat kidney.

Zinquin; zinc transporter; polarized cells; zinc homeostasis; renal epithelium; Madin-Darby canine kidney cells

ZINC IS AN ESSENTIAL DIETARY factor required for enzyme catalysis and as a structural element of metalloproteins. Adequate intake of this trace element is therefore required for normal functioning of cells and tissues. Zn(II) balance is obtained through a controlled rate of intestinal uptake as well as renal reabsorption.

Zn(II) is taken up from the diet and transported across the absorptive epithelium of the small intestine into the bloodstream. Intestinal absorption of all trace elements is tightly regulated, and excess Zn(II) is eliminated through the feces together with the endogenous Zn(II) contained in pancreatic secretions (5). Both saturable and nonsaturable components were shown to be involved in zinc uptake at the level of the intestinal mucosa (34), whereas zinc efflux into the bloodstream has not been fully characterized. The majority of Zn(II) in plasma is bound to albumin, while a small portion circulates, associated with amino acids. This latter portion enters the glomerular filtrate, becoming a source of Zn(II) destined for urinary excretion. Most of the filtered Zn(II) is reabsorbed along kidney proximal tubules; therefore, in nonpathological conditions its urinary loss is minimized. The onset of dietary zinc deficiency induces a decline in urinary Zn(II) concentrations, while plasma levels tend to be constant (2). On the contrary, renal insufficiency and other syndromes, such as diabetes, result in reduced serum Zn(II) concentrations and increased urinary excretion (Ref. 4 and references therein). These observations point to the kidney as being a major organ involved in zinc homeostasis in the body. Despite this, proteins involved in Zn(II) transport, excretion, and reabsorption have been poorly studied in this tissue (28).

Along with other metal ions, Zn(II) is highly charged and cannot cross biological membranes by passive diffusion. Intracellular homeostasis of this ion is achieved by the activity of specific proteins involved in its uptake, efflux, and intracellular compartmentalization. These transport systems are tightly regulated to avoid deficiency or overload. Insights into how cells handle Zn(II) are emerging after the recent cloning of genes encoding several mammalian Zn(II) transporters. Computer-aided analysis of protein sequences, derived both from cloned cDNAs and from genomic sequencing, indicates that Zn(II) transporters can be subdivided.
ZnT proteins belong to the cation diffusion facilitator family and comprise six characterized members in mammals. All ZnTs, with the exception of ZnT5, have six transmembrane domains, with NH2 and COOH termini located on the cytoplasmic side of the membrane (reviewed in Ref. 8). They also contain a conserved histidine-rich domain between transmembrane segments IV and V that at least in the case of ZnT4, was shown to be capable of binding metal ions (29). ZnT1 and ZnT2 are involved in Zn(II) detoxification likely using different mechanisms, because ZnT1 is localized at the plasma membrane whereas ZnT2 was visualized in the membrane of intracellular vesicles (30, 32). The expression of ZnT3 is restricted to the nervous system, and its function is related to Zn(II) transport into synaptic vesicles (6). ZnT4 is developmentally regulated in intestinal epithelial cells (3) and represents the molecular basis of the lethal milk (lm) mouse syndrome (18); dams with the lm genotype produce milk with insufficient Zn(II) content, and pups of any genotype suckling on lm dams die within a few days (1, 13). Such a phenotype suggests an important role for ZnT4 in Zn(II) secretion in the mammary epithelium. Because the ZnT4 gene is ubiquitously expressed and the encoded protein is localized in the membrane of intracellular vesicles (29), it is likely that this protein plays a broader role in maintaining cellular zinc homeostasis, which is yet to be elucidated. The recent isolation of ZnT5 has been independently reported by two laboratories (10, 20). This protein is longer than the other ZnTs, but only its COOH-terminal portion displays homology to ZnT proteins. It appears to associate with secretory granules in pancreatic \( \beta \) cells (20) and with the apical membrane of intestinal enterocytes (10). The last ZnT transporter to be described, ZnT6, has a vesicular localization in normal rat kidney cells, partially overlapping with the Golgi apparatus and becoming more peripheral after zinc addition to the culture media (19).

Total cellular Zn(II) is decreased only slightly in the case of severe zinc deficiency, whereas cell function is deeply impaired. Cellular Zn(II) not tightly associated with metalloproteins is concentrated in discrete subcellular pools and plays a crucial role in a number of important biological processes, including gene expression, DNA synthesis, cell proliferation, and apoptosis (28, 37, 39). This important fraction of free or loosely bound Zn(II) is easily exchanged and can be revealed using ethyl-(2-methyl-8-p-toluenesulfonylamo-6-quin-olxyloxy) acetate (Zinquin), a specific probe that reacts with nanomolar concentrations of labile Zn(II), giving a strong fluorescent signal. Zinquin was employed to image intracellular pools of Zn(II) in several cell types and tissues, where it highlights the intracellular vesicles rich in this metal. The number and shape of such vesicles depend on cell type (37, 39, 40). An independent technique revealing labile pools of Zn(II) is autometallography (AMG). This method is an improved version of Timm’s sulfide silver stain, in which zinc is specifically localized by formation of a complex with sodium selenite that is developed by silver staining (11). Understanding which set of proteins is involved in Zn(II) fluxes in different cell types, how they work to regulate intracellular Zn(II) levels, and how Zn(II) is delivered to metalloproteins is central to understanding the normal physiology of zinc, as well as the abnormalities that might arise in zinc-related diseases. Regulation of zinc transporter expression has been investigated in detail in the yeast Saccharomyces cerevisiae, but not as extensively in mammalian cells (reviewed in Ref. 14). The emerging picture points to both the transcriptional and the posttranscriptional level as key regulatory steps in the expression of proteins involved in zinc metabolism. Transcription of ZnT1 and ZnT2 mRNAs was shown to be upregulated in the small intestine, kidney, and liver by dietary zinc, as well as in response to short-term acute increases in zinc intake (24). ZnT1 transcription appears to depend on metallothionein transcription factor 1 (MTF1), a transcription factor that also regulates metallothionein transcription in response to zinc (22). In the case of ZnT1, however, the zinc-induced increase in mRNA level did not lead to a corresponding increase in protein level, which was only mildly induced in the small intestine (27). ZnT4 mRNA levels, on the contrary, were unchanged under the same experimental conditions (24). Constructs containing fragments up to 3 kb from the ZnT4 promoter region, driving expression of a reporter gene, are not regulated by zinc in transfected cells of different types (unpublished observations). However, recent reports indicate that zinc supplementation of cultured cells induces intracellular relocalization of endogenous ZnT4-containing vesicles from the Golgi apparatus to the cell periphery (Ref. 19 and unpublished observations). Zinc-dependent regulation of the expression of hZIP transporters has not yet been thoroughly characterized. The available data indicate that zinc uptake is a regulated process in some cell types, but only in the case of hZIP1 was a decrease in mRNA level demonstrated in zinc-supplemented cells (7).

In the present paper, we have characterized the expression of proteins involved in Zn(II) transport in rat kidney and have investigated Zn(II) distribution in...
renal tissue and in the Madin-Darby canine kidney (MDCK)-derived cell line. Comparison of the intracellular localization of the ZnT4 transporter, in relation to that of labile Zn(II) pools, indicates that the majority of ZnT4-containing vesicles are distinct from those containing free Zn(II) both in vivo and in vitro.

**MATERIALS AND METHODS**

**RNA extraction and RT-PCR analysis.** Kidneys were removed from adult Sprague-Dawley rats previously anesthetized by intraperitoneal injection of 20 mg/100 g body wt of Farmotal (Farmitalia-Carlo Erba, Milan, Italy). The dissected tissue was immediately frozen in liquid nitrogen, and RNA was extracted from pulverized tissue using TRIzol reagent (GIBCO BRL Life Technologies) and random hexamer primers. First-strand cDNA was PCR amplified with poliTaq enzyme (Polymed), using the following oligonucleotides as primers: ZnT1: AGTGGCTTCTCCACGGCCGCGTCC (sense), ATGGCGCGCGCTCCAGGGC (antisense); and GADPH: AAACCCATCACCATCTTCCAGCCTGTTG (sense), CTAGCCGCATTCCTACACCAAACACGGCAGT (antisense); hZIP2: ACTTGGCTGCCTGTTTGAGACAATTTGCACA (antisense); hZIP1: TACAAGGAGCAGTTCCGAGGGACCCTGGCACTCCTG (antisense); ZnT4: ATGAAAGCTTGTTTCTCCACAGCGCCGCTCC (sense), ATTGCGATCCTCCATGGACCGGACGG (antisense).

**PCR reactions were performed in a PerkinElmer Gene Amp PCR System 9700.**

**Plasmid cDNA constructs, transfection, and cell culture.** Full-length rat ZnT4-myc cDNA was excised from the pcDNA3 vector (Invitrogen, Groningen, The Netherlands) (29) by digestion with BamHI and XbaI and subcloned into the pTRE Not/hyg plasmid (kindly provided by Dr. Michael Francis, Oxford, UK). This vector, containing the tetracycline-responsive element, was modified from the original pTRE vector (Tet-Off system, Clontech Laboratories, Palo Alto, CA) by insertion of the hygromycin resistance gene into the NotI site. Plasmid pTRE-ZnT4-myc was transfected into a stable clone of Tet-off MDCK II cells, expressing the tTa tetracycline repressor (kindly provided by Dr. Keith Mostov, Univ. of California, San Francisco, CA). Cells were transfected using Transfast TM E2432 transfecting agent (Promega, Madison, WI) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were split and selected with 0.2 mg/ml hygromycin B. Resistant colonies were isolated with glass cylinders and amplified. An aliquot of the selected clones was plated on glass coverslips in doxycycline-free medium, and positive clones expressing the ZnT4-myc protein were screened by immunofluorescence with anti-myc monoclonal antibodies (Sigma, St. Louis, MO). Expressing clones, corresponding to ~10% of hygromycin-resistant clones, were maintained in high-glucose DMEM supplemented with 10% FBS, 0.1 mg/ml hygromycin, and 20 ng/ml doxycycline. Transgene expression was turned on by passing cells in fresh medium lacking doxycycline. Cells were subcultured twice a week at a split ratio of 1:20, and the culture medium was replaced every 2–3 days. As a negative control of Zinquin staining, cells were either incubated with the intracellular Zn(II) chelator [N,N'-N'-tetakis(2-pyridylmethyl)ethylenediamine] (TPEN; 25 μM; Sigma) at 37°C for 30 min in complete culture medium or starved overnight in DMEM without serum. Cells treated with TPEN for up to 2 h were stained with bisbenzimide (Roche, Basel, Switzerland). Zinc uptake was observed by incubating starved cells with increasing concentrations of ZnSO₄, up to 100 μM, in Hanks’ buffer (Flow Laboratories, Irvine, UK) at 37°C for 30 min.

**Zinc influx.** Rats were killed by asphyxiation with CO₂. For frozen tissues, the entire kidney was excised and transverse sections of the tissue were quickly placed into plastic embedding trays containing OCT and snap-frozen on dry ice. Tissue sections (5 μm) were left to adhere on coated glass slides at room temperature for 20 min. Sections were fixed in 100% chilled acetone for 10 min at room temperature before tissue staining. Cells were grown on glass coverslips or on transparent filters (Transwell-Clear; Corning Life Sciences) where indicated. All methods for immunolocalization have been described previously (29). Primary antibodies used in this study were the following: mouse mAb anti-c-myc (1:200; clone 9E10 Sigma); mouse mAb anti-ZO1 (1:300; Zymed Laboratories, South San Francisco, CA); rat mAb anti-α₁ integrin subunit (1:200; clone GoH3, Pharmingen, San Diego, CA); rabbit polyclonal anti-c-myc (Santa Cruz, CA); rabbit polyclonal anti-ZO1 (1:300; Zymed Laboratories, South San Francisco, CA); rat mAb anti-α₁ integrin subunit (1:200; clone GoH3, Pharmingen, San Diego, CA); rabbit polyclonal anti-c-myc (Santa Cruz, CA); and rabbit polyclonal anti-ZO1 (1:300; Zymed Laboratories, South San Francisco, CA). Secondary antibodies, labeled with either tetramethylrhodamine isothiocyanate or FITC, were affinity-purified and species specific (Jackson ImmunoResearch Laboratories, West Grove, PA). Total protein extracts from induced and uninduced cells were prepared by lysing the same number of cells in 1% SDS, 5 mM Tris-HCl, pH 6.8 (26). Equal volumes of lysates were tested for protein content, subjected to SDS-PAGE, and transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany). Antibody-antigen complexes were detected with horseradish peroxidase-conjugated secondary Abs by enhanced chemiluminescence SuperSignal (Pierce, Rockford, IL).

**Zinquin staining and AMG.** Cells fixed in 4% paraformaldehyde for 15 min or cryostat sections were incubated for 30 min in 25 μM Zinquin (Dept. of Chemistry, Univ. of Adelaide, Adelaide, Australia), freshly diluted in 1× PBS. Where indicated, cells and tissue were processed for immunofluorescence before Zinquin staining. Fluorescence microscopy was performed on a Zeiss Axioskop II. A Bio-Rad MRC-1000 ultraviolet laser scanning confocal microscope system, equipped with ultraviolet-argon laser, was used in combination with a Nikon Diaphot 300 inverted microscope in fluorescence mode for Zinquin; fluorescence excitation was at 351/5 nm and emission at 460LP, whereas for FITC fluorescence excitation was at 488/10 nm and emission at 522/35. Images were collected using a ×40 water-immersion objective lens with a numerical aperture of 1.35. Each image was averaged over six scans by Kalman filtering. Where dual staining was performed, fluorescence images were merged using the Confocal Assistant (version 4.02) software package. For the AMG experiments, rats received either sodium selenite (20 mg/kg ip, Sigma) for 45 min or no treatment (control). Rats were anesthetized with Nembutal and then
maintained on halothane. The abdomen was opened, and kidneys were fixed by retrograde perfusion via the aorta with 3% glutaraldehyde in 1× PBS. The kidneys were removed, postfixed for 1 h, cut into three pieces, and fixed further overnight at room temperature. Vibratome sections (100 μm) were cut and transferred to a beaker of 1× PBS and then distilled water, dipped in a 0.5% gelatin (Merck, Rahway, NJ) solution, placed in AMG developer (11), and developed in distilled water, dipping in a 0.5% gelatin (Merck, Rahway, NJ) solution, placed in AMG developer (11), and developed in distilled water. These were examined by light microscopy (AMG) of rat kidney section. Zn(II) pools are stained with dark particles. Moreover, our results of RT-PCR analysis show that ZnT4 mRNA is expressed in rat kidney (24, 29).

RESULTS

Zn(II) distribution and expression of Zn(II) transporters in kidney epithelium. To image the pool of labile Zn(II), cryosections of rat kidney were stained with Zinquin. Zinquin is a highly specific fluorescent probe able to detect nanomolar concentrations of intracellular stores of Zn(II). It has been used to map pools of labile Zn(II) in several cells and tissues (9, 37). To date, there are no data reported for the kidney intracellular Zn(II) store. The results in Fig. 1, A and B, show that epithelial cells lining convoluted tubules are strongly positive for Zinquin staining, consistent with the active role of these cells in Zn(II) reabsorption. This pattern was confirmed using AMG (Fig. 1C), which also shows the lack of Zn(II) in the glomeruli. Reabsorption of Zn(II) requires the activity of specialized proteins; therefore, we next sought to determine which of the best characterized proteins responsible for Zn(II) uptake and transport were expressed in kidney cells. Four ZnT and three hZIP transporters have been characterized in mammals, and we compared the expression of their transcripts in the kidney by RT-PCR. The results in Fig. 1D show that with the exception of hZIP2, all mRNAs examined (ZnT1, T2, T4, and hZIP1) appear to be simultaneously expressed in the kidney. ZnT3 was not tested as its expression is known to be restricted to brain and testis (31).

Zn(II) transport and ZnT4 expression in MDCK cells. The canine kidney cell line MDCK is a widely used tissue culture model of kidney epithelium (25). To ascertain the feasibility of this tissue culture model for our studies of zinc transport and ZnT4 expression, we initially investigated the main features of zinc metabolism in this cell line. As shown in Fig. 2A, MDCK cells express endogenous ZnT4, as determined by RT-PCR. We then used Zinquin staining to study the distribution of intracellular labile Zn(II). Figure 2B shows a fluorescence microscopic image of the characteristic stary sky pattern of Zinquin labeling in fully differentiated MDCK cells. As shown in Fig. 2B, left, significant amounts of intracellular labile Zn(II) were concentrated within intracellular vesicles in these cells. Treatment with the chelating agent TPEN, which tightly binds intracellular Zn(II), resulted in almost a complete loss of Zinquin fluorescence (Fig. 2B, middle). Bisbenzimide staining of nuclei assessed a lack of apoptosis in this experimental condition (Fig. 2B, right). To characterize Zn(II) uptake in these cells, Zinquin staining was performed in Zn(II)-starved cells, before and after addition of increasing concentrations of ZnSO4 within the physiological range. Overnight incubation in medium lacking serum led to a dramatic loss of fluorescence from zinc-containing vesicles (Fig. 2C, left). Zn(II)-starved cells, incubated in HBSS buffer with 25 or 100 μM ZnSO4 for 30 min, efficiently accumulated zinc in intracellular bodies, showing a concentration-dependent increase in Zinquin fluorescence (Fig. 2C, middle and right). Taken together, these results demonstrate that MDCK cells are capable of efficient Zn(II) uptake, efflux, and intracellular compartmentalization. Therefore, this cell line represents a good in vitro model for the study of Zn(II) metabolism in kidney epithelium.

Creation of a stable MDCK cell line overexpressing ZnT4. The transmembrane Zn(II) transporter ZnT4 localizes in the membrane of intracellular vesicles of different cell types. The Zn(II) vesicular pattern that we observed in these cells is closely reminiscent of the vesicular distribution of ZnT4. Our laboratory and others have previously shown by Northern hybridization that ZnT4 mRNA is expressed in rat kidney (24, 29). Moreover, our results of RT-PCR analysis show that...
the ZnT4 mRNA is transcribed in kidney-derived MDCK cells (Fig. 2A). For these reasons, we sought to investigate whether ZnT4 was involved in vesicular sequestration of Zn(II) and whether its overexpression affects zinc homeostasis. Using a constitutive expression vector driving ZnT4 transcription from a viral promoter, we were unable to select stable transfectants expressing ZnT4, suggesting that long-term overexpression of this transporter is probably toxic to cells. To circumvent this problem, we resorted to an inducible expression system, by placing the open reading frame of a myc-tagged rat ZnT4 cDNA construct under the control of the bacterial tetracycline promoter (17). This construct was transfected into a clone of MDCK cells that stably expresses the bacterial tetracycline repressor (MDCK Tet-off; kindly provided by Dr. Keith Mostov). Several lines of stable clones were selected and monitored for the expression of ZnT4. Figure 3 shows how withdrawal or addition of the tetracycline analog doxycycline to the culture medium tightly regulates ZnT4 expression in independent clones of stably transfected MDCK Tet-off (ZnT4-myc) cells. Using Western blotting (Fig. 3A) and indirect immunofluorescence (Fig. 3B), we observed that addition of doxycycline completely abolishes the expression of the transfected cDNA. Anti-myc antibodies revealed the

Fig. 2. Expression of endogenous ZnT4 and vesicular localization of free Zn(II) pool in Madin-Darby canine kidney (MDCK) cells. A: RT-PCR analysis was performed using oligonucleotides specific for ZnT4 cDNA on total RNA extracted from MDCK cells. C, negative control reaction lacking reverse transcribed template RNA but containing ZnT4-specific oligonucleotides. B: Zinquin staining of a monolayer of confluent, fully polarized MDCK cells. To better visualize the morphology of the cells, the ultraviolet fluorescence was superimposed on the bright field (left). Middle: cells were treated with the zinc chelator TPEN before Zinquin staining. Cells were treated with TPEN for 2 h and stained with bisbenzimide (Hoechst) to visualize the morphology of nuclei (right). C: MDCK cells were zinc depleted by overnight serum starvation (left) and incubated with the indicated concentrations of ZnSO₄ for 30 min before Zinquin staining (middle and right).

Fig. 3. Inducible expression of ZnT4-myc in stably transfected MDCK subclones. A: Western blotting of total protein extracts from 3 independent subclones of MDCK Tet-off (ZnT4-myc) cells, in which the expression of ZnT4 was induced (−dox) or uninduced (+dox), probed with monoclonal anti-myc primary antibody. B: indirect immunofluorescence of a fourth independent subclone of MDCK Tet-off (ZnT4-myc), performed with monoclonal anti-myc primary antibody and TRITC-labeled secondary antibody.
characteristic vesicular localization of ZnT4 (Fig. 3B) in expressing clones cultured without doxycycline. Ten independent subclones were screened both by Western blotting and by immunofluorescence with anti-myc antibodies, and no significant differences were detected in the expression levels or in the intracellular localization of the transfected protein. Therefore, further experiments were carried out in three independent MDCK Tet-off (ZnT4-myc) subclones.

ZnT4 accumulates in a perinuclear vesicular compartment. We have previously demonstrated that in nondifferentiated epithelial cells, ZnT4 is localized in a subset of endosomes also containing transferrin receptor and the β-subunit of the clathrin adaptor complexes AP-1/2 (29). To further investigate whether such vesicles had a polarized distribution within the cell, we performed confocal laser scanning microscopy analysis in confluent, fully polarized MDCK subclones overexpressing ZnT4. The results shown in Fig. 4 revealed that ZnT4-containing vesicles were distributed around the nuclei, the majority of them being clustered on the basal side. Figure 4A shows a basal section of the cell monolayer with strongly stained vesicles superimposed on a less intense signal of reticular appearance, most likely representing endoplasmic reticular staining. Figure 4, B–D, shows that in progression toward the apical domain of the cells, vesicles tend to decrease in number while concentrating around the nuclei. Such a distribution is clearly evident in Fig. 4E, showing a vertical section of the monolayer along the X-Z axis.

Fig. 4. Confocal microscopy analysis of fully differentiated MDCK Tet-off (ZnT4) cells after 24-h induction of ZnT4-myc expression. The ZnT4 protein was detected with an anti-myc primary antibody and TRITC-labeled secondary antibody and viewed by confocal laser scanning microscopy (continuous scan). A–D: 4 consecutive laser sections along the X-Y axis taken at 1.2-μm intervals. E: laser section along the X-Z axis. AP, apical; BL, basolateral membrane.

Fig. 5. Intracellular localization of ZnT4-containing vesicles relative to apical and basolateral markers. Confocal laser scanning microscopy of indirect immunofluorescence of fully differentiated MDCK Tet-off (ZnT4-myc) cells in which the expression of ZnT4-myc had been induced by doxycycline withdrawal for 24 h. A: double staining of ZnT4-myc and ZO-1. ZnT4-myc was stained with monoclonal anti-myc and TRITC-labeled secondary antibody. ZO-1 was detected with a monoclonal primary and FITC-labeled secondary antibody. B: ZnT4-myc was detected with monoclonal anti-myc and an FITC-labeled secondary antibody. α6-Integrin subunits were stained with a specific rat monoclonal and TRITC-labeled secondary antibody. C: single section through the X-Z axis of the immunofluorescence shown in A.
ZnT4-containing vesicles are not superimposed on Zn(II)-containing vesicles. To assess whether MDCK cells concentrate labile Zn(II) in the same vesicles that contain ZnT4, double-staining experiments were performed with anti-myc antibodies (FITC immunofluorescence) and Zinquin (ultraviolet fluorescence). The results are shown in Fig. 6 for both subconfluent, nonpolarized cells (Fig. 6, A and B) and fully polarized cells (Fig. 6, C–H). Although both Zinquin and ZnT4 stainings showed a similar pattern of vesicular cytoplasmic distribution, a close observation failed to reveal any extensive overlapping of the two sets of vesicles when the only source of Zn(II) was that normally present in complete culture medium (Fig. 6, A–D). To

Fig. 6. Double staining of ZnT4-myc and Zn(II)-containing vesicles in stably transfected, ZnT4 expressing MDCK subclones. Expression of the transgene was induced by doxycycline withdrawal for 24 h. A, C, E, and G: indirect immunofluorescence whereby ZnT4 protein was detected with a monoclonal anti-myc primary antibody and FITC-labeled secondary antibody. B, D, F, and H: Zinquin staining of the corresponding fields. A and B: subconfluent, undifferentiated cells. C and D: fully differentiated cells 48 h after reaching confluence. E and F: zinc-depleted, differentiated cells after overnight serum starvation. G and H: serum-starved cells after supplementation with 100 μM ZnSO4 for 30 min.
verify whether depletion or addition of zinc could affect ZnT4 localization relative to Zn(II)-loaded vesicles, cells overexpressing ZnT4 were incubated overnight in medium lacking serum. TPEN was not used to chelate Zn(II) in this set of experiments because it interferes with Zinquin staining. Serum-starved cells were then incubated with 100 μM ZnSO4 to stimulate Zn(II) uptake. The results showed that neither Zn(II) starvation (Fig. 6, E and F) nor Zn(II) supplementation (Fig. 6, G and H) would induce colocalization of ZnT4-containing vesicles with intracellular Zn(II) bodies highlighted by Zinquin.

Consistent with the in vitro results, double staining of rat kidney sections with the anti-ZnT4 polyclonal antibody and Zinquin shows that the epithelial cells lining convoluted tubules accumulate significant amounts of Zn(II) (Fig. 7A) and express ZnT4 (Fig. 7B), although ZnT4 immunofluorescence and Zinquin staining do not overlap (Fig. 7C). ZnT4 appears to be concentrated at the basal side of renal epithelial cells, whereas the free Zn(II) pool is evenly distributed throughout the cytoplasm. As expected from its physiological role, the central glomerulus is only weakly labeled with Zinquin (Fig. 7D), although it displays high levels of expression of ZnT4 (Fig. 7E). In the epithelial cells lining the capsule, where both stainings are present, no colocalization was observed (Fig. 7F).

**DISCUSSION**

The kidney epithelium is the site of urinary zinc excretion and reabsorption. This tissue plays a key role in the maintenance of the homeostasis of this essential trace element in the body. The physiological role of renal epithelial cells within this context is zinc reabsorption from the glomerular filtrate to minimize body loss of this essential trace element. At the cellular level, this is achieved through the expression of proteins specifically involved in zinc uptake, intracellular compartmentalization, transport, and efflux, some of which have been cloned and characterized at the molecular level. In this paper, we attempt to answer some questions concerning the mechanism by which kidney epithelial cells handle intracellular Zn(II).

We initially sought to investigate which of the zinc transporters might be responsible for maintaining zinc homeostasis in kidney cells by performing an expression analysis of zinc transporters belonging to the ZnT and hZIP protein families in rat kidney. The results of RT-PCR analysis show that ZnT1, ZnT2, and ZnT4, as well as hZIP1, are all expressed in this tissue, whereas hZIP2 mRNA is not present. Coexpression of different ZnT proteins is not surprising, because, despite their high degree of sequence and structural homology, they display distinct intracellular localizations and are likely to play different roles in zinc handling. On the contrary, both hZIP1 and hZIP2 were shown to participate in cellular Zn(II) uptake (15, 16). The expression of hZIP2 in different tissues and cell lines was postulated to be either very low or absent as it was not possible to detect it by Northern hybridization (15). Our results, obtained with a more sensitive technique such as RT-PCR, indicate that hZIP2 mRNA is indeed absent in kidney cells. Therefore, it seems more likely that this gene is expressed only in a restricted number

Fig. 7. Double staining of rat kidney with ZnT4 antibody and Zinquin. Confocal microscopy analysis of Zinquin-stained cryosections from rat kidney showing a group of convoluted tubules (A) and a typical glomerulus (D). B and E: immunofluorescence of ZnT4 distribution of the corresponding fields. C: merge of A and B. Zinquin ultraviolet fluorescence is shown in blue, and ZnT4 immunofluorescence is revealed with an FITC-labeled secondary antibody. F: merge of D and E. The colors are the same as in C. C, Bowman’s capsule; T, convoluted tubules; US, urinary space.
of tissues, whereas hZIP1 is ubiquitously expressed (16). The scenario that has emerged is that the apparent redundancy of zinc transport functions is probably counterbalanced by their distinct expression profiles in each tissue. Expression of the two vesicular transporters ZnT2 and ZnT4 overlaps only in a restricted number of cell types (31), and the same is true for hZIP1 and hZIP2, both responsible for Zn(II) uptake (16).

ZnT1 was localized to the plasma membrane and postulated to act as a zinc efflux transporter (27). On the contrary, both ZnT2 and ZnT4 appear to be inserted in the membrane of intracellular vesicular compartments (30). Moreover, ZnT4 represents the molecular basis of the lethal milk mouse phenotype (18), characterized by a zinc secretion defect in mammary epithelial cells (1, 23). Given the intracellular localization of ZnT2 and ZnT4, both transporters have been hypothesized to play a role in vesicular sequestration of excess cytoplasmic Zn(II). According to this model, zinc-loaded vesicles could eventually fuse with the plasma membrane and complete the detoxification process by extruding Zn(II) ions into the extracellular space. However, colocalization of ZnT4-containing vesicles with Zn(II) bodies was never demonstrated.

In this paper, we show that MDCK cells, derived from kidney epithelium, express ZnT4 mRNA. We have imaged the intracellular zinc pool in these cells using Zinquin, a fluorescent probe specific for Zn(II) ions. Zinquin binds free Zn(II) as well as Zn(II) loosely bound to proteins such as metallothionein (9). When compared with other cell types, including airway epithelial cells (37) and intestinal cells (unpublished observations), MDCK cells, cultured in standard medium without further zinc supplementation, show intense Zinquin staining concentrated in intracellular vesicles. This observation suggests the presence of a very efficient, constitutively expressed system for Zn(II) uptake and compartmentalization that could be further stimulated by the addition of 25–100 μM ZnSO₄ to the culture medium. To compare these results with the in vivo distribution of free Zn(II), which had not been previously determined for the kidney, cryosections of rat tissue were stained with Zinquin. The results revealed a strong signal in the epithelium of the convoluted tubules, whereas the central glomerulus was only weakly labeled. This was shown independently by AMG staining. The ability of kidney tubule cells to accumulate free Zn(II) is consistent with their role in reabsorption of this ion from the glomerular filtrate. Overall, the results of Zn(II) imaging in vivo and in vitro show the ability of differentiated renal epithelial cells to accumulate free Zn(II) and store it in intracellular bodies dispersed throughout the cytoplasm.

In the past few years, our laboratory has been involved in cloning and in the molecular characterization of a rat ZnT4 cDNA (3, 33). In this paper, we sought to investigate whether there was a relationship between the vesicular pools of free Zn(II) and ZnT4 localization in this kidney-derived cell system. This question was approached by creating stably transfected clones of MDCK cells overexpressing a myc-tagged rat ZnT4 protein in a regulated fashion. Intracellular localization of free Zn(II) and of the ZnT4 protein was compared in the stably transfected subclones of MDCK cells overexpressing ZnT4. The experiments were conducted under different conditions of zinc loading to take into account a possible zinc-dependent regulation of Zn(II) bodies or ZnT4 localization. We have shown that in both fully polarized and in undifferentiated cells, Zn(II)-containing vesicles are interspersed within the cytoplasm with a pattern resembling that of ZnT4. Double staining with anti-myc antibodies and Zinquin, however, did not show extensive overlapping between the two sets of vesicles. This result was confirmed by double staining of kidney sections with Zinquin and anti-ZnT4 antibodies. Neither zinc depletion nor zinc loading of MDCK cells with up to 150 μM ZnSO₄ affected ZnT4 distribution relative to Zn(II)-loaded vesicles.

In the MDCK Tet-off subclones, ZnT4 displays the same vesicular localization that we had previously observed in Caco-2 and COS cells (29). Our laboratory has also previously shown that ZnT4 partly colocalizes with the transferrin receptor and the β-subunit of the clathrin adaptor complexes AP-1 and AP-2 in a subpopulation of endosomal vesicles (29). Because in sections of rat small intestine ZnT4 vesicles appear to concentrate in the basal side of differentiated enterocytes, we have exploited the capacity of MDCK cells to fully differentiate into a monolayer of polarized epithelium to further investigate by confocal microscopy whether ZnT4-containing vesicles were differentially distributed into the apical and the basal domains in kidney epithelium. The position of ZnT4-containing vesicles within polarized cells was assessed by confocal microscopic analysis of double-stained cells with antibodies specifically recognizing ZnT4 and the polarity markers ZO-1 (identifying apical tight junctions) or the α6-integrin subunit (identifying the basolateral plasma membrane). These experiments have shown that the majority of ZnT4-containing vesicles are found in the basal domain of polarized cells, with perinuclear localization. Fewer vesicles appear scattered throughout the cytoplasm up to the level of tight junctions. Such intracellular distribution, which is compatible with a role in basolateral secretion, does not rule out a function for ZnT4 in regulating the intracellular distribution of Zn(II).

Overall, our results indicate that despite the apparently analogous vesicular localization of the two transporters, ZnT4 is distinct from ZnT2, which was shown to fully colocalize with free Zn(II) (30). It is interesting to note, however, that ZnT2 and ZnT4 are coexpressed in intestinal and kidney epithelial cells, whereas only ZnT4 is present in the mammary epithelium (24), where the most dramatic phenotype is manifested in the lethal milk mouse syndrome. From a functional viewpoint, the mammary gland-restricted phenotype in adult lm mice lacking a functional ZnT4 protein indicates that although the two vesicular transporters do not appear to colocalize in Zn(II)-loaded vesicles, they might still be able to play partially overlapping...
roles in handling the vesicular Zn(II) pool. However, while ZnT2 was shown to confer zinc resistance to transfected cells (30), overexpression of ZnT4 in our in vitro cell model did not alter the cell sensitivity to the addition of increasing Zn(II) concentrations to the culture medium. When ZnT4-transfected subclones and the parental cell line are compared for the effect of Zn(II) toxicity on tight junctional integrity (as measured by transepithelial electrical resistance and insulin passage) and cell survival (evaluated by neutral red uptake), no differences were detected (data not shown). A possible explanation for the lack of a zinc transport-related phenotype in ZnT4-transfected MDCK cells could be that overexpression of ZnT4 alone is not sufficient to enhance its function. In support of this latter hypothesis, we have previously demonstrated with pull-down experiments that the NH2-terminal segment of ZnT4 specifically interacts with at least one yet unknown partner that might be necessary for ZnT4 function and required at stoichiometric concentrations. Further research is presently ongoing in our laboratory to uncover the identity of the ZnT4-interacting protein(s).

Overall, the results of our study underline the key role of kidney cells in zinc metabolism, as we demonstrate very efficient Zn(II) uptake and intracellular accumulation both in vivo and in vitro. The fate of vesicular free Zn(II), once it has entered renal epithelial cells, as well as which of the zinc transporters might be responsible for delivery of vesicular zinc to the bloodstream remain to be established and will represent a challenge for future research in this field.

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