Differential subcellular localization of hZip1 in adherent and non-adherent cells

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Abstract Two human divalent cation transporters of the ZIP family, hZip1 and hZip2, homologous to Irt1 (*Arabidopsis thaliana*), the first identified member, have been described. They were shown by transfection into K562 cells to be localized at the plasma membrane and to mediate zinc uptake. Here we report a differential subcellular localization of hZip1 according to cell type. By transient expressions of EGFP-hZip1, FLAG-tagged or native hZip1, we observed that hZip1 has a vesicular localization in COS-7 cells or in several epithelial cell lines, corresponding partially to the endoplasmic reticulum. Using anti-hZip1 antibodies, we confirmed the intracellular localization of the endogenous protein in PC-3, a prostate cancer cell line. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Zinc; hZip1; ZIP family; Endoplasmic reticulum; Plasma membrane

1. Introduction

Zinc is an essential trace element for organisms. It plays a role as a cofactor for many enzymes like alkaline phosphatase or Cu–Zn superoxide dismutase. Moreover, it stabilizes some structures found in transcription factors ('zinc-finger' domains for example). Several new zinc transporters have been described recently, which fall into two different families.

The ZnT family includes proteins with six predicted transmembrane domains, the amino- and carboxy-terminus residing intracellularly, as well as a histidine-rich loop between transmembrane domains IV and V. Four members have been characterized: ZnT1 ubiquitously expressed is implicated in zinc efflux [1], ZnT2 and ZnT3 allow vesicular sequestration of the cation in specific tissues [2,3] and ZnT4 exports zinc especially from gland and brain cells [4].

The ZIP family (ZRT, IRT-like proteins family) includes both iron and zinc transporters, and is highly conserved throughout evolution. Most ZIP proteins have eight transmembrane domains with the amino- and carboxy-terminus residing on the outer surface of the plasma membrane. An intracellular loop between transmembrane domains III and IV varies in size between the different proteins of the family and contains a putative metal binding site with a variable number of (H-X) motif [5]. Two human proteins, hZip1 and hZip2, have been described. hZip1 is ubiquitously expressed. It is a 324 amino acid protein with 30% identity to Irt1, a cation transporter expressed in the root of iron deficient plant and the first member of the ZIP family to be identified [6,7]. hZip2 is expressed at a low level in epithelial cells and is inducible by growth arrest. It is a 309 amino acid protein with 22% identity to Irt1. Both hZip1 and hZip2 have been shown to be zinc transporters and both are localized at the plasma membrane when transfected into K562 cells [8,9].

In this paper, we report that hZip1 has an intracellular vesicular localization in adherent cells as opposed to a cytoplasmic membrane localization in K562 cells.

2. Materials and methods

2.1. DNA constructs

The hZip1 open reading frame, followed by a poly(A) sequence, was subcloned into the *Hin*dIII and *Eco*RI cloning sites of pBluescript SK. This vector obtained for in vitro transcription and translation was called pBS-hZip1.

To generate a mammalian expression vector, the hZip1 open reading frame was excised from pBS-hZip1 and subcloned into the pEGFP-C1 vector (Clontech, Palo Alto, CA, USA) digested by *Bgl*II and *Sal*I to obtain an in-frame fusion protein between the green fluorescent protein (EGFP) and hZip1. The resulting construct was verified by sequencing and called pEGFP-hZip1.

To generate a FLAG®-tagged sequence, the coding sequence of hZip1 was first excised from pBS-hZip1 and subcloned into pcDNA3 (Invitrogen, Groningen, The Netherlands). This construct was called pcD-hZip1. Subsequently, the FLAG® sequence was generated by PCR. The upstream oligonucleotide maps to an internal hZip1 sequence overlapping a *Cvn*I restriction site (CACCTTAGGGCACAGGTG). The downstream primer contained the final 21 nucleotides of the hZip1 coding sequence, without the STOP codon and in frame with the FLAG coding sequence (GACCTCGAGCTATTTGTCATCGTCATCTTTGTAGTCGATTTGGATGAAGAGCAGGCC). This primer also introduced a *Xho*I site at the 3′ end of the PCR fragment. The *Cvn*I–*Xho*I-digested PCR fragment was inserted in place of the *Cvn*I–*Xho*I 3′ end of hZip1. The construction was verified by sequence and called pcD-hZip1-FLAG.

2.2. Production of anti-hZip1 antibodies

Rabbit polyclonal antibodies were raised against a recombinant peptide corresponding to the third loop of hZip1 (residues 137–179) fused to glutathione-*S*-transferase and affinity purified using the same peptide immobilized on NHS-activated HiTrap column (Amersham).

2.3. Cell culture and transfections

All cells were grown at 37°C under a 5% CO_2 atmosphere. COS-7 (ATCC CRL 1651) and HepG2 (ATCC) cells were cultured as described [10,11]. PC-3 cells (ECACC, Salisbury, UK), were grown in DMEM supplemented with 10% heat-inactivated FCS, 25 mM HEPES and PS. Before transfection, cells were seeded onto coverslips in six-well culture plates. The following day, they were transiently transfected by lipofection with 2 μg of plasmid. Lipofectamine PLUS reagent (Life Technologies) was used for COS-7, Fugene 6

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(Boehringer, Meylan, France) for HepG2 and transfection reagent selector kit (Life Technologies) for PC-3 cell transfections. K562 were cultured as previously described [9] and transfected by electroporation.

2.4. Immunofluorescence and confocal microscopy

One day after transient transfections, adherent cells or K562 cells coated on poly-L-lysine slides were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. The hZip1-FLAG fusion protein was detected by the anti-FLAG® M2 monoclonal antibody (Kodak). Expressed native hZip1 as well as endogenous hZip1 were detected by a specific polyclonal antibody. Adequate Alexa Fluor® 488 secondary antibody conjugates (Molecular Probes) were used as fluorescent probes. The nuclei were counterstained with propidium iodide. The stained cells were mounted in Slow Fade™ Light antifade solution (Molecular Probes, Interchim, Asnières, France) and the fluorescence was observed by confocal microscopy (Leica TCS, Rueil-Malmaison, France).

For colocalization studies, the fixed and permeabilized cells transiently transfected with pEGFP-hZip1 were incubated with different antibodies. To identify the endoplasmic reticulum (ER) compartment, we used a mouse antibody directed against grp78 (anti-BIP, StressGen Biotechnologies Corp.). To localize the Golgi apparatus, we used a mouse antibody directed against an uncharacterized Triton X-100 extractable antigen of the Golgi medium compartment (a gift of M. Bornens, Institut Curie, France). Cells were then incubated with TRITC-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA). To study the endosomal-lysosomal pathway, the transfected COS-7 cells were incubated in DMEM containing 1 mg/ml of dextran labeled with Texas red (lysine fixable, $M_r = 10\,000$, Molecular Probes) for 15 min. The cells were then washed and chased with DMEM (not containing dextran) for 15, 30, 60 and 120 min. In all cases, cells were fixed by paraformaldehyde and processed for confocal microscopy study as described above.

2.5. In vitro transcription translation

In vitro transcription translation was performed with the TNT® Coupled Reticulocyte Lysate Systems (Promega, Charbonnieres, France). Transcripts were prepared from pBS-hZip1 using T7 polymerase and peptides were synthesized in the presence of amino acids minus methionine with [^{35}S]methionine (Amersham, Les Ulis, France). Incubation was at 30°C for 90 min in the presence or absence of canine pancreatic microsomal membranes. Samples were denatured in SDS sample buffer containing 5% β-mercaptoethanol and 2% SDS and were analyzed on a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel.

2.6. RT-PCR

PC-3 cells were cultured for 18 h in DMEM without FCS and further incubated for 2 h with or without 5 nM prolactin. After incubation, the cells were washed and total RNA was extracted from the cells using RNA Plus (Quantum Biotechnologies). For RT-PCR, 2 μg RNA and 250 ng random primers were incubated at 80°C for 15 s and then 65°C for 4 min and 30 s. 1×Reverse transcriptase buffer, 10 mM DTT, 500 μM dNTP, 10 μg/ml BSA, 32U RnaseOUT (Life Technologies) and 200 U MMLV reverse transcriptase superscript II

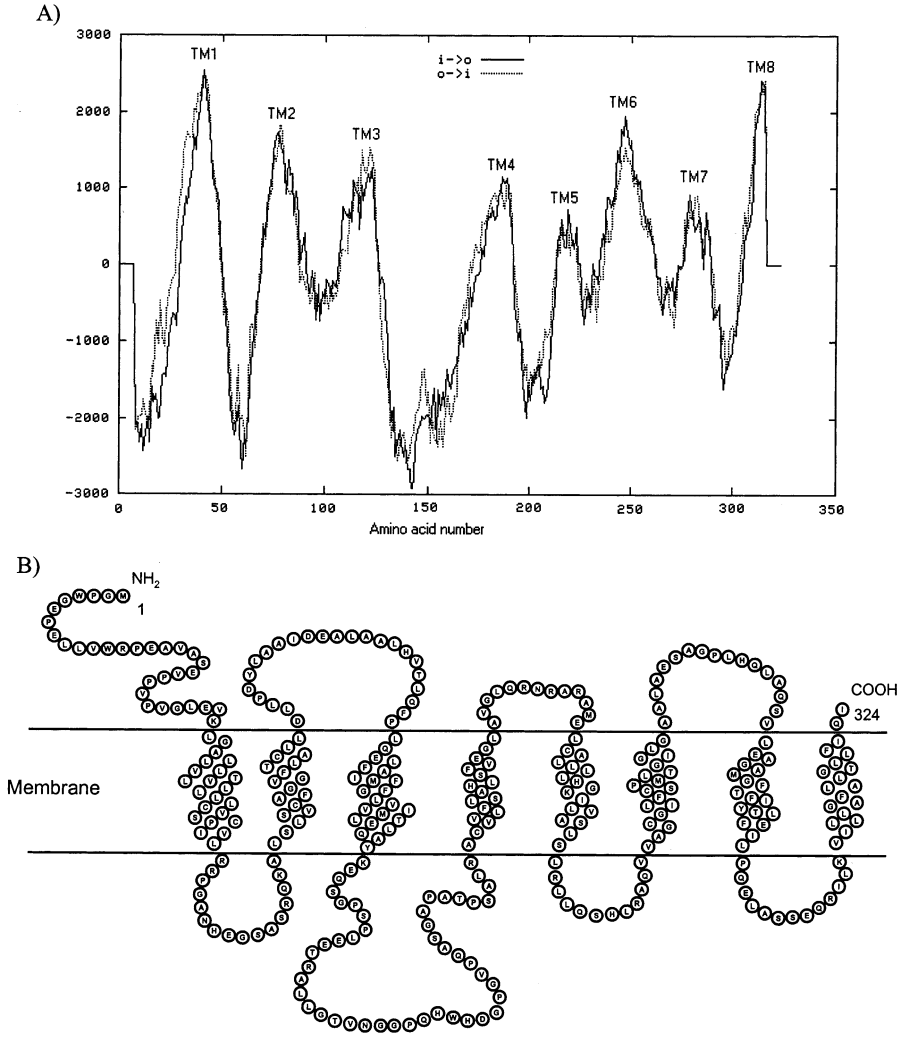

Fig. 1. Predicted secondary structure of hZip1. A: Hydropathy analysis of hZip1 by the method of Kyte and Doolittle. B: Putative molecular structure of hZip1.

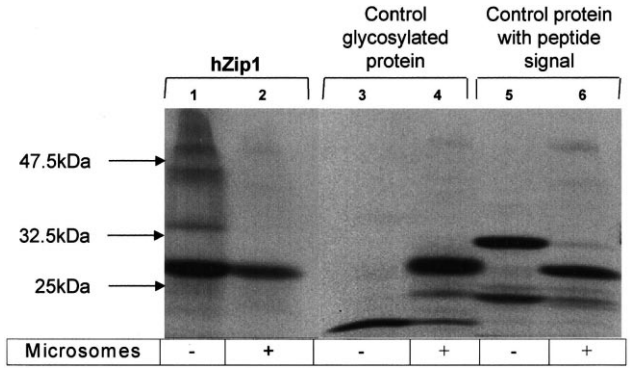

Fig. 2. In vitro transcription/translation of hZip1 cDNA. The hZip1 transcribed mRNA and two control mRNAs for post-translational modification were translated in a reticulocyte lysate in the presence of [^{35}S]methionine, with (lanes 2, 4 and 6) or without (lanes 1, 3 and 5) addition of canine pancreatic microsomal membranes. Products were electrophoresed on a 12% SDS–polyacrylamide gel. The two controls were the α-factor (lane 3), which has a slower mobility following glycosylation (lane 4) and β-lactamase (lane 5) which has a lower molecular weight following cleavage of the peptide signal (lane 6).

(Life Technologies) were then added and incubated for 30 min at 42°C and for 3 min at 95°C. PCR was performed using 2 μl of cDNA, 1.5 mM $MgCl_2$ and 10 pmol of hZip1-specific primers (CCTGGCTTTGCTGCTCCACAAGGGCAT and TCCTATTCCCCACAACTGGGGGAGGGA), with denaturation at 95°C for 3 min followed by a hot start at 80°C (Taq Life technologies) and 10, 15, 20 or 25 cycles consisting of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C. PCR fragments were visualized on an EtBr-stained agarose gel.

3. Results

The human cation transporter hZip1 is a 324 amino acid protein with a calculated molecular mass of 34 kDa. Prediction structure and hydrophobicity plot analyses by the TMpred program suggest that it contains eight putative transmembrane domains, with highly conserved motifs characteristic of the ZIP family (Fig. 1).

3.1. Absence of post-translational modification of hZip1 protein

In order to study post-translational modifications, hZip1 cDNA was transcribed in vitro and the resulting mRNA was translated in a reticulocyte lysate in the presence of [^{35}S]methionine, with or without pancreatic microsomal membranes (Fig. 2). The native protein migrates as a 30 kDa marker (lane 1) and is not modified in the presence of microsomal membranes (lane 2), unlike the control protein α-factor whose molecular weight increases following glycosylation (lanes 3, 4) or β-lactamase which has a lower molecular weight following cleavage of the signal peptide (lanes 5, 6). This

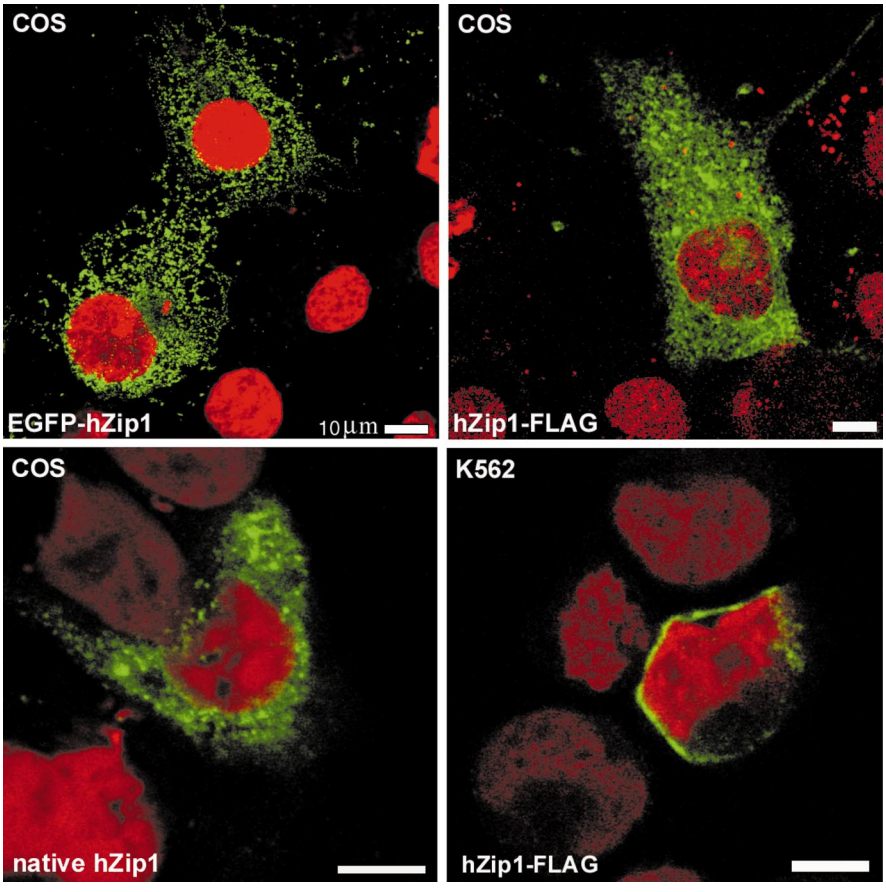

Fig. 3. Subcellular localization of hZip1 in transiently transfected cells. COS-7 cells were transfected with a vector expressing the EGFP-hZip1 (upper left) or the hZip1-FLAG fusion protein (upper right), or the native protein (lower left). K562 cells were transfected with the hZip1-FLAG fusion protein (lower right). hZip1-FLAG and expressed native hZip1 were revealed using anti-FLAG or affinity-purified anti-hZip1 antibodies, respectively. GFP fluorescence or fluorescent anti-IgG antibodies (pseudo-colored in green) were visualized using a confocal microscope. The nuclei were counterstained with propidium iodide (pseudo-colored in red) (bar = 10 μm).

suggests that hZip1 contains no *N*-glycosylation sites nor peptide signal.

3.2. Subcellular localization

In order to study the subcellular localization of hZip1, we first subcloned the hZip1 coding sequence in frame with the green fluorescent protein (EGFP) in pEGFP expression vectors. Two different hZip1-EGFP constructs were generated, with EGFP either in the N-terminal or in the C-terminal part of the construct. Both constructs were tested by transient transfection into different cell lines and analyzed by confocal microscopy. Results obtained with both constructs were very similar, and only the results obtained with the EGFP in the N-terminal part of the fusion protein (EGFP-hZip1) are shown.

Confocal microscopy reveals a bright punctate vesicular

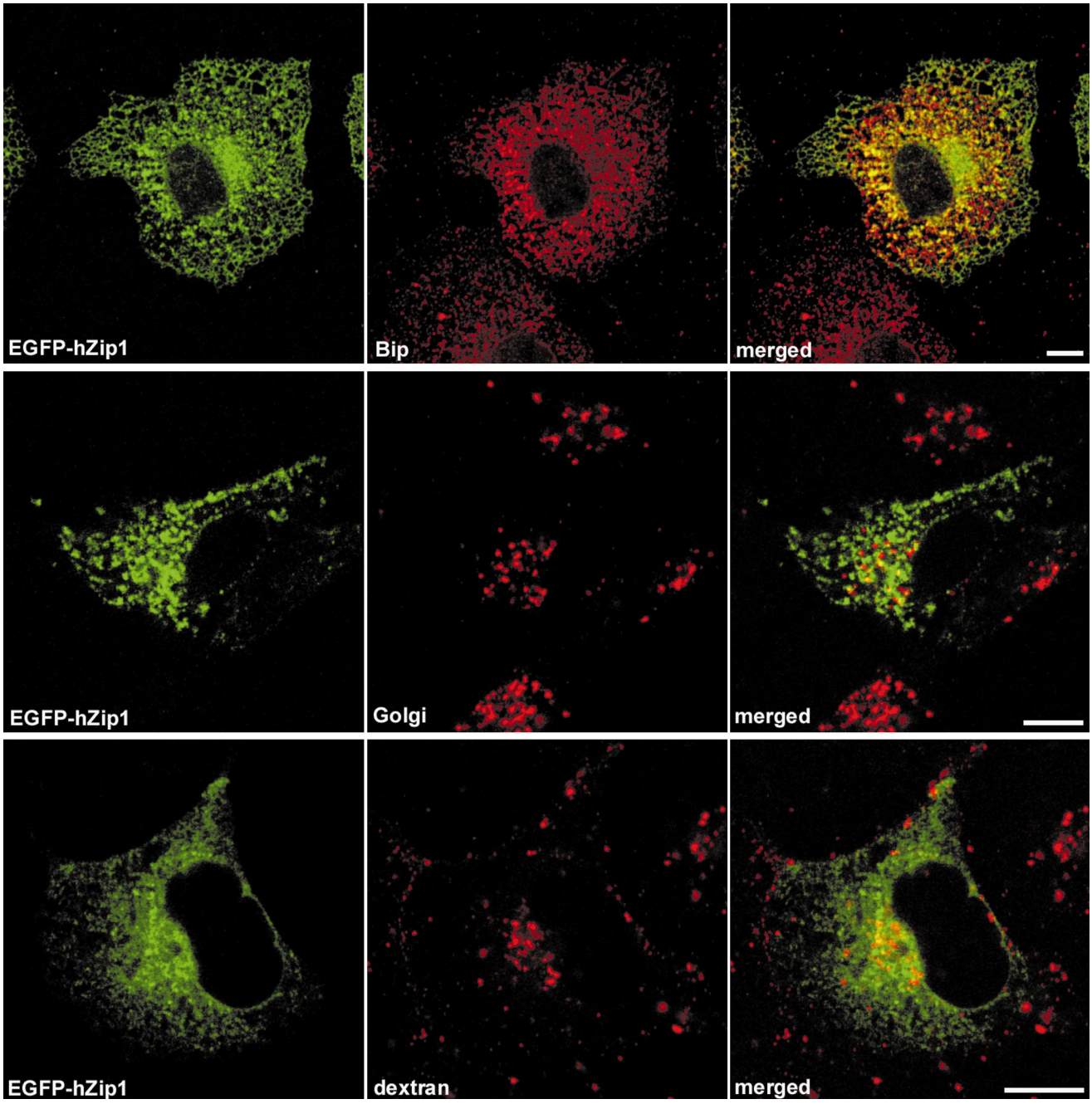

Fig. 4. Colocalization of hZip1 with ER and Golgi markers and with the late endosomal pathway. In COS-7 cells (upper panel), the overlapping of the EGFP-hZip1 staining (pseudo-colored in green) and of the ER marker stained by rhodamine-conjugated anti-mouse IgG (pseudo-colored in red) results in a yellow staining. In HepG2 cells (middle panel), the staining patterns of EGFP-hZip1 (green) and of medium Golgi compartment marker stained by rhodamine-conjugated anti-mouse IgG (red) were mostly different and the only few yellow spots found in the merged image cannot account for a significant colocalization. In the lower panel, COS-7 cells were chased with dextran-free medium for 120 min after incubation with Texas red-dextran beads. There was a partial colocalization (yellow spots) of EGFP-hZip1 (green) with a limited number of the endocytic vesicles containing dextran beads (red). This partial colocalization appeared restricted to a perinuclear area (bar = 10 μm).

staining with a perinuclear and cytoplasmic pattern in COS-7 cells (Fig. 3). A similar pattern was also observed in several epithelial cell lines, including HepG2 (Fig. 4), PC-3 (Fig. 5) and Caco-2 (not shown). To confirm that this intracellular localization was not an artifact due to the presence of the GFP in the fusion protein, we made a construct expressing a hZip1-FLAG fusion protein (pcD-hZip1-FLAG) and another one expressing the native hZip1 (pcD-hZip1). Using anti-FLAG antibodies or anti-hZip1 antibodies on transfected COS-7 cells, we observed a very similar punctate and vesicular localization (Fig. 3). In contrast, K562 cells transfected with the pcD-hZip1-FLAG vector showed a strong staining of the plasma membrane (Fig. 3) as it has been recently reported [8].

The vesicular staining pattern that we observed in COS-7 and HepG2 cells suggested an ER localization (Figs. 3 and 4). To confirm this hypothesis, we performed colocalization studies using monoclonal antibodies directed against the immunoglobulin heavy chain binding protein (BIP) which is a resident protein of the ER. In COS-7 cells, the staining of the EGFP-hZip1 fusion protein showed partial colocalization with that for BIP (35%), mostly in the perinuclear area (Fig. 4). Similar results were obtained with the protein disulfide isomerase (PDI), another resident protein of the ER (not shown). We repeated the same experiments with antibodies directed against antigen of the Golgi apparatus. Since the antibody we used recognizes only the human protein, we performed this colocalization study on human HepG2 cells [12]. As shown in Fig. 4, there was not a significant colocalization of the EGFP-hZip1 fusion protein with the Golgi marker. Additional experiments were performed with labeled dextran to study the endosomal-lysosomal pathway (Fig. 4). When the cells were chased with dextran-free medium for 15 or 60 min, no EGFP-hZip1 signals overlapped in distribution with labeled dextran (data not shown). After 120 min chase, a few EGFP-hZip1 signals partly colocalized with some of the endocytic vesicles containing labeled dextran into a perinuclear compartment.

3.3. *hZip1 subcellular localization and expression in prostate cancer cells PC-3*

Costello et al. [13] have shown that prolactin treatment increases zinc uptake at the plasma membrane in PC-3, a prostate cancer cell line and they proposed that this increase resulted from activation of hZip1 expression, at odds with the localization we report in this paper. Indeed, the subcellular localization of the EGFP-hZip1 fusion protein transiently transfected into PC-3 cells (Fig. 5, lower right), as well as that of the endogenous hZip1 (Fig. 5, upper) appeared iden-

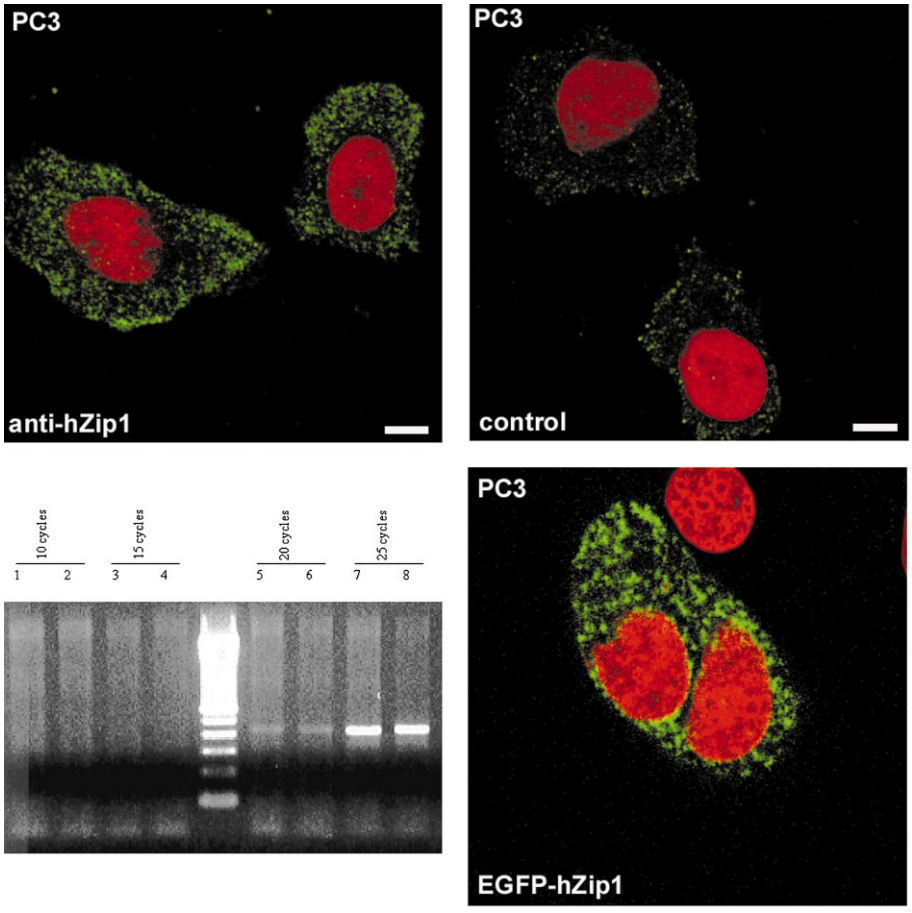

Fig. 5. Subcellular localization of hZip1 and expression of the mRNA in PC-3 cells. PC-3 cells were fixed and incubated with affinity-purified anti-hZip1 antibody (upper left) and Alexa Fluor 488 anti-rabbit IgG. The control was performed by incubating the anti-hZip1 antibodies with an excess of the recombinant peptide for 1 h at 37°C prior to incubating with the cells (upper right). Localization of the transfected EGFP-hZip1 fusion protein was indicated by GFP fluorescence (lower right). hZip1 mRNAs were quantified in prolactin-stimulated PC-3 cells by RT-PCR (lower left). At different cycles, RT-PCR from non-treated (lanes 1, 3, 5 and 7) and prolactin-treated (lanes 2, 4, 6 and 8) cells were loaded on agarose gel and visualized by ethidium bromide staining.

tical to those observed in COS-7 or HepG2 cells, without significant plasma membrane labeling. In addition, we performed a semi-quantitative RT-PCR on mRNA from control or prolactin-stimulated PC-3 cells, using oligonucleotides specific for hZip1 (Fig. 5, lower left). No obvious difference could be seen in hZip1 mRNA levels between prolactin-treated and non-treated cells, suggesting that under our conditions, hZip1 is not up-regulated by prolactin.

4. Discussion

In this paper, we report that hZip1 can have a different subcellular localization according to cell type and show that this protein is not glycosylated and does not contain a peptide signal.

The EGFP-hZip1 and the hZip1-FLAG fusion proteins, as well as the expressed native hZip1 and the endogenous hZip1 repeatedly gave an intracellular distribution in epithelial and COS-7 cell lines and colocalization studies suggested that hZip1 partly resides in ER. The very partial colocalization with endocytosed dextran beads appeared restricted to a perinuclear compartment which could correspond to late/recycling endosomes. In contrast, the hZip1-FLAG fusion protein is sorted to the plasma membrane in K562 cells (erythroïd cell line).

The use of an EGFP tag can create misfolding of hZip1 and abnormal retention in ER compartment, but the use of a much smaller tag (FLAG) and studies of the endogenous hZip1 gave identical results. Moreover, this intracellular staining pattern was obtained in four different epithelial cell lines whereas both we and Gaither and Eide [8] observed a plasma membrane localization of the FLAG-tagged protein in the erythroïd cell line K562.

We have also found that hZip1 has an intracellular localization in the prostate cancer cell line PC-3. This rules out the possibility that hZip1 mediates the hormone-inducible zinc transport activity that has been reported in these cells. Accordingly, we failed to reproduce the up-regulation of hZip1 mRNA by prolactin treatment described by Costello et al. [13]. It is possible that the probe they used in Northern blot experiment cross-hybridized with hZip2 mRNA which has been shown by Gaither and Eide to localize at the plasma membrane and to stimulate zinc uptake [9].

The intracellular vesicular localization of hZip1 in epithelial cell lines suggests that it might have a role in regulating cytoplasmic accumulation of zinc. Zinc homeostasis in cells relies on several mechanisms. The first one implies increase in zinc efflux mediated by ZnT1. A second mechanism involves transport and sequestration of zinc into vesicular compartment depending on ZnT2 and ZnT3 expression [14]. Considering that hZip1 stimulates zinc uptake when it is expressed at the plasma membrane, it is possible that, in cells where it has a vesicular localization, it could contribute to transfer of stored zinc into the cytoplasm in condition of zinc depletion. In yeast, Zrt3, a new member of the ZIP family, has recently been shown to mobilize zinc from the vacuole [15]. Further functional studies will be necessary to elucidate these points.

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