Constitutive expression of hZnT4 zinc transporter in human breast epithelial cells

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Zinc is an essential trace element required by all living organisms. An adequate supply of zinc is particularly important in the neonatal period. Zinc is a significant component of breast milk, which is transported across the maternal epithelia during lactation. The mechanisms by which zinc becomes a constituent of breast milk have not been elucidated. The function of the zinc transporter ZnT4 in the transport of zinc into milk during lactation was previously demonstrated by studies of a mouse mutant, the 'lethal milk' mouse, where a mutation in the *ZnT4* gene decreased the transport of zinc into milk. In the present study, we have investigated the expression of the human orthologue of *ZnT4* (*hZnT4*) in the human breast. We detected *hZnT4* mRNA expression in the tissue from the resting and lactating human breast, using reverse-transcriptase PCR. Western-blot analysis using antibodies to peptide sequences of hZnT4 detected a major band of the predicted size of 47 kDa and a minor band of 77 kDa, in extracts from the resting and lactating breast tissues. There was no difference in the hZnT4 expression levels between lactating and resting breasts. The hZnT4 protein was present in the luminal cells of the ducts and alveoli where it had a granular distribution. A cultured human breast epithelial cell line PMC42 was used to investigate the subcellular distribution of hZnT4 and this showed a granular label throughout the cytoplasm, consistent with a vesicular localization. The presence of zinc-containing intracellular vesicles was demonstrated by using the zinc-specific fluorphore Zinquin (ethyl-[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate). Double labelling indicated that there was no obvious overlap between Zinquin and the hZnT4 protein, suggesting that hZnT4 was not directly involved in the transport of zinc into vesicles. We detected expression of two other members of the *hZnT* family, *hZnT1* and *hZnT3*, in human breast epithelial cells. We conclude that *hZnT4* is constitutively expressed in the human breast and may be one of the several members of the ZnT family involved in the transport of zinc into milk.

Key words: epithelial cells, lactation, zinc gene, zinc transport.

INTRODUCTION

Zinc is an essential trace element required by all living organisms. The numerous functions of zinc in cells are related to its structural and catalytic roles in biological processes. Zinc has a unique and extensive role in key biological processes including growth, development and reproduction. In humans, the significance of zinc is illustrated by the devastating effects of zinc deficiency, which include dermatitis, diminished immune response, decreased healing and neurological changes [1]. Nutritional zinc deficiency in a newborn or growing animal can be fatal; it is most probably a consequence of enzyme defects, as it is known that over 300 mammalian enzymes are zinc-dependent (see [2] for a review). The cellular and molecular processes by which zinc is incorporated and transported within living organisms are not clear. Recently, several genes which encode proteins that are involved in the uptake and efflux of zinc have been identified in bacterial, yeast and rodent cells [3–9]. The identification of these genes has provided opportunities for the isolation and study of the human orthologues. Gene sequences belonging to the *ZnT* family of genes predict proteins with multiple membrane-spanning regions and histidine-rich domains that are thought to be involved in zinc binding and transport. *ZnT4* was initially identified from a cDNA clone called Dri27, which was obtained from rat intestine by subtractive hybridization [10]. A function for the ZnT4/Dri27 protein in zinc intake in the gut was suggested by its upregulation in differentiated enterocytes and localization on the basal side of intestinal cells in the rat. The histidine residues of rat Dri27/ZnT4 were demonstrated to bind zinc [11]. ZnT4 was found to be defective in the 'lethal milk' mouse that produces zinc-deficient milk [6]. Newborn mice suckling 'lethal milk' dams develop zinc deficiency and die within a week. The defect in this disease was shown to be the result of impaired transfer of zinc into milk [12]. This finding, together with the capacity of mouse ZnT4 to confer zinc resistance when expressed in a zinc-sensitive Δ*zrc1* yeast strain [6], suggests that ZnT4 plays a role in the transport of zinc from the breast into milk.

Although ZnT4 has been implicated in zinc transport in the rodent mammary gland, expression of ZnT4 in the human breast has not been reported. In the present study, we show that the *hZnT4* gene is expressed in both resting and lactating human breast epithelial cells and that the expression of the hZnT4 protein is not increased as a result of lactation. Using a cell-cultured model of the human breast [13], we show for the first time the intracellular localization of endogenously expressed hZnT4 protein in human breast epithelial cells.

MATERIALS AND METHODS

Breast tissue

Tissue from normal non-lactating and normal lactating breast was obtained from breast biopsies performed for diagnosis of breast disease. The tissues were immediately frozen at −80 °C until use.

Abbreviations used: FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; RT–PCR, reverse-transcriptase PCR; TBS, Tris-buffered saline; Zinquin, ethyl-[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate.

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Table 1 Primers to *ZnT4*, *ZnT1* and *ZnT3*

Primers were designed according to GenBank® Nucleotide Sequence accession nos. AF025409, 1419271/AA252544 and HSU76010, respectively.

Forward primer	Reverse primer	PCR product length (bp)
ZnT4-C GGAAGCGCCTCAAATCTATGCT	*ZnT4*-D TACATTCAAAATGGCTTGGCACA	1004
ZnT4-E GATAGCCTGGCAGTGAGAGCTG	*ZnT4*-F ATACGGACACAGCTGTCAGGGA	610
ZnT4-E GATAGCCTGGCAGTGAGAGCTG	*ZnT4*-G TGCTTTGGACTGTACTTCC	381
ZnT4-E GATAGCCTGGCAGTGAGAGCTG	*ZnT4*-G45 TGCTTTGGACTGTACTTCCTCCTGGCAGTAGATTTTCCTGAAG	336
ZnT1-A CCTGGGCTTCTTCTCTAGATTG	*ZnT1*-B TTGTCTTGGAAAGGTTGTTCTG	634
ZnT3-A GAGTCTCTTCACAGAGCCCTCA	*ZnT3*-B CTGGTGCAGCACAAAGGCCATT	508

Cell culture

The cell line PMC42, originally derived from a pleural effusion [14], was cultured in Nunclon 25 or 80 cm² culture flasks in RPMI 1640 (Trace Biosciences, Melbourne, Australia) supplemented with 10 % fetal bovine serum (FBS) (CSL, Melbourne, Australia). The cells were passaged when confluent by using 0.05 % trypsin/versene solution in PBS (Sigma–Aldrich, Sydney, Australia). Cultures were viewed with an Olympus CK2 inverted phase-contrast microscope. PMC42 cells resistant to 500 μM zinc were obtained by growth in RPMI 1640 with 10 % FBS containing increasing concentrations of $ZnCl_2$ from 50 to 500 μM over a period of 4 months.

Exposure of PMC42 cells to zinc

To determine the effect of increased extracellular zinc levels on *hZnT4* expression, confluent PMC42 cells were incubated in 100 μM $ZnCl_2$ in RPMI 1640 with 10 % FBS for 3 h or overnight. The cells were then rinsed three times in PBS and harvested.

Treatment of PMC42 cells with sodium pyrithione

To increase intracellular zinc concentrations, confluent PMC42 cells were treated with 1 μM sodium pyrithione for 3 h (Sigma–Aldrich) in RPMI 1640 with 10 % FBS containing 25 μM $ZnCl_2$. Cells were then rinsed three times in PBS before harvesting.

RNA preparation

Cultured PMC42 cells from a 80 cm² culture flask were rinsed three times in PBS and trypsinized. After rinsing twice in PBS, total RNA was prepared by using a Qiagen RNeasy Mini Kit according to the manufacturer's instructions. For preparation of RNA from resting and lactating human breast tissue, 20 mg of frozen tissue was disrupted in liquid nitrogen with mortar and pestle and homogenized by using a needle and syringe. Tissue lysate was centrifuged for 3 min at 18000 ***g*** and the supernatant was used for RNA preparation according to the manufacturer's instructions in the Qiagen RNeasy Mini Kit.

Reverse transcription and PCR

Reverse transcription was carried out at 41 °C with the following: 5 μg of total RNA from cultured PMC42 cells or from breast tissue, 4 μl of 40 mA_{250} units random hexamer primer (Roche Diagnostics, Sydney, Australia), 4 μl of 5 × RT buffer, 3 μl of 2 mM dNTP, 0.5 μl of 40 units RNase inhibitor/μl (Roche Diagnostics), 40 units of avian myeloblastosis virus RT (Roche Diagnostics), for 1.5 h. cDNA (5 μl) was used as a template for PCR amplification, to which was added 55 pmol of forward *hZnT4* primers C or E and 55 pmol of reverse *hZnT4* primers D or F (Table 1), 5 μl of 2 mM dNTP, 5 μl of 10 × buffer (Sigma–Aldrich), 1.5 mM $MgCl_2$, and 2.0 units of *Taq* DNA (Sigma–Aldrich). Amplification was performed by using 34 cycles of 95, 58 and 72 °C for 60, 30 and 45 s, respectively. PCR products were viewed after agarose gel electrophoresis. A similar protocol was used to amplify *hZnT1* (forward-primer *ZnT1*-A, reverse-primer *ZnT1*-B) and *hZnT3* (forward-primer *ZnT3*-A, reverse-primer *ZnT3*-B) (Table 1).

Competitive reverse-transcriptase PCR (RT–PCR)

A competitor DNA fragment for *hZnT4* was generated by using a forward primer *hZnT4*-E, a reverse primer that contained *hZnT4*-G primer sequences plus 23 additional bases complementary to a region 45 bases upstream on the template cDNA, *hZnT4*-G45 (Table 1). This primer was used in a standard PCR reaction to create a competitor fragment with a deletion of 45 bp. The competitor fragments were purified and their concentrations measured in a UV spectrophotometer at 260 nm. They were diluted to known quantities and 1 μl of the template DNA was added to competitive PCR reactions. The *hZnT4*-E and *ZnT4*-G primers were used for competitive RT–PCR of both competitor and target *hZnT4* cDNA templates. A master mixture containing 2 mM dNTP, 10 × buffer, 25 mM $MgCl_2$, 2.5 units of *Taq* polymerase, primers and sterile Millipore water was prepared as described above and aliquoted into tubes containing 2.5 μl of test cDNA and 1 μl of competitor DNA. After electrophoresis of the PCR products, the ethidium bromide-stained bands of the competitor and normal fragments were recorded.

Antibodies

Two synthetic peptides were prepared for antibody production, the MAG peptide consisting of the N- and C-terminal sequences of hZNT4 (MAGSGAWKRCQSSSP) and the CSK peptide consisting of internal hydrophilic sequences of hZnT4 (CSKQREILKQRKVKARLT) (Chiron Technologies, Melbourne, Australia). The MAG and CSK peptides were used to raise CSK and MAG antibodies to human hZnT4 protein in two separate rabbits (Institute of Medical and Veterinary Science, Adelaide, South Australia). MUC1 (BC2 murine monoclonal anti-human [15]) antiserum was kindly supplied by Professor Ian McKenzie (Austin Research Institute, Melbourne, Australia). Monoclonal anti-human β-casein antibody (MAS-447b) and goat anti-human lactoferrin (AES216) were purchased from Harlan Sera-Lab (Melbourne, Australia). Monoclonal antibody to β-actin was obtained from Sigma.

Affinity purification of hZnT4 antisera

A solution of powdered MAG and CSK antigens was prepared in sterile 0.01 M PBS at a concentration of 200 μg/400 μl and 400 μl of the diluted antigen was placed into an Eppendorf tube

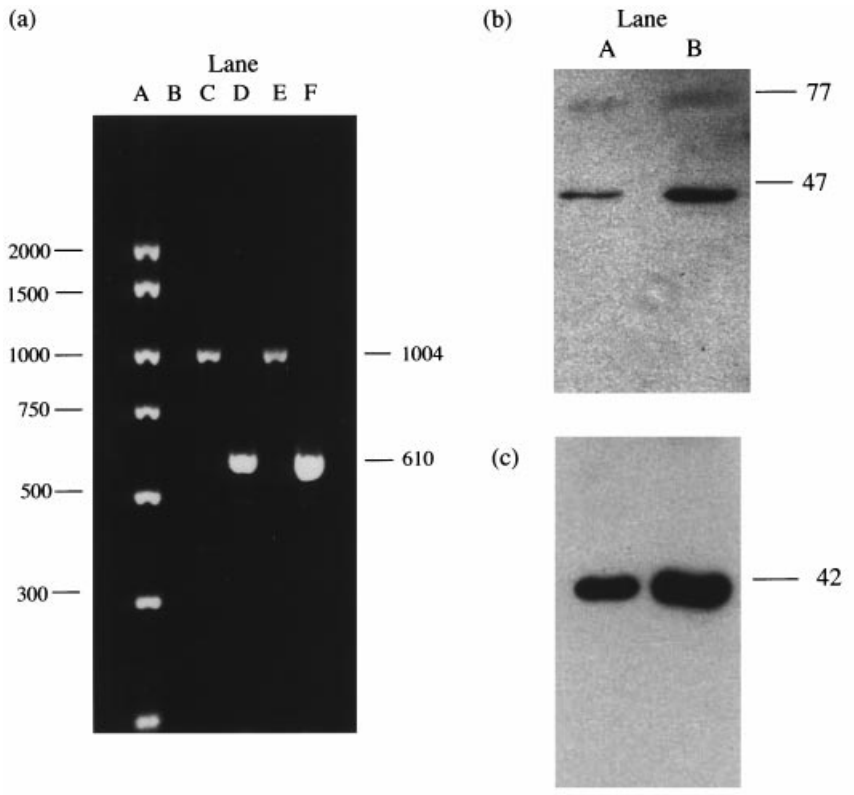

Figure 1 RT–PCR and Western-blot analyses of human breast tissue

(**a**) RT–PCR using two forward primer and reverse primer sets (Table 1) to detect *hZnT4* expression in human breast tissue extracts. Lane A, MW markers; lane B, control with no cDNA; lane C, resting breast with *hZnT4*-C and *hZnT4*-D primers; lane D, resting breast with *hZnT4*-E and *hZnT4*-F primers; lane E, lactating breast with *hZnT4*-C and *hZnT4*-D primers; lane F, lactating breast with *hZnT4*-E and *hZnT4*-F primers. (**b**) Western-blot analysis of resting and lactating human breast tissue. hZnT4 protein was detected by using CSK and MAG antibodies, raised to two different peptide sequences: lane A, 300 μg of resting human breast tissue extract showing a strong 47 kDa band and a weak 77 kDa band; lane B, 300 μg of lactating human breast tissue extract showing a strong 47 kDa band and a weak 77 kDa band. (**c**) Western-blot analysis of samples from (**b**), probed with antibody to β-actin to indicate relative amounts of protein loaded.

with a 1 cm^2 piece of crumpled nitrocellulose membrane (Amersham). The tubes were incubated overnight at 4 °C on a rocker and the antigen mixture was removed and 1 ml of 3 % BSA diluted in PBS was added to prevent non-specific binding of antibody. The tubes were incubated overnight at 4 °C on a rocker and the BSA was then removed and the membranes were washed eight times with 0.1 % BSA in PBS. Antiserum (400 μl of crude serum diluted with 800 μl of PBS) was added to the drained membrane and left overnight on a rocker at 4 °C to ensure binding of antibodies to the peptide. The antiserum was removed and the subsequent washes were completed for 2 min each at 4 °C. Three 1 ml washes with 0.1 % BSA in Tris-buffered saline (TBS); two 1 ml washes with 0.1 % BSA in TBS and 0.1 % Nonidet P40 in TBS; three 1 ml washes with 0.1 % BSA in TBS. The last wash was completely removed and 150 μl of 0.2 M glycine/HCl (pH 2.5) was added to each tube for 1 min to strip the antibody from the membrane. The solution was neutralized by the addition of 75 μl of 1 M KPO_4 (pH 9.0) in 5 % BSA in PBS. The mixture was then transferred to a new sterile Eppendorf tube, the acid and neutralization steps were repeated and the mixtures were added to the first preparation. The mixtures from both elutions were then placed together in Centricon filters (Millipore, Australia) and centrifuged for 10 min at 5000 ***g*** at 4 °C. The Centricon was then inverted and spun for 2 min at 1000 ***g*** at 4 °C. The final solution contained the concentrated, purified antibody.

Western-blot analysis

PMC42 cells grown in 80 cm^2 plastic flasks were washed twice with PBS (5 ml at 23 °C), trypsinized (0.05 % trypsin/versene in PBS) and washed twice with 3 ml of PBS and collected as a cell pellet by centrifugation at 789 ***g*** for 5 min at 23 °C. Cells were lysed by sonication (15 pulses, 40 % power output, 30 % duty cycle) in 1 ml of EDTA-free inhibitor cocktail (Roche Diagnostics), 20 mg SDS/ml and 5 mM 2-mercaptoethanol (Sigma–Aldrich) and centrifuged for 5 min at 18000 ***g***. Breast tissue (100 mg) was disrupted in liquid nitrogen, homogenized in a Dounce homogenizer (30 strokes) and sonicated as described in the previous step. The cell extract and breast-tissue supernatants (300 μg protein) were fractionated by SDS/PAGE (12 % gel) by using a Bio-Rad Mini Protean Gel system according to the manufacturer's instructions. Proteins were transferred to nitrocellulose membranes at 10 V for 25 min by using a Trans-Blot SD semi-dry Transfer Cell in 25 mM Tris, 192 mM glycine (pH 8.3) and 20 % methanol. After overnight blocking with 1 % casein in TBST [0.05 M Tris, 0.15 M NaCl and 0.1 % (v/v) Tween 20], the membrane was treated with the hZnT4 CSK or MAG antibody 1/200 or β-actin antibody 1/2000, diluted in the blocking buffer for 2 h and then washed in the blocking buffer (4 × 15 min). hZnT4 protein was detected by using a 1 in 1000 dilution of a horseradish peroxidase-conjugated sheep anti-rabbit (hZnT4) or sheep anti-mouse (β-actin) antibody

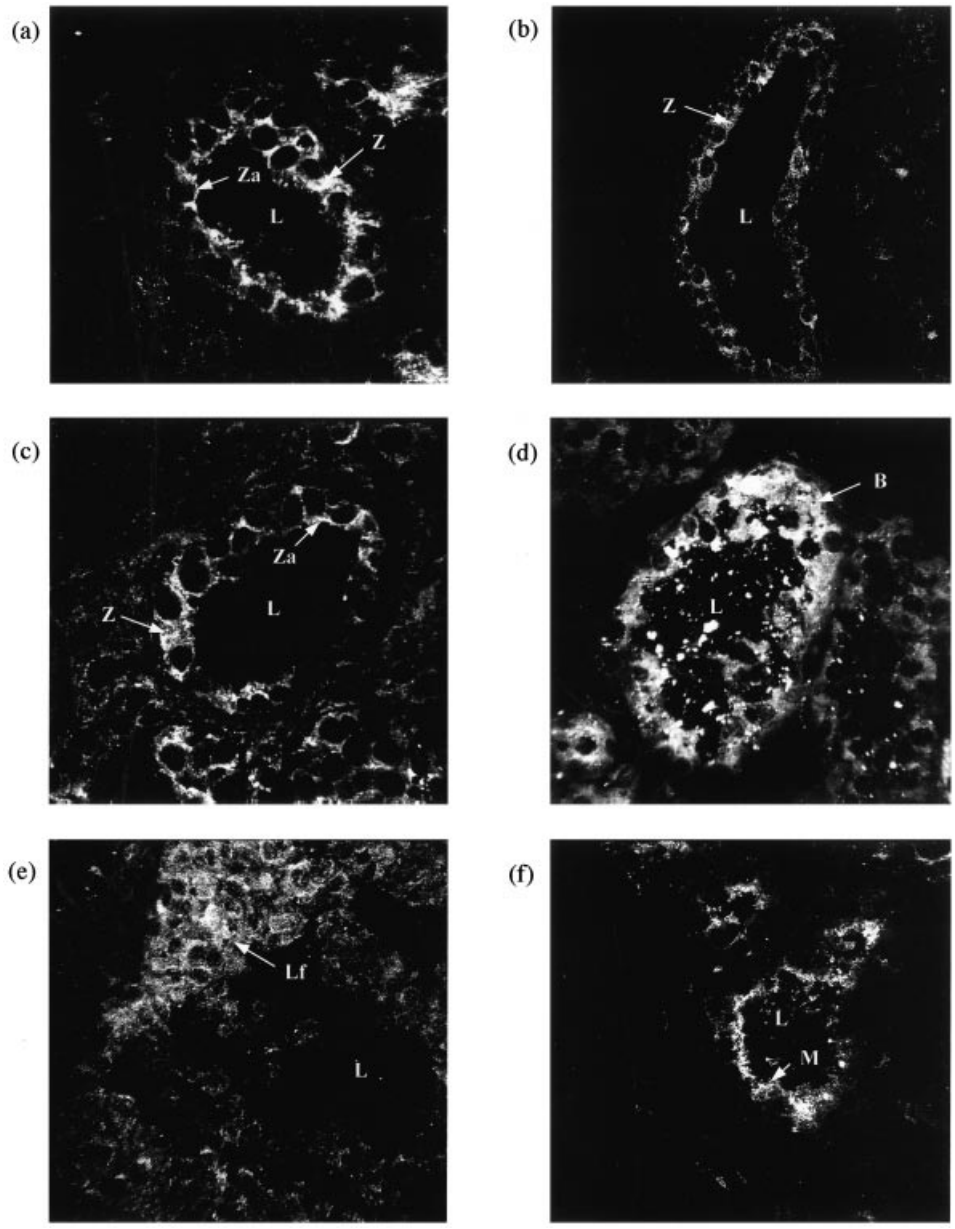

Figure 2 Immunohistochemical analysis of human breast tissue

(**a**) MAG antibody to hZnT4 protein shows granular distribution of hZnT4 (Z) in luminal epithelial cells of a single alveolus in resting breast tissue. Some label is found on the apical surface (Za), adjacent to the lumen (L) of the alveolus. (**b**) Localization of hZnT4 in luminal epithelial cells of a duct in resting breast tissue, showing granular distribution of ZnT4 throughout luminal epithelial cells (Z) adjacent to the lumen of the duct (L). (**c**) In lactating human breast tissue, ZnT4 is located in the luminal epithelial cells of a secretory alveolus (Z) where it has a granular distribution similar to that found in the resting breast tissue. Some label is found on the apical surface (Za), adjacent to the lumen (L) of the alveolus. (**d**) Immunohistochemical localization of the major human milk protein β-casein (B) in lactating breast tissue, showing strong label throughout the cytoplasm of luminal epithelial cells and within the secretory lumen itself (L). (**e**) Immunohistochemical localization of the milk protein lactoferrin (Lf) in lactating breast, showing a granular, diffuse label in the luminal epithelial cells adjacent to the lumen (L). (**f**) Immunohistochemical localization of MUC1 glycoprotein (M) in lactating breast, showing a predominantly apical distribution in the luminal epithelial cells adjacent to the lumen (L). Magnification, $\times 600$.

and a chemiluminescence detection kit (Roche Diagnostics) according to the manufacturer's instructions. The membranes were placed in contact with a Kodak XAR X-ray film for 1–15 min.

Immunocytochemistry and immunohistochemistry

PMC42 cells grown on 1 cm² glass coverslips were rinsed three times in PBS and fixed in 4 % (w/v) paraformaldehyde for 10 min. They were then rinsed twice in PBS, permeabilized with 0.1 % (v/v) Triton X-100 in PBS for 10 min and blocked with 3 % BSA in PBS for 90 min. Breast tissue blocks (1 cm^3) were immersed in OCT (Tissue Tek, Sydney, Australia), frozen in liquid nitrogen for 3 min, and sectioned (Leica CM 1800 cryostat) in the temperature range -17 to -20 °C. Sections 8–10 μm thick were collected on 5 % gelatin-coated slides. The sections were dried and fixed in paraformaldehyde as described above, rinsed twice in PBS, permeabilized with 5 % (v/v) Triton X-100 in PBS for

5 min and blocked with 3 % BSA in PBS for 90 min. Primary antibodies diluted in 1 % BSA in PBS (1 in 50 for hZnT4, 1 in 2000 for β-actin, 1 in 50 for β-casein antibody, 1 in 200 for lactoferrin and 1 in 200 for MUC-1) were applied to cells and sections overnight at 4 °C. Control cells were incubated with a mixture of 1 μM hZnT4 antibody with 4 μM hZnT4 CSK or MAG peptides overnight. After three PBS washes, a secondary antibody AlexaFluor 488 goat anti-rabbit IgG (Molecular Probes, OR, U.S.A.) or AlexaFluor 488 goat anti-mouse, 1 in 2000 dilution in 1 % BSA in PBS was applied for 2 h at 23 °C. Cells were washed three times for 10 min in PBS and a drop of Fluoroguard (Bio-Rad, Sydney, Australia) was added to the sections and a coverslip applied. Epifluorescence was viewed with an Olympus BX50 microscope with a PlanApo 60 × 1.4 oil objective. Confocal images were collected by using an Optiscan F900e system in a 512 × 512 pixel format.

Detection of free zinc pools by using Zinquin (ethyl-[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate)

Zinquin was kindly supplied by Dr Peter Zalewski (Department of Medicine, University of Adelaide, Adelaide, Australia). Cells on coverslips were rinsed three times in PBS and Zinquin [100 μM in Hanks balanced salt solution (HBSS)] was added for 30 min at 37 °C. The cells were rinsed three times in PBS, a drop of Fluoroguard was added and the coverslip sealed. For double labelling of cells with hZnT4 and Zinquin, the cells were processed as described above for hZnT4 and treated with Zinquin after the second antibody had been removed.

RESULTS

RT–PCR to detect expression of *hZnT4* in human breast tissue

By using RT–PCR, we detected expression of *hZnT4* in human breast tissue (Figure 1a). With two sets of overlapping primers, we obtained fragments of the predicted sizes of 1004 and 610 bp from resting breast (lanes C and D) and lactating breast tissue (lanes E and F). Together, the PCR products represented 98 % of the *hZnT4* reading frame. The identity of the fragments was confirmed by partial sequencing. No size differences in the *hZnT4* cDNA fragments were detected between the resting and lactating breast tissues.

Western-blot analysis to detect hZnT4 protein

Expression of hZnT4 protein was measured by Western-blot analysis using the CSK and MAG antibodies. Both antibodies produced a major band of size 47 kDa and a weaker band of 77 kDa in extracts from the resting and lactating breasts (Figure 1b). A control protein β-actin was used to indicate the relative levels of protein loaded on the gel (Figure 1c). Although similar levels of protein were loaded on the gel, more β-actin was present in the lactating tissue relative to the resting tissue, due to the increased proportion of glandular tissue relative to the extracellular matrix, seen in the lactating tissue. The ratio of the band intensities of the lactating tissue/resting tissue measured by densitometry was 1.5 for hZnT4 and 1.3 for β-actin. Similar values for these ratios indicate that levels of hZnT4 protein were similar in resting and lactating human breast tissue, relative to the level of the housekeeping protein β-actin.

Immunohistochemical localization of hZnT4 protein

Immunofluorescence using CSK and MAG antibodies showed that hZnT4 was localized to the luminal epithelial cells of the resting and lactating breasts in both the alveolus (Figure 2a) and the duct (Figure 2b), where it had a cytoplasmic distribution. In the lactating breast tissue, hZnT4 had a more extensive, granular cytoplasmic localization compared with the resting breast (Figure 2c). The subcellular localization of hZnT4 was compared with that of two major milk proteins β-casein and lactoferrin, which are secreted during lactation. In lactating human breast tissue, β-casein (Figure 2d) and lactoferrin (Figure 2e) showed a strong, punctate label in the luminal epithelial cells, with a cytoplasmic distribution similar to that of hZnT4. hZnT4 had considerably more dispersed cytoplasmic localization compared with MUC1 (Figure 2f), a mucoprotein which is known to be located exclusively on the apical domain in human breast epithelial cells.

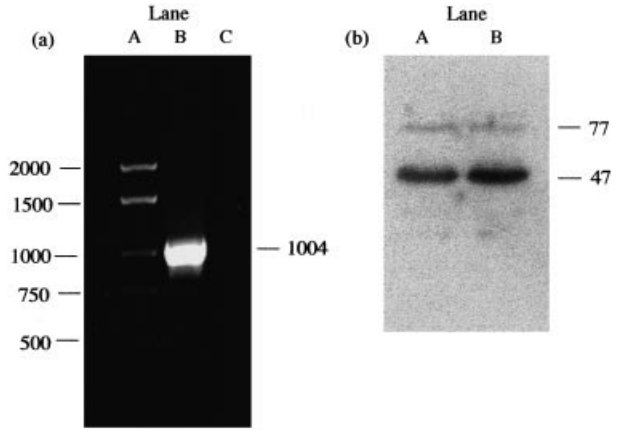

Figure 3 RT–PCR and Western-blot analyses of cultured human breast epithelial-cell extracts

(**a**) RT–PCR to detect *hZnT4* expression in PMC42 cultured human breast epithelial-cell extracts. Lane A, molecular mass markers; lane B, *hZnT4*-C and *hZnT4*-D primers (Table 1), which detected a 1004 bp product; lane C, control with no cDNA. (**b**) Western-blot analysis of PMC42 cell extract. hZnT4 protein was detected by using CSK antibody to hZnT4, which detected a strong 47 kDa band and a weak 77 kDa band from 300 μg of cell extract of untreated PMC42 cells (lane A) and cells which were resistant to 500 μM $ZnCl_2$ (lane B).

RT–PCR detection of *hZnT4* expression in cultured human breast epithelial cells

To investigate further the expression of *hZnT4* in breast epithelial cells we used a human breast epithelial-cell culture model PMC42 in which we have recently been able to induce synthesis of milk proteins [13]. *hZnT4* expression was detected in PMC42 cells by using RT–PCR with *hZnT4*-C and *hZnT4*-D primers (Figure 3a), showing a band of 1004 bp in lane B, similar in size to that found in breast tissue.

Expression of ZnT4 protein in control PMC42 cells and PMC42 cells, which were resistant to 500 μM $ZnCl_2$, was measured by Western-blot analysis using the CSK antibody to hZnT4. A major band of the expected size of 47 kDa and a weaker band of size 77 kDa were seen in untreated PMC42 cells (lane A) and in cells which were resistant to 500 μM $ZnCl_2$ (lane B) (Figure 3b). The MAG antibody to hZnT4 produced similar results, a strong band at 47 kDa and a weak band at 77 kDa.

Immunolocalization of hZnT4 protein in cultured human breast cells

By using indirect immunofluorescence confocal microscopy with the MAG antibody to ZnT4, the maximum-brightness projection image showed hZnT4 localized to the cytoplasm of PMC42 cells where it had a granular appearance with some label above the

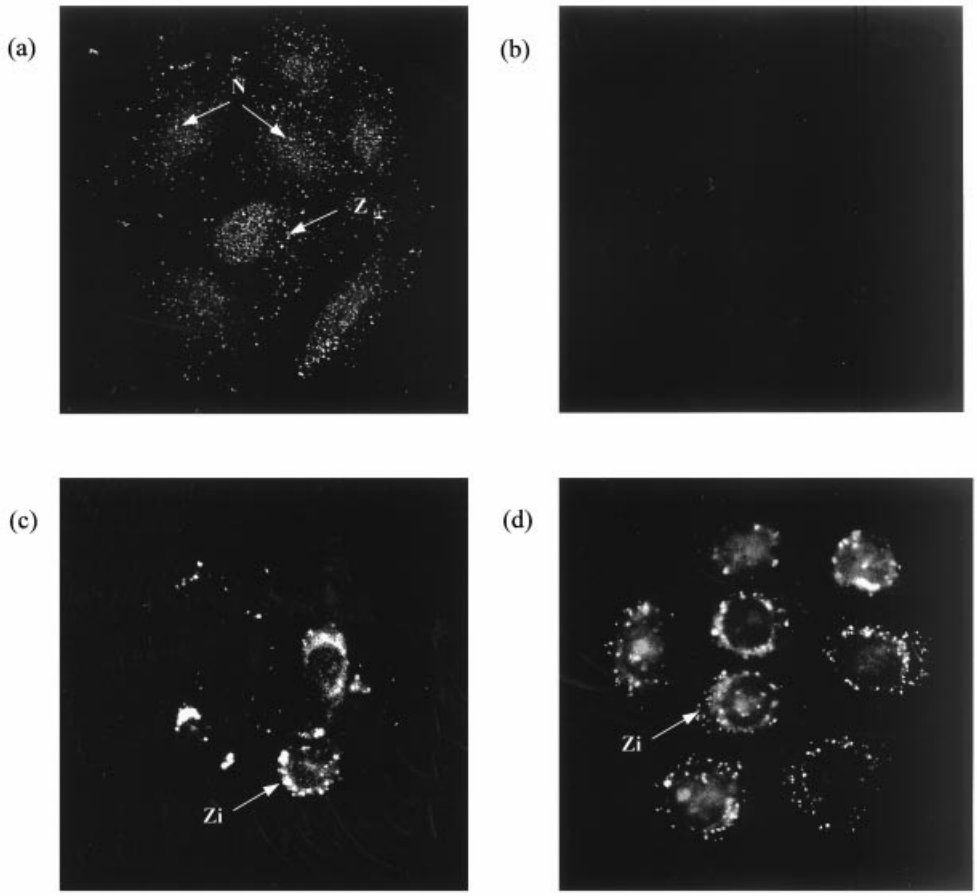

Figure 4 Immunocytochemical analysis of MAG antibody to hZnT4 protein in cultured PMC42 cells

(**a**) Maximum-brightness image using MAG antibody to hZnT4 (Z) protein shows granular distribution of hZnT4 throughout the cytoplasm including a label on the apical side of the nucleus. Nuclei (N) were lightly stained with ethidium bromide. (**b**) Control in which ZnT4 antibody was incubated with 4 μM ZnT4 antigen to compete with binding sites in cells. (**c**) PMC42 cells incubated in Zinquin (100 μM in HBSS) for 30 min showed a punctate label (Zi) located in the region around the nucleus. (**d**) PMC42 cells were incubated with 1 μM of the zinc ionophore sodium pyrithione in RPMI 1640 with 10% FBS containing 25 μM $ZnCl_2$, stained with 100 μM Zinquin. It resulted in a strong punctate label around the nucleus, which extended further into the cytoplasm.

nucleus (Figure 4a). The CSK antibody gave similar results. As a control, 1 μM hZnT4 antibody was mixed with 4 μM hZnT4 antigen and then applied to the cells, with no detectable signal (Figure 4b).

Detection of intracellular free zinc in cultured human breast cells using Zinquin

We used Zinquin to detect pools of free and loosely bound zinc. Zinquin had a perinuclear localization in PMC42 cells (Figure 4c). The intensity of the Zinquin label was increased by incubation of the cells with 1 μM of the zinc ionophore pyrithione in the presence of 25 μM zinc (Figure 4d).

To determine whether the hZnT4 protein was co-localized with free zinc pools, we performed double labelling with Zinquin and the hZnT4 antibody. A granular pattern throughout the cytoplasm of the cells was seen for hZnT4 (Figure 5a), whereas Zinquin showed a more perinuclear distribution (Figure 5b).

Competitive PCR to determine the effect of zinc treatment on hZnT4 expression in cultured human breast cells

To determine whether hZnT4 in breast epithelial cells was influenced by zinc treatment, we used competitive PCR to measure the effect of overnight exposure of PMC42 cells to zinc. There was no significant change in the levels of *hZnT4* mRNA after overnight treatment of PMC42 cells with 100 μM $ZnCl_2$ (Figure 6a), as the equilibrium concentration of 25 fg was seen in lanes C and I for both control (lanes A–E) and zinc-treated cells (lanes G–K). *hZnT4* expression was also measured in PMC42 cells which were resistant to 500 μM zinc. No difference in the levels of *hZnT4* expression was detected between cDNA from control cells (lanes A–E) and zinc-resistant cells (lanes G–K), as the equilibrium occurred at 12.5 fg in both extracts.

Expression of other members of the ZnT family in cultured human breast epithelial cells

Since we did not find differences in hZnT4 expression levels between the resting and lactating breast tissues, and *hZnT4* was refractile to zinc treatment, we used RT–PCR to determine whether other members of the ZnT gene family might be expressed in human breast epithelial cells, including *hZnT1*, *hZnT2* and *hZnT3*. With primers *hZnT1*-A and *hZnT1*-B we obtained a fragment of the predicted length of 634 bp from PMC42 cells (Figure 7a, lane A). The identity of the *ZnT1*

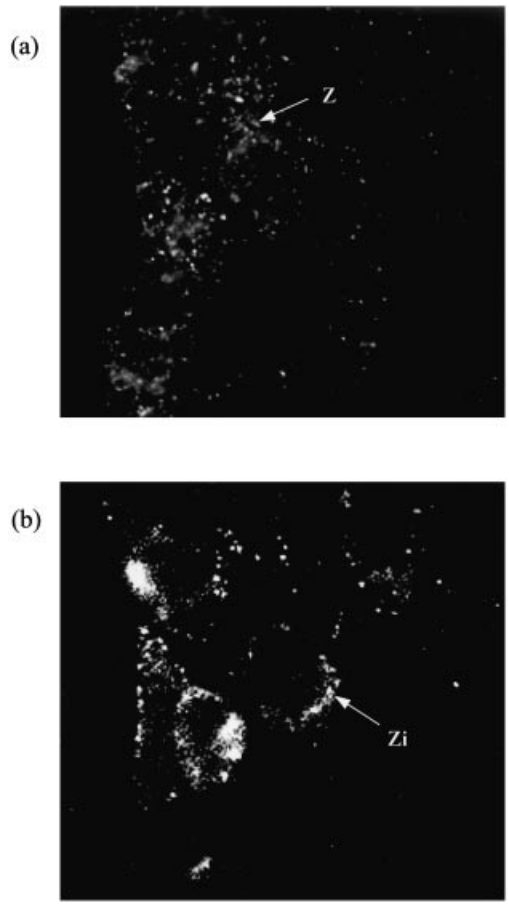

Figure 5 Double-label immunocytochemical analysis of hZnT4 protein and Zinquin in cultured PMC42 cells

(**a**) PMC42 cells incubated with MAG antibody showed a granular distribution of hZnT4 (Z) throughout the cytoplasm. (**b**) PMC42 cells incubated in Zinquin [(Zi) 100 μM] in HBSS showed a punctate label located in the region around the nucleus.

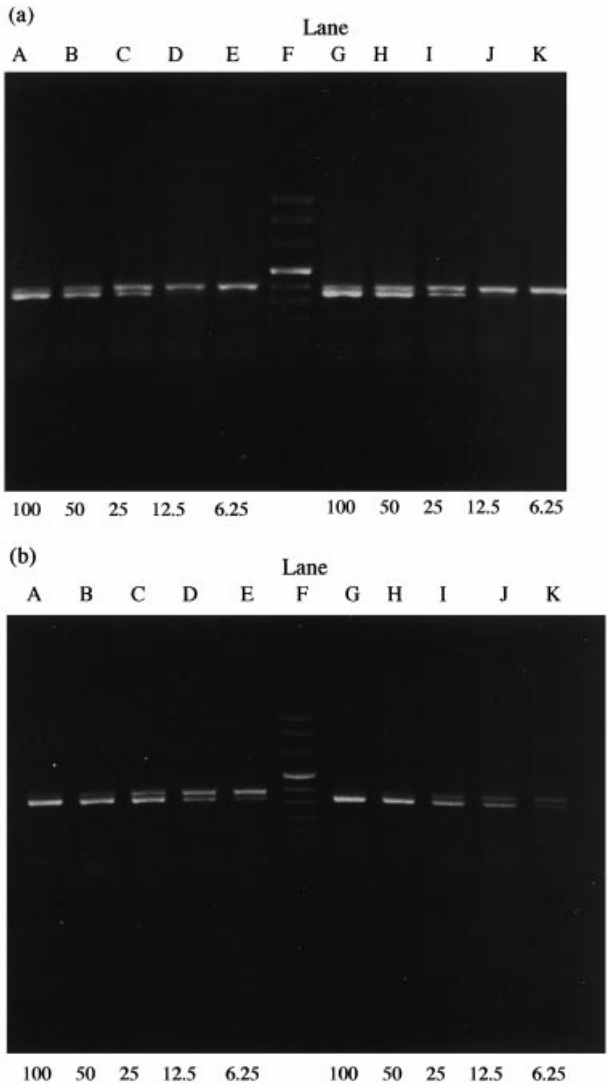

Figure 6 Competitive RT–PCR using forward-primer *hZnT4*-E and reverse-primer *hZnT4*-G (Table 1) to detect *hZnT4* expression

(**a**) Untreated PMC42 cells and cells treated with 100 μM $ZnCl_2$ overnight. Equilibrium between the amount of cDNA in extracts from untreated cells and competitor was seen at 25 fg of competitor (lane C), which was similar to that seen for zinc-treated cells (lane I). (**b**) Zinc-resistant cells cultured in 500 μM $ZnCl_2$. Equilibrium between amount of cDNA in extracts from untreated cells and competitor was seen at 12.5 fg of competitor (lane D), which was similar to that seen for zinc-resistant cells (lane J). See the text for details of other lanes.

fragment was confirmed by partial sequencing. Although we designed several sets of primers for *hZnT2*, this cDNA was not detected in PMC42 cells. With primers *hZnT3*-A and *hZnT3*-B we obtained a fragment of the predicted size of 508 bp from PMC42 cells (Figure 7b, lane A). The identity of the hZnT3 fragments was confirmed by partial sequencing.

DISCUSSION

The importance of zinc for a diverse range of cellular processes has been well documented [1]. Recently, several families of zinc transport proteins have been found, including the ZnT family, which appear to transport zinc out of the cytoplasm [7–9]. One of the members of the ZnT family, ZnT4, was found to be ubiquitously expressed in mouse and rat tissues, including stomach, small intestine, colon, liver, kidney, brain, testes, lung and skeletal muscle, heart and spleen [6,11,16]. It was also highly expressed in lactating rat mammary gland [16] and in two mouse mammary gland cell lines [6]. In a previous study [6], a premature termination codon in the *hZnT4* gene was reported to be responsible for the impaired transport of zinc into milk seen in the 'lethal milk' mouse. The 'lethal milk' dam produces milk containing approx. 50 % less zinc than the control [17], shown by ^{65}Zn studies [12,18] to be due to impaired transport of zinc through the mammary gland. Although ZnT4 is found to be essential for the normal transport of zinc into milk in mice, the significance of ZnT4 in the human breast has not been established.

In the present study, we investigated the expression of the human orthologue of ZnT4, hZnT4, in human breast cells. By using two antibodies raised to different fragments of ZnT4 peptide sequences, we detected bands of the predicted size of 47 kDa in both the resting and lactating breast tissues. Increased levels of ZnT4 protein in the breast might be expected during lactation, given that in 1 day the lactating human breast may secrete up to 1 litre of milk containing approx. 50 μM zinc [19]. Interestingly, we found no significant differences in ZnT4 protein levels between the resting and lactating tissues, relative to the housekeeping protein β-actin. It is possible that ZnT4 plays a

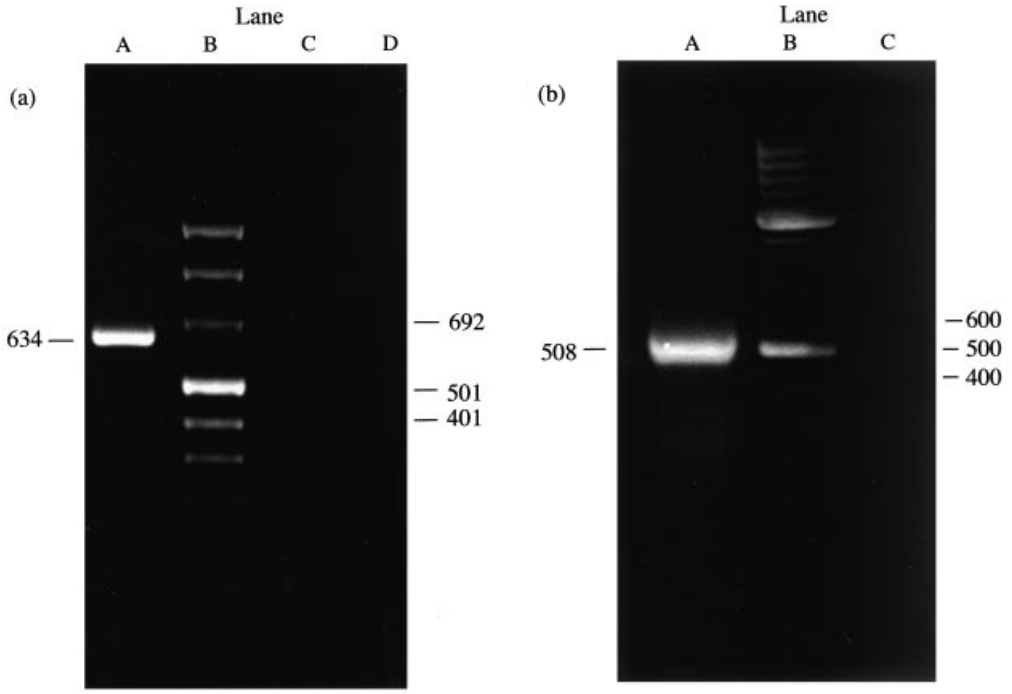

Figure 7 RT–PCR using forward and reverse primers (Table 1)

(**a**) Detection of *hZnT1* expression. Lane A, PMC42 cell extracts using *hZnT1*-A and *hZnT1*-B primers; lane B, molecular-mass markers; lane C, no cDNA; lane D, no enzyme. (**b**) Detection of *hZnT3* expression. Lane A, PMC42 cell extracts using *hZnT3*-A and *hZnT3*-B primers; lane B, molecular-mass markers; lane C, no cDNA.

role in zinc transport in the human breast through increased activity without increased protein levels. A precedent for this is seen in the mouse mammary gland where the copper transporter ATP7B functions to transport copper into milk. Our previous studies on the mouse mammary gland showed that the levels of ATP7B were similar in the resting and lactating mammary glands. Lactation, however, induced trafficking of ATP7B protein from the *trans*-Golgi apparatus to a more cytoplasmic region in the mammary gland [20]. The significance of transporter trafficking was also shown in our previous work [20] with the toxic milk mouse, where a mutation of the ATP7B gene prevented trafficking and consequently impaired transfer of copper into milk. It is therefore possible that trafficking of hZnT4 in the human breast epithelial cells is required for zinc transport and that increased secretion of zinc during lactation is achieved by increased trafficking of the transporter. The extensive distribution of hZnT4 in the cytoplasm of the cells from the lactating breast, relative to the more perinuclear distribution seen in cells from the resting breast, could be a consequence of increased trafficking of the protein during lactation.

The pattern of distribution of hZnT4 in lactating human breast epithelial cells was similar to that of the milk proteins β-casein and lactoferrin. Both these milk proteins and hZnT4 had a granular cytoplasmic distribution, but β-casein and lactoferrin labelling was stronger and more extensive than that of hZnT4. Thus hZnT4 could be associated with vesicles containing milk proteins. Interestingly, β-casein and lactoferrin were not located predominantly on the apical side of the epithelial cells as might be expected if they were being secreted into the lumen during lactation. We used the BC2 antibody to MUC1 [15] to mark the apical surface of the breast luminal epithelial cell. MUC1 is a member of the mucin family of highly glycosylated proteins found in normal breast tissue and produced in excessive amounts in breast carcinomas (see [21] for a review). The MUC1 glycoprotein was localized on the apical surface of the breast epithelial cell, distinctly different in localization from hZnT4. Our results showing a granular cytoplasmic label of hZnT4 suggest that it may be associated with vesicles that may follow a pathway from the Golgi similar to those taken by the major milk proteins [22] and calcium [23,24].

To visualize more clearly the subcellular distribution of hZnT4 protein, we used a human breast epithelial-cell culture model PMC42 in which, recently, we were able to induce synthesis of milk proteins [13]. Immunofluorescence showed a granular label consistent with a vesicular distribution. Some label was detected in the apical region of the PMC42 cells above the nucleus. But because of the low level of expression of the ZnT4 protein, we could not determine whether there was any membrane label on the apical surface of the cells consistent with delivery of vesicles to the apical surface of the cells. The vesicular localization of hZnT4 in the cultured breast cells is consistent with that found for a c-Myc-tagged Dri27/ZnT4 rat construct introduced into Caco-2 cells, where the ZnT4 was detected in vesicles concentrated around the nucleus and sparsely distributed throughout the cytoplasm [11].

To determine whether breast epithelial cells contained free or loosely bound pools of free zinc, we used the fluorophore Zinquin, which was developed to localize labile zinc in metabolically active cells and is highly specific for Zn^{2+} compared with other metal ions [25]. Zinquin has been used to detect free or loosely bound zinc in pancreatic islet cells [26], rat hepatocytes [27], primary respiratory epithelial cells from sheep and pigs and in cultured human respiratory epithelial-cell lines [28]. Intracellular pools of zinc, thought to be vesicular, have been termed 'zincosomes' [29]. Cultured PMC42 cells treated with Zinquin showed a punctate label, consistent with zinc present in vesicles.

Double-label immunofluorescence showed no overlap of ZnT4 protein with Zinquin, suggesting that ZnT4 is not involved in the accumulation of zinc in PMC42 zincosomes.

Our results show that other members of the ZnT4 family are expressed in breast epithelial cells. We detected expression of hZnT1 and hZnT3 using RT–PCR. Our previous studies on the 'lethal milk' mouse [12] provide evidence for alternative zinc transporters apart from ZnT4. The milk produced by the 'lethal milk' mouse has a 50 % reduction in zinc concentrations relative to the control. Thus 50 % gets through presumably by other transporters, possibly by ZnT1 or ZnT3. Zinc in milk is bound to a number of different components including casein 14 %, albumin 28 %, low-molecular-weight ligands 29 % and fat 29 % [30]. It is possible that different zinc transporters are involved in different secretory pathways taken by zinc.

In both breast tissue and cultured PMC42 cells, we observed the presence of a larger protein band of 77 kDa in addition to that of the 47 kDa band of the predicted size, using both the CSK and the MAG antibodies. The RT–PCR results using overlapping primers to amplify 98 % of the hZnT4 open-reading frame show a single band, suggesting that no alternative splicing of the mRNA occurs. The 77 kDa band seen on the Western blot was stronger in the absence of reducing agents, suggesting that the hZnT4 protein may interact with another protein. ZnT4 protein contains a leucine zipper motif, which was previously shown to bind several proteins of different sizes [11]. In this study [11], it was postulated that the leucine zipper in the amino-terminal cytoplasmic domain may interact with other proteins to form a multisubunit transporter complex or regulate the ZnT4 transporter. Our results support this hypothesis.

Our cell culture studies using the PMC42 human breast epithelial lines showed that zinc treatment did not increase the expression of hZnT4 and nor was *hZnT4* mRNA expression elevated in cells resistant to 500 μM zinc. In rats, *ZnT4* mRNA in the liver, small intestine, kidney and mammary gland was not increased in response to dietary zinc supplements [16].

In conclusion, we have detected expression of hZnT4, the human orthologue of the rodent ZnT4 transporter, in the secretory epithelium of resting and lactating breasts. Expression levels of hZnT4 were not altered with lactation or after zinc treatment of cultured cells. Our studies showing expression of ZnT1 and ZnT3 suggest the presence of alternative pathways for transport of zinc into milk.

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REFERENCES

1 Aggett, P. J. and Harries, J. T. (1979) Current status of zinc in health and disease states. Arch. Dis. Child **54**, 909–917
2 Vallee, B. L. and Auld, D. S. (1990) Zinc coordination, function, and structure of zinc enzymes and other proteins. Biochemistry **29**, 5647–5659
3 Zhao, H. and Eide, D. (1996) The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc. Natl. Acad. Sci. U.S.A. **93**, 2454–2458
4 Zhao, H. and Eide, D. (1996) The ZRT2 gene encodes the low affinity zinc transporter in *Saccharomyces cerevisiae*. J. Biol. Chem. **271**, 23203–23210
5 Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L. and Hediger, M. A. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature (London) **388**, 482–488
6 Huang, L. and Gitschier, J. (1997) A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nat. Genet. **17**, 292–297
7 Palmiter, R. D. and Findley, S. D. (1995) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J. **14**, 639–649
8 Palmiter, R. D., Cole, T. B. and Findley, S. D. (1996) ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. EMBO J. **15**, 1784–1791
9 Palmiter, R. D., Cole, T. B., Quaife, C. J. and Findley, S. D. (1996) ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc. Natl. Acad. Sci. U.S.A. **93**, 14934–14939
10 Barila, D., Murgia, C., Nobili, F., Gaetani, S. and Perozzi, G. (1994) Subtractive hybridization cloning of novel genes differentially expressed during intestinal development. Eur. J. Biochem. **223**, 701–709
11 Murgia, C., Vespignani, I., Cerase, J., Nobili, F. and Perozzi, G. (1999) Cloning, expression, and vesicular localization of zinc transporter Dri27/ZnT4 in intestinal tissue and cells. Am. J. Physiol. **277**, G1231–G1239
12 Ackland, M. L. and Mercer, J. F. (1992) The murine mutation, lethal milk, results in production of zinc-deficient milk. J. Nutr. **122**, 1214–1218
13 Ackland, M. L., Michalczyk, A. and Whitehead, R. H. (2001) PMC42, a novel model for the differentiated human breast. Exp. Cell. Res. **263**, 14–22
14 Whitehead, R. H., Bertoncello, I., Webber, L. M. and Pedersen, J. S. (1983) A new human breast carcinoma cell line (PMC42) with stem cell characteristics. I. Morphological characterization. J. Natl. Canc. Inst. I **70**, 649–661
15 Xing, P. X., Tjandra, J. J., Stacker, S. A., Teh, J. G., Thompson, C. H., McLaughlin, P. J. and McKenzie, I. F. (1989) Monoclonal antibodies reactive with mucin expressed in breast cancer. Immunol. Cell. Biol. **67**, 183–195
16 Liuzzi, J., Blanchard, R. and Cousins, R. (2001) Differential regulation of zinc transporter 1,2, and 4 mRNA expression by dietary zinc in rats. J. Nutr. **131**, 46–52
17 Piletz, J. E. and Ganschow, R. E. (1978) Zinc deficiency in murine milk underlies expression of the lethal milk (lm) mutation. Science **199**, 181–183
18 Lee, D. Y., Shay, N. F. and Cousins, R. J. (1992) Altered zinc metabolism occurs in murine lethal milk syndrome. J. Nutr. **122**, 2233–2238
19 Casey, C. E., Hambridge, K. M. and Neville, M. C. (1985) Studies in human lactation: zinc, copper manganese and chromium in human milk in the first month of lactation. Am. J. Clin. Nutr. **41**, 1193–1200
20 Michalczyk, A., Rieger, J., Allen, K., Mercer, J. and Ackland, M. (2000) Defective localisation of the Wilson disease protein (ATP7B) in the mammary gland of the toxic milk mouse and the effects of copper supplementation. Biochem. J. **352**, 565–571
21 McKenzie, I. F. and Xing, P. X. (1990) Mucins in breast cancer: recent immunological advances. Cancer Cells **2**, 75–78
22 Linzell, J. L. and Peaker, M. (1971) Mechanism of milk secretion. Physiol. Rev. **51**, 564–597
23 Baumrucker, C. R. and Keenan, T. W. (1975) Membranes of mammary gland. X. Adenosine triphosphate dependent calcium accumulation by Golgi apparatus rich fractions from bovine mammary gland. Exp. Cell. Res. **90**, 253–260
24 Neville, M. C. and Peaker, M. (1979) The secretion of calcium and phosphorus into milk. J. Physiol. Lond. **290**, 59–67
25 Zalewski, P. D., Forbes, I. J. and Betts, W. H. (1993) Correlation of apoptosis with change in intracellular labile Zn(II) using zinquin [(2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II). Biochem. J. **296**, 403–408
26 Zalewski, P. D., Millard, S. H., Forbes, I. J., Kapaniris, O., Slavotinek, A., Betts, W. H., Ward, A. D., Lincoln, S. F. and Mahadevan, I. (1994) Video image analysis of labile zinc in viable pancreatic islet cells using a specific fluorescent probe for zinc. J. Histochem. Cytochem. **42**, 877–884
27 Coyle, P., Zalewski, P. D., Philcox, J. C., Forbes, I. J., Ward, A. D., Lincoln, S. F., Mahadevan, I. and Rofe, A. M. (1994) Measurement of zinc in hepatocytes by using a fluorescent probe, zinquin: relationship to metallothionein and intracellular zinc. Biochem. J. **303**, 781–786
28 Truong-Tran, A. Q., Ruffin, R. and Zalewski, P. D. (2000) Visualization of labile zinc and its role in apoptosis of primary airway epithelial cells and cell lines. Am. J. Physiol. Lung Cell Mol. Physiol. **279**, L1172–L1183
29 Chai, F., Truong-Tran, A. Q., Ho, L. H. and Zalewski, P. D. (1999) Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: a review. Immunol. Cell. Biol. **77**, 272–278
30 Lonnerdal, B., Hoffman, B. and Hurley, L. S. (1982) Zinc and copper binding proteins of human milk. Am. J. Clin. Nutr. **36**, 1170–1176

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