Identification and Cloning of a β-Cell–Specific Zinc Transporter, ZnT-8, Localized Into Insulin Secretory Granules

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***SLC30A8*, a novel member of the zinc transporter (ZnT) family, was identified by searching the human genomic and expressed sequence tag (EST) databases with the amino acid sequence of all known human ZnT. The protein (369 amino acids) predicted from this gene, ZnT-8, contains six transmembrane domains and a histidine-rich loop between transmembrane domains IV and V, like the other ZnT proteins. We demonstrated by RT-PCR on cDNA libraries and human tissue extracts that the *ZnT-8* gene is solely transcribed in the pancreas, mainly in the islets of Langerhans. The gene, named *SLC30A8*, was cloned and sequenced. Confocal immunofluorescence analysis revealed that a ZnT-8-EGFP (enhanced green fluorescent protein) fusion product colocalized with insulin in the secretory pathway granules of the insulin-secreting INS-1 cells. Exposure of the ZnT-8-EGFP stably expressing HeLa cells to 75 μmol/l zinc caused an accumulation of zinc in intracellular vesicles compared with cells expressing EGFP alone. These results identified ZnT-8 as a ZnT specific to the pancreas and expressed in β-cells. Because ZnT-8 facilitates the accumulation of zinc from the cytoplasm into intracellular vesicles, ZnT-8 may be a major component for providing zinc to insulin maturation and/or storage processes in insulin-secreting pancreatic β-cells.**
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In the pancreas, the islets of Langerhans, mainly insulin-secreting β-cells, accumulate very high amounts of zinc (1). Insulin is generally believed to be stored inside secretory vesicles as a solid hexamer bound with two Zn^{2+} ions per hexamer (2–4). Insulin, produced and stored in pancreatic β-cells, is released by exocytosis in response to external stimuli, such as elevated glucose concentrations or arginine. When exocytosis of insulin occurs, insulin granules fuse with the β-cell plasma membrane and release their contents, insulin as well as zinc, into the circulation (5). Moreover, there appears to be a complex relationship between zinc and both type 1 and type 2 diabetes because several complications of diabetes may be mediated through oxidative stress, which is amplified in part by zinc deficiency (6). Hence, zinc is an important mediator of insulin storage and secretion, and β-cells undoubtedly need very efficient and specialized transporters to accumulate zinc in secretion vesicles.

In the body, intracellular zinc concentration and localization are strictly regulated (7,8). The two main components involved in zinc homeostasis are metallothioneins and membrane transporters (9,10). Metallothioneins are implied in intracellular zinc storage and trafficking, whereas membrane transporters ensure zinc carriage across biological membranes. Zip proteins allow intracellular uptake of zinc (11,12), whereas zinc transporter (ZnT) proteins permit cellular efflux of zinc into extracellular matrix or into intracellular vesicles. In mammalian cells, seven homologous zinc export proteins, named ZnT-1 to -7, have been described (for review see Chimienti et al. [13]). These proteins are members of the *SLC30* (solute carrier) subfamily of the CDF (cation diffusion facilitator) family and share the same predicted structure, with six membrane-spanning domains and a histidine-rich intracellular loop between helices IV and V, except for ZnT-6, which retains a serine-rich loop from prokaryotic sequences. ZnT-1 and -2, located either in the plasma membrane or in acidic endosomal/lysosomal vesicles, respectively, are ZnTs that confer zinc resistance by ensuring zinc efflux from the cytoplasm (14,15). ZnT-3 and -4, cloned in humans, are also localized in intracellular vesicles, but are more dedicated to secretion pathways (16,17). ZnT-3 is tissue specific and mainly located in brain, in the membranes of zinc-rich synaptic vesicles within the mossy fiber boutons of the hippocampus (18), and in testis (16). Conversely, ZnT-4 is largely expressed (19). However, higher levels of ZnT-4 were found in brain and epithelial cells, and this transporter has been shown to be essential in mammary epithelia for regulating milk zinc content (20). ZnT-5 is a ZnT mainly localized in the Golgi apparatus and expressed in pancreas (21). However, it is also strongly expressed in liver, kidney, muscle, and heart (21). Moreover, ZnT-5 null mice have been shown to suffer osteopenia and male-specific sudden cardiac death in mice lacking the *SLC30A5* gene, suggesting that the ZnT-5 protein plays an important role in the maintenance of cells involved in the cardiac conduction system (22). Two other transporters, ZnT-6 and -7, described very recently (23,24), are expressed in various tissues and both localized to the Golgi apparatus. The intracellular distribution of ZnT-6

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EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; ZnT, zinc transporter.

and ZnT-4 has been shown to be regulated by zinc, whereas exposure of ZnT-7–expressing cells to zinc causes an accumulation of zinc in the Golgi apparatus.

The zinc content in the pancreatic β-cell is among the highest of the body, but little information about which proteins might handle zinc in the β-cell is available. Previous studies (21,25) have determined that some ZnT genes are involved in zinc uptake and storage in pancreatic cells and that their expression is developmentally regulated in the islet.

We report here for the first time the identification and cloning of a pancreatic-specific ZnT belonging to the ZnT protein family, named ZnT-8, and localized in insulin secretory granules. This new ZnT may be a major component for providing zinc to insulin maturation and/or storage processes in insulin-secreting pancreatic β-cells.

RESEARCH DESIGN AND METHODS

All chemicals were of reagent grade and from Sigma (St. Quentin-Fallavier, France) or Merck (Grenoble, France). Zinquin ethyl ester was purchased from TRC (North York, Canada).

Data mining and bioinformatics. BLASTN or TBLASTN (26) analysis was performed on the National Center for Biotechnology Information (NCBI) web server (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and the ENSEMBL web server (http://www.ensembl.org/) with human ZnT-1 (AF323590 and Q9Y6M5), mouse ZnT-1 (U17132 and Q60738), rat ZnT-1 (U17133 and Q62720), rat ZnT-2 (U50927 and Q62941), human ZnT-3 (NM_003459 and Q99726), mouse ZnT-3 (U76007 and P97441), human ZnT-4 (AF025409 and O14863), mouse ZnT-4 (O35149), rat ZnT-4 (O55174), human ZnT-5 (AY089991 and AAM09099), human ZnT-6 (NM_017964 and NP_060434), and human ZnT-7 (AY094606 and AAM21969) DNA or protein sequences. The predicted ZnT-8 sequence (open reading frame and 3′ untranslated region) was used for a BLASTN search of the human expressed sequence tag (EST) database (release 030802). The significant ESTs (a bit score >50 and an *E* value <0.0001) were sorted, and information regarding each cDNA library was retrieved from either the human Unigene databank (http://www.ncbi.nlm.nih.gov/Unigene) or from the respective company catalog. The ESTs were also analyzed by the Gene2EST program (http://woody.embl-heidelberg.de/gene2est) to examine the 5′ region and the putative splicing variants (27). *Homo sapiens* ZnT protein sequences were aligned using the ClustalW program (28). For phylogenetic analysis, bootstrapping (2,000 replicate sets) and calculation of the consensus tree were performed with the DAMBE program by the neighbor-joining method (29). Bootstrap analysis is based on multiple resampling of the original data and is the most common method for estimating the degree of confidence in the topology of phylogenetic trees. The TMpred computer program (http://www.ch.embnet.org/software/TMPRED_form.html) was used to analyze and predict the transmembrane potential of the predicted protein sequence (30).

Cell culture methods. HeLa epithelial cells (American *Type Culture* Collection [ATCC] number CCL-2) were grown in Opti-MEM medium (modified Eagle's medium; Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated FCS, 2 mmol/l glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Aliquots of the INS-1 cell line (31) were a gift from P.Y. Benhamou (Hospital of Grenoble, Grenoble, France), with the courtesy of Dr. W. Pralong (Lausanne, Switzerland). INS-1 cells were grown at 37°C, in a 5% CO_2-enriched atmosphere, in RPMI 1640 medium (Invitrogen), including 10% heat-inactivated fetal bovine serum (Invitrogen), 50 μmol/l 2-mercaptoethanol, 1 mmol/l Na-pyruvate, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and routinely split at a 1:5 ratio.

Plasmid construction and generation of stable cell lines. Full-length ZnT-8 cDNA was isolated by RT-PCR amplification from human islet total RNA with specific primers containing 5′-*Eco*RI and 3′-*Xba*I restriction sites. The

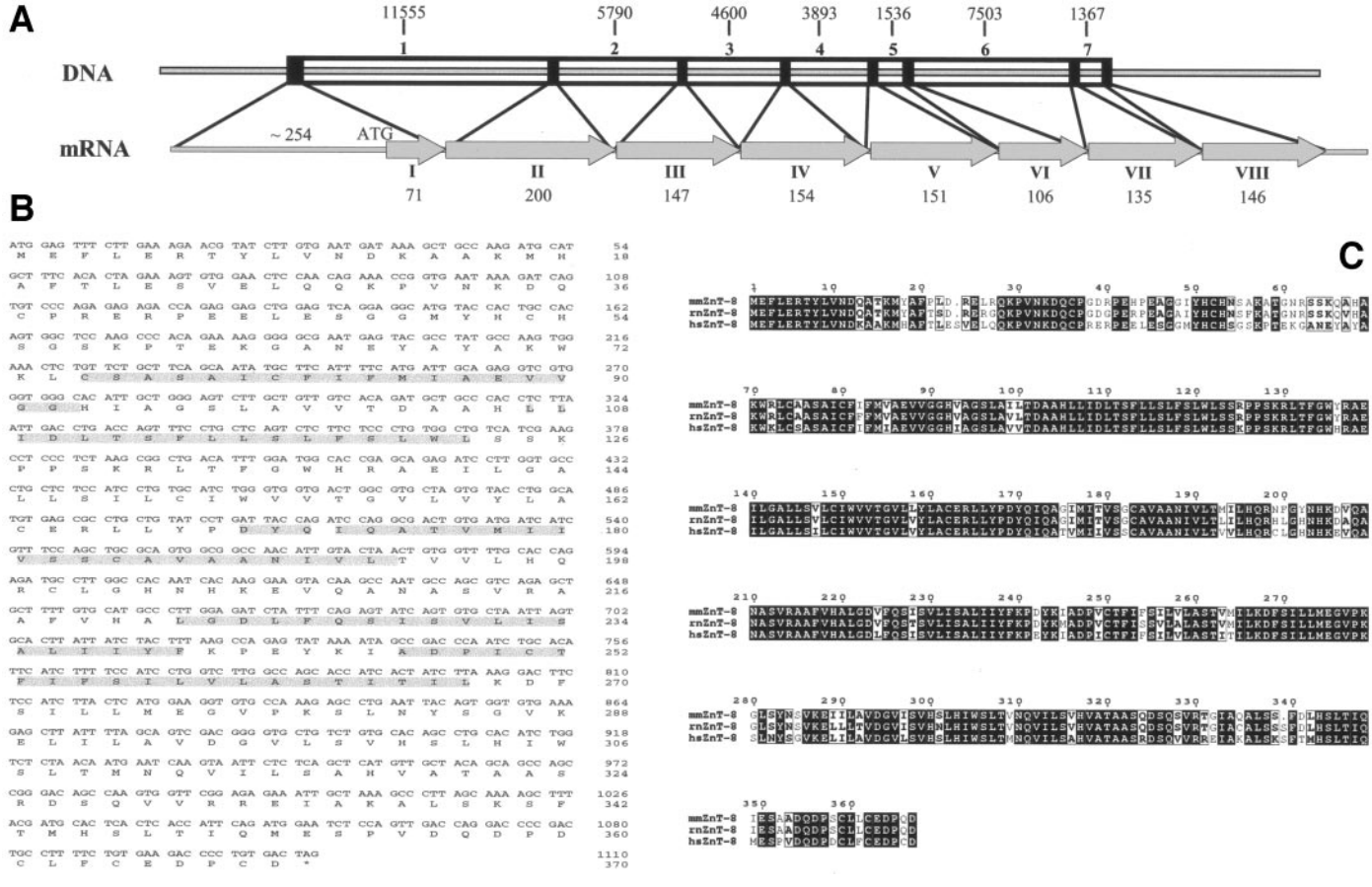

FIG. 1. Genomic organization of *SLC30A*8, sequence of ZnT-8 cDNA, and protein. *A*: Gene structure, chromosomal localization, and putative splicing of human SLC30A8. The gene was located on chromosome 8q24.11, contained eight exons, and spanned 37 kb. *B*: cDNA sequence with deduced amino acid sequence of human ZnT-8 protein. The predicted transmembrane domains are indicated by gray boxes (▨). *C*: Comparison of the hsZnT-8, mmZnT-8, and rnZnT-8 amino acid sequences. Identical residues are indicated by black boxes (■).

Intron number	Donor sequence	Acceptor sequence	Intron sizes (bp)
1	F T L E S	V E L Q	11555
	TTCACACTAGAAAGgtaatagatgtctgtg	gtcaaactcatccatagTGTGGAACTCCAA	
2	A E V V	G G H I A	5790
	TGCAGAGGTCGTGGgtgagtctttctgcag	tattttgcattctctagGTGGGCACATTGC	
3	W H R A	E I L G A	4600
	ATGGCACCGAGCAGgtacggttcatagagt	tgtgtttgaattcctagAGATCCTTGGTGC	
4	A A N I V	L T V V	3893
	GCGGCCAACATTGTgtaagtcatcccctgg	attgttctctctttcagACTAACTGTGGTT	
5	I I Y F K	P E Y K	1536
	TTATCTACTTTAAGgtgagtttgagtttac	cctgtttttttttctagCCAGAGTATAAAA	
6	L L M E	G V P K S	7503
	CTTACTCATGGAAGgtaggagtgattttat	ctcttccctttgtcagGTGTGCCAAAGAG	
7	H V A T	A A S R D	1367
	TCATGTTGCTACAGgtcagtgagttttgta	tgcttctttatcaacagCAGCCAGCCGGGA	

FIG. 2. Residues consistent with the splice site consensus sequence (MAG/GTRAG and YAG/G) are underlined.

amplified product was cloned into *Eco*RI- and *Xba*I-digested pEGFP (enhanced green fluorescent protein; Clontech, Palo Alto, CA) to generate the pZnT-8-EGFP vector. Positive clones were amplified, sequenced, and used for transfection experiments. Stably expressing ZnT-8-EGFP INS-1 cells were obtained by transfecting INS-1 cells with pZnT-8-EGFP and Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Expressing cells were selected using a medium containing 350 μg/ml G418 (Roche, Meylan, France). The ZnT-8-EGFP– and control EGFP-expressing HeLa stable cell lines were generated by transfecting pZnT-8-EGFP or pEGFP into HeLa cells using Lipofectamine Plus (Invitrogen). The stable cell lines were selected by culturing the cells in the medium containing 700 μg/ml G418 (Roche) and cloned using a cell sorter. After amplification of clones, the expression of the fusion protein was controlled under fluorescence microscopy, and the size of the fusion product was verified by Western blotting using a specific anti-EGFP antibody (Invitrogen). For Golgi apparatus localization, transient transfections with pECFP-Golgi (Clontech) were performed as described above on the ZnT-8-EGFP–expressing HeLa stable cell line.

Islet of Langerhans and cell RNA extraction. Total RNA isolated from human islets using the RNaid Plus extraction kit (Qbiogene, Carlsbad, CA) and total fetal pancreas RNAs (Stratagene, La Jolla, CA) were kindly provided by Dr. F. De Fraipont (Hospital of Grenoble, Grenoble, France). Prior to RNA extraction, INS-1 or HeLa cells were washed twice with cold PBS, scraped, and centrifuged for 3 min at 2,000*g*. Total RNA was extracted from the cell pellet by the RNA extraction kit (Roche) as described by the manufacturer. The RNA was quantified and stored in aliquots at −80°C.

ZnT-8 expression in different tissues. The presence and level of ZnT-8 mRNA were determined by PCR amplification of cDNA libraries from different human tissues (Rapid-Scan; Origene Technologies, Rockville, MD) or by RT-PCR on total RNAs prepared from islets of Langerhans or cultured cells. The specific primers were: 5′-GAT GCT GCC CAC CTC TTA ATT GAC and 5′-CCA AGA CCA GGA TGG AAA AGA TGA for ZnT-8 and 5′-CCA AGG CCA ACC GCG AGA AGA TGA C and 5′-AGG GTA CAT GGT GGT GCC GCC AGA C for β-actin. The products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

Immunofluorescence. pZnT-8-EGFP–expressing INS-1 cells were seeded onto glass coverslips and allowed to attach for 72 h. Cells were fixed in 4% formaldehyde in PBS for 15 min and permeabilized in PBS containing 0.1% Triton X-100. Cells were then blocked in 2% BSA in PBS for 1 h, followed by the addition of a monoclonal anti-insulin antibody (Sigma, Lyon, France) diluted 1:2,000 in blocking buffer. Cells were washed in blocking buffer, incubated in donkey anti-mouse IgG-Cy3 conjugate (Jackson ImmunoResearch Laboratory, West Grove, PA), and diluted in blocking buffer at 1:300. Cells were washed in PBS, followed by a final wash in ddH_2O. Coverslips were mounted onto glass slides using FluorSave (Calbiochem, La Jolla, CA) and photographed using a Leica confocal fluorescence microscope.

Zinquin staining. Zinquin staining was performed using pEGFP- or pZnT-8-EGFP–transfected cells. Cells were grown in Labtek II chambered cover glass systems (Nunc, Rochester, NY) for 48 h and then treated with 0 or 75 μmol/l $ZnSO_4$ for 3 h in the culture medium. After $ZnSO_4$ treatment, cells were rinsed three times with PBS, pH 7.4, and then incubated in Ca^{2+}-Mg^{2+} PBS containing 5 μmol/l zinquin ethyl ester for 20 min. The cells were then washed with PBS, pH 7.4, and examined under a Zeiss Axiovert 200 fluorescence microscope (λ_{exc} 365 nm; λ_{em} 420 nm). Digital images were captured with a cooled charge-coupled device camera (Hamamatsu C5935; Hamamatsu, Hamamatsu City, Japan).

Accession numbers. The sequence data reported for human ZnT-8 was submitted to GenBank under the following accession number: AY117411.

RESULTS

Genomic organization of *ZnT-8* gene. We used a data-mining strategy using the different already-known ZnT cDNA and protein sequences: human, mouse, and rat ZnT-1; rat ZnT-2; human ZnT-3; human, mouse, and rat ZnT-4; human ZnT-5; human ZnT-6; and human ZnT-7. These sequences were used in a BLASTN or TBLASTN search of the Human Genome Project databank. Among the results, we discovered a new sequence of a putative ZnT, named ZnT-8. The gene coding for ZnT-8 was designated *SLC30A8* by the Human Genome Organization (HUGO), and applications were patented (32). A human ZnT-8 DNA coding sequence was found in the contig AC027419, which allowed us to localize *SLC30A8* to human chromosome 8 at the position q24.11. The gene contained eight exons, spanned 37 kb, and encoded a 369–amino acid protein (Fig. 1*A* and *B*). RT-PCR analysis and examination of EST sequences were used to confirm the structure of the human *SLC30A8* gene (Fig. 2), and the transcription start site was mapped on EST BM565086. We also identified orthologues of *SLC30A8* in mouse and rat genomes. Mouse ZnT-8 gene was located at the position 15D1 in the contig AC127562.3.1.173351. The predicted protein shares 80% identity with human ZnT-8 (Fig. 1*C*). The rat ZnT-8 gene was located at the position 7q31 in the contig RNOR03308537.1.41192, and the predicted protein shares a 76% amino acid identity with human ZnT-8 (Fig. 1*C*).

Primary structure analysis of Znt-8 protein. The transmembrane topology of the protein was assessed using the TMPRED computer program (30). ZnT-8 was predicted to adopt the same topology as the other ZnT proteins with six transmembrane helixes (Fig. 3*A*). In addition, the predicted sequence contains a histidine-rich domain between the fourth and the fifth helix (Fig. 3*B*). Figure 3*C* shows the alignment of the ZnT histidine-rich domains. It is noteworthy that ZnT-6 retains serine residues from a prokaryotic ancestor instead of a histidine-rich domain. These two features are highly conserved among members of the ZnT family. A phylogenetic tree for all identified human ZnT family proteins was generated using the program DAMBE (29) (Fig. 3*D*). Analysis of the tree indicated that ZnT-5, -6,

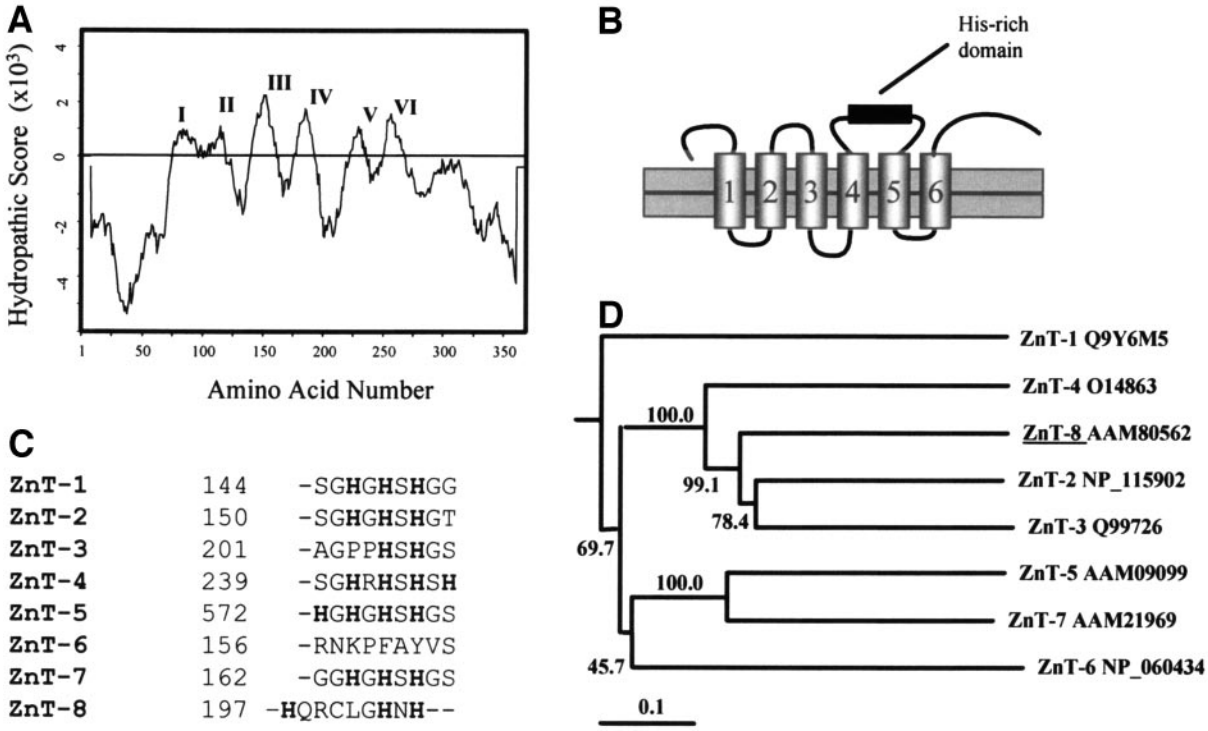

FIG. 3. In silico analysis of ZnT-8 protein. *A*: The hydropathic scores of ZnT-8 amino acid sequence were determined by the TMPred program (http://www.ch.embnet.org/software/TMPRED_form.html). The predicted membrane-spanning domains are designated with roman numerals. *B*: Transmembrane topology of ZnT-8. The protein was predicted to carry the six transmembrane regions, with a long COOH-terminal tail. The black box (■) indicates the histidine-rich domain. These structural motifs are conserved in rat and mouse ZnT-8. *C*: A partial amino acid alignment of the histidine-rich loop domain from ZnT-1 to -8 proteins identified in *Homo sapiens* was performed with ClustalW. The histidine residues are in boldface. *D*: For phylogenetic analysis on ZnT proteins, *Homo sapiens* ZnT protein sequences were aligned using the ClustalW program. Bootstrapping (2,000 replicate sets) and calculation of the consensus tree were done with the DAMBE program. The numbers indicate bootstrapping values as a percentage at internal nodes. The scale of the branch length is given in amino acid substitutions per site. Accession numbers are indicated for protein sequences in the Entrez databank.

and -7 cluster closely together because they may all be involved in Golgi accumulation of zinc in many cell types. The plasma membrane transporter ZnT-1 is more distant from all other ZnT proteins. ZnT-8 is closely related to ZnT-2, -3, and -4, all of which have already been involved in secretory/synaptic vesicle transport and lysosomal/endosomal zinc storage in different cells. In this subfamily, ZnT-2 and -3 are closer to ZnT-8 than ZnT-4. A PROSITE database scan indicated that the human ZnT-8 protein contains several putative signals: three potential protein kinase C phosphorylation sites, one casein kinase II phosphorylation site, one tyrosine kinase phosphorylation site, one cAMP- and cyclic guanosine monophosphate (cGMP)-dependent protein kinase phosphorylation site, two *N*-glycosylation sites, and two *N*-myristoylation sites.

Specific tissue expression of ZnT-8 mRNA. To confirm the expression of this new ZnT, we designed oligonucleotides from the predicted mRNA sequence and performed PCR amplification on cDNA prepared from different human tissues. A PCR product was amplified and detected only from a pancreas cDNA sample (Fig. 4*A*). This product had the expected size of 475 bp and was sequenced to confirm the specificity of the PCR amplification. We also designed oligonucleotides for full-length cDNA amplification and sequenced the PCR product to confirm the predicted mRNA splicing for ZnT-8 (data not shown). Since the ZnT-8 sequence was only detected in pancreas cDNA, we performed the same reaction on total RNAs isolated from the HeLa and insulin-secreting INS-1 cell lines, as well as total RNAs isolated from human fetal pancreas and human islets of Langerhans (Fig. 4*B*). ZnT-8 mRNA was not detected in HeLa cells, but was present in other pancreatic RNAs, from human tissue to cultured cells, thus confirming the tissue specificity of ZnT-8 mRNA expression.

Localization of ZnT-8 to the insulin granules of INS-1 cells. Previous studies have demonstrated that other ZnT proteins are localized to specific subcellular compartments for zinc transport in mammalian cells. Therefore, we sought to determine the subcellular localization of ZnT-8 in cells relevant to its pancreatic expression. For this purpose, we expressed the ZnT-8 protein as a fusion with the EGFP in the insulin-secreting INS-1 cells. After transfection and selection, we observed in live cells a strong punctuate staining pattern in the cytoplasm, an indication of cytoplasmic vesicle localization (Fig. 5*A*). To further determine the exact nature of these ZnT-8–containing granules, we compared the subcellular staining patterns of a ZnT-8-EGFP fusion protein with those of insulin in pancreatic insulinoma INS-1 cells. To this end, ZnT-8-EGFP–expressing INS-1 cells were fixed, and insulin was detected using a monoclonal anti-insulin antibody. As demonstrated by confocal immunofluorescence microscopy assay, the punctuate staining of ZnT-8 was almost completely coincident with that of insulin (Fig. 5*B*), strongly suggesting that ZnT-8 is associated with the insulin granule secretory pathway.

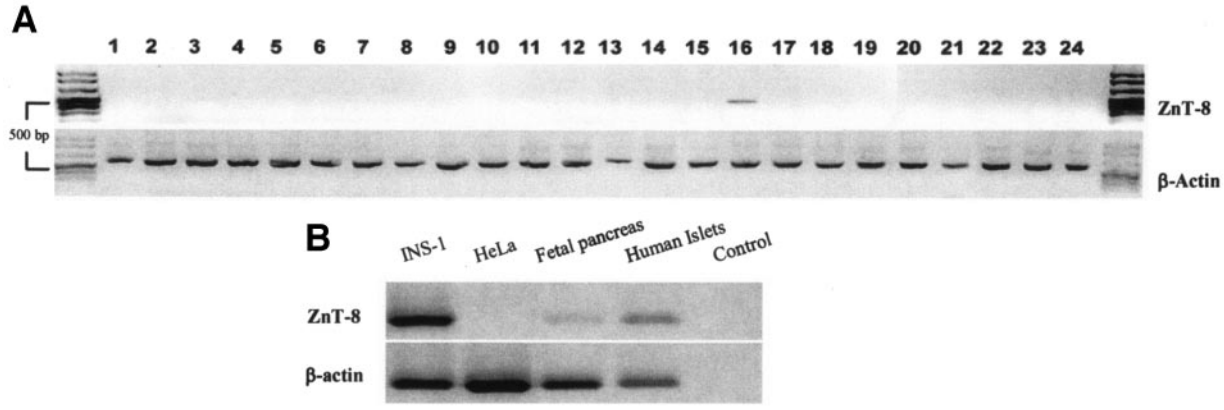

FIG. 4. Expression of ZnT-8 mRNA in human tissues. *A*: ZnT-8 mRNA expression was assessed by PCR using cDNA libraries prepared from different human tissues: 1, brain; 2, heart; 3, kidney; 4, spleen; 5, liver; 6, colon; 7, lung; 8, small intestine; 9, muscle; 10, stomach; 11, testis; 12, placenta; 13, salivary; 14, thyroid; 15, adrenal; 16, pancreas; 17, ovary; 18, uterus; 19, prostate; 20, skin; 21, peripheral blood lymphocyte; 22, bone marrow; 23, fetal brain; and 24, fetal liver (*upper panel*). Amplified products were visualized on an agarose gel after ethidium bromide staining. Specific β-actin primers were used as a control (*lower panel*). *B*: ZnT-8 mRNA expression was assessed by RT-PCR using total RNAs prepared from INS-1 or HeLa cells, fetal pancreas, and human islets. The negative control was performed without RNA.

Accumulation of zinc in ZnT-8-EGFP–expressing HeLa cells. To confirm the expression of this new ZnT and possibly localize ZnT-8 to a specific compartment, we stably expressed the EGFP-tagged ZnT-8 protein in HeLa cells. In these cells, which lack the insulin secretory pathway, ZnT-8-EGFP sited mainly into intracellular vesicles and to the plasma membrane, with a stronger signal at cell-cell contact. Whereas, to the contrary, control (EGFP-alone) cells displayed a diffuse staining (Fig. 6*A*). Some ZnTs have been recently shown to be expressed in specific subcellular compartments. In particular, ZnT-5 was found to be abundantly expressed in the Golgi apparatus in HeLa cells. To assess a potential colocalization between these two ZnTs, we transiently transfected ZnT-8-EGFP–expressing HeLa cells with the Golgi marker pECFP (enhanced cyan fluorescent protein)-Golgi. The fluorescence microscopy assay revealed that in HeLa cells, the ZnT-8-EGFP staining differs completely from the Golgi apparatus (Fig. 6*B*), which is strictly restricted to a perinuclear region. Hence the ZnT-8 protein is not part of the Golgi apparatus, contrary to what has been found for ZnT-5, but is found in intracellular vesicles and at the plasma membrane.

To further examine the function of ZnT-8 in the translocation of cytoplasmic zinc into the ZnT-8-EGFP–expressing HeLa cells, we used zinquin, a zinc-specific fluorescent dye to detect under fluorescence microscopy the accumulation of labile zinc. To compare signal intensity between different conditions, all digital images were captured with the same integration time. When control HeLa cells were grown in the culture medium, a signal was hardly detected (Fig. 7*A*), whereas ZnT-8-EGFP–expressing HeLa cells displayed membrane and intracellular staining (Fig. 7*C*). Signal intensity was slightly increased when all HeLa cells were treated with 75 μmol/l $ZnSO_4$ for 3 h (Fig. 7*B*), but this increase was dramatically more pronounced in ZnT-8-EGFP–expressing HeLa cells (Fig. 7*D*). Hence, the level of zinc accumulation is dependent on the expression of ZnT-8-EGFP and the concentration of zinc in the culture medium.

Taken together, these results strongly suggest that the ZnT-8-EGFP protein is functional in INS-1 and HeLa cells

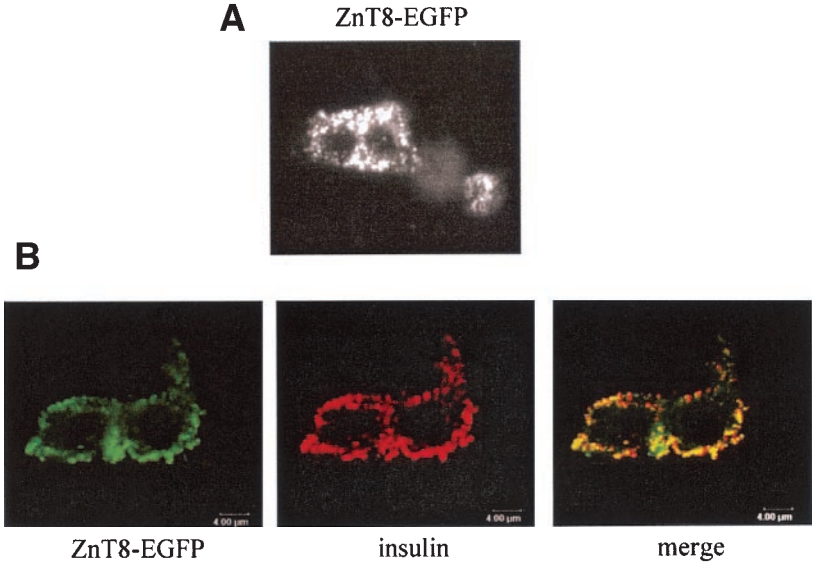

FIG. 5. Localization of ZnT-8-EGFP to insulin granules in INS-1 cells. *A*: ZnT-8-EGFP–expressing INS-1 cells display a strong punctuate staining pattern, thus suggesting a vesicular localization of the fusion protein. *B*: Immunofluorescence staining of INS-1 cells was performed using anti-insulin antibody, and fluorescence was observed under a confocal fluorescence microscope. ZnT-8-EGFP staining (green, *left*) was present in granules near the plasma membrane, as well as insulin (red, *center*). Superimposition of these two images demonstrates the almost complete colocalization between ZnT-8-GFP and insulin (yellow, *right*).

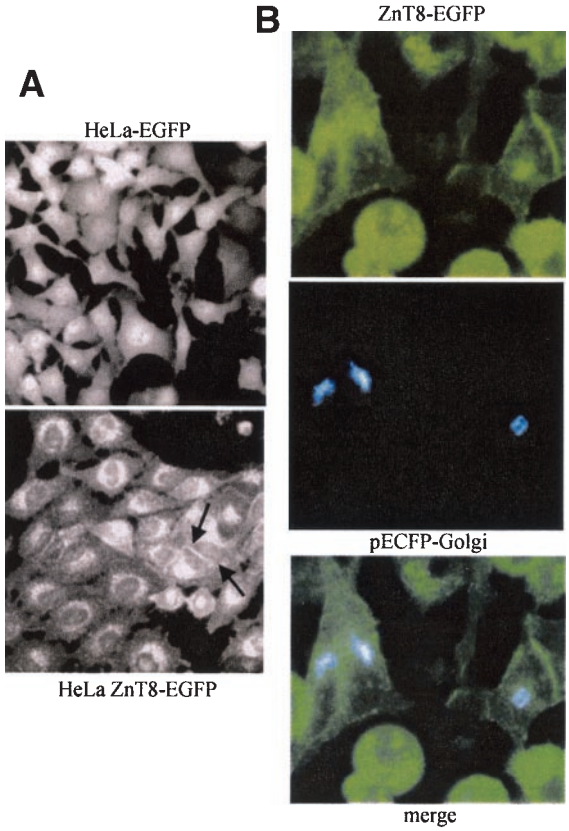

FIG. 6. Localization of ZnT-8-EGFP in HeLa cells. *A*: HeLa cells were grown in LabTek II chambered cover glass systems for 48 h and observed with a fluorescence microscope. Although EGFP-alone–expressing HeLa cells display a diffuse staining (*top*), ZnT-8-EGFP–expressing cells display a strong signal (*bottom*), mainly into intracellular vesicles and to the plasma membrane, with a stronger signal at cell-cell contact (arrows). *B*: Localization of ZnT-8-EGFP (green, *top*) and Golgi apparatus (blue, *middle*) in pECFP-Golgi–transfected, ZnT-8-EGFP–expressing HeLa cells. The Golgi apparatus is strictly restricted to a perinuclear region, whereas ZnT-8-EGFP is expressed in intracellular vesicles and in the plasma membrane. Superposition of these to images (*bottom*) clearly shows that ZnT-8-EGFP staining does not coincide with that of Golgi.

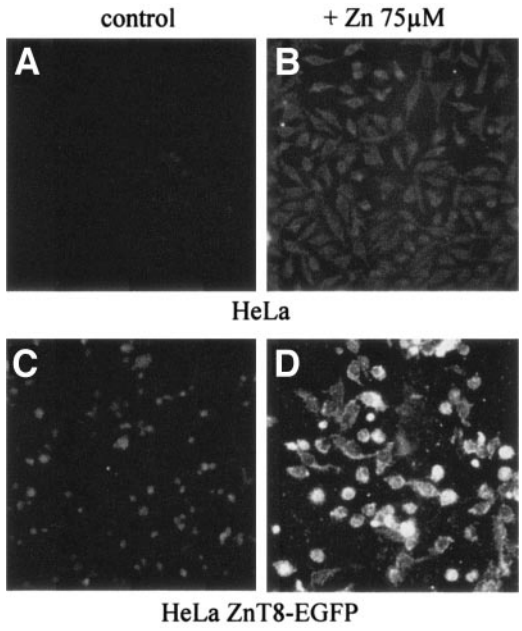

FIG. 7. Zinc-dependent zinquin staining in ZnT-8-EGFP–expressing HeLa cells. Control and ZnT-8-EGFP stably expressing HeLa cells were cultured for 48 h before treatment with either new medium (*A* and *C*) or new medium supplemented with 75 μmol/l $ZnSO_4$ (*B* and *D*). Three hours after treatment, cells were rinsed and loaded with the zinc-specific fluorescent dye zinquin-ethyl-ester. Cells were then observed under a fluorescence microscope, and all digital images were captured with the same integration time.

and is a ZnT that facilitates the rapid zinc accumulation in intracellular vesicles in these cells.

DISCUSSION

To date, genetic and genomic studies (14–16,20,21,23,24) have identified seven proteins involved in zinc extrusion in mammalian cells. In this study, we describe the discovery of a new gene, *SLC30A8*, which encodes ZnT-8, a novel member of the ZnT family. ZnT-8 shares many properties with the other members of the ZnT family, including six predicted transmembrane domains and a histidine-rich loop between transmembrane domains IV and V. We also demonstrate that the expression of the *SLC30A8* gene is strictly restricted to the pancreas islet cells and that a fusion protein was colocalized with insulin secretory granules in the rat insulin-secreting INS-1 cell line, one of the few existing models of the β-cell. The ZnT proteins have been shown to play roles in transporting zinc out of cells or sequestrating zinc into intracellular compartments. We show that ZnT-8 leads to zinc accumulation in intracellular vesicles when overexpressed in HeLa cells. This finding, combined with the similarity in the amino acid sequence and predicted secondary structures to the previously described ZnT proteins, strongly indicates that ZnT-8 is a ZnT involved in the translocation of the cytoplasmic zinc into intracellular vesicles.

Contrary to that of ZnT-1, ZnT-5 and -7 (14,15,21,24), which localized on the plasma membrane or in the Golgi apparatus, respectively, in different cell lines, the vesicular compartment where ZnT-8-EGFP localized appears different in the insulinoma INS-1 cell line and in HeLa cells, which lack the insulin secretion pathway. Furthermore, ZnT-5 is widely expressed in tissues but is specifically detected in insulin-containing β-cells and not in other endocrine cell types (21). ZnT-8 is specifically expressed in the pancreas and localized to insulin secretory granules in INS-1 cells. Therefore, it may act in synergy with ZnT-5 in the insulin synthesis/secretion pathway, as well as possibly participate in intracellular free-zinc homeostasis. The presence of more than one ZnT in pancreatic cells is not surprising. They are present in different cell compartments, i.e., ZnT-1 to the plasma membrane and ZnT-5 in the Golgi apparatus, and are essential to buffer cytosolic zinc and to transport zinc to correct sites in the cell.

In addition, phylogenetic analysis demonstrates that ZnT-8 is closely related to ZnT-2, -3, and -4, a subfamily of ZnT proteins that has already been involved in secretory/synaptic vesicle transport and lysosomal/endosomal zinc storage in different cells (for review see Kambe et al. [21]). Taken together, these data strongly suggest that ZnT-8 is involved in transporting zinc into a unique vesicular compartment, the insulin secretory granules of pancreatic β-cells. Therefore, ZnT-8 may play an important role in insulin synthesis, storage, and/or secretion.

Zinc is known to play a key role in the synthesis and

action of insulin, both physiologically and in the pathogenic state of diabetes. Zinc is required for insulin synthesis and storage (3), and thus large amounts of zinc are released with insulin after glucose stimulation (2). Additionally, considering the anatomical structure of the islets of Langerhans, it is likely that the β-cells also modulate the function of neighboring cells via paracrine/autocrine interactions. Indeed, a recent report indicates that zinc released from activated insulin-secreting β-cells is implied in the suppression of glucagon-secreting activity of neighboring α-cells (33), an effect comparable with that of zinc in neurons, where synaptically released zinc functions as a conventional synaptic neurotransmitter or neuromodulator (34). Thus, zinc is of prime importance for both insulin secretion by β-cells and islet cell paracrine/autocrine communication.

Regarding pathological states, there appears to be a complex interrelationship between zinc and both type 1 and type 2 diabetes. Modulations of the zinc content in the whole body induced in the pathological state of diabetes can have primary effects and secondary complications (for review see Chausmer [6]). The predominant effect of diabetes on zinc homeostasis is hypozincemia, which may result from hyperzincuria, decreased absorption, or both. Forty years ago, Quarterman et al. (35) demonstrated that zinc-deprived rats had an impaired response of insulin secretion to glucose stimulation, and other studies (36) indicated a decreased islet cell insulin content in zinc-deficiency states. Conversely, zinc supplementation markedly ameliorated the hyperglycemia of diabetic mice (37). Moreover, the intracellular zinc depletion induced by diabetes may have other effects. Excessive apoptosis of the pancreatic β-cell has been associated with diabetes (for review see Chandra et al. [38]). Because zinc is a potent inhibitor of apoptosis (8,39), zinc depletion by itself can enhance apoptosis in β-cells.

In conclusion, zinc plays a significant role in all processes of insulin trafficking, i.e., synthesis, storage, and secretion, and is also important in islet of Langerhans cell communication. Additionally, zinc modulations can have deleterious effects in diabetes. We describe here for the first time a islets of Langerhans–specific ZnT, expressed in β-cells, which facilitates the accumulation of zinc from the cytoplasm into intracellular vesicles. Thus, ZnT-8 may be a major component for providing zinc to insulin maturation and/or storage processes in insulin-secreting pancreatic β-cells.

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