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In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion

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Summary

Insulin-secreting pancreatic beta cells are exceptionally rich in zinc. In these cells, zinc is required for zinc-insulin crystallization within secretory vesicles. Secreted zinc has also been proposed to be a paracrine and autocrine modulator of glucagon and insulin secretion in pancreatic alpha and beta cells, respectively. However, little is known about the molecular mechanisms underlying zinc accumulation in insulin-containing vesicles. We previously identified a pancreas-specific zinc transporter, ZnT-8, which colocalized with insulin in cultured beta cells. In this paper we studied its localization in human pancreatic islet cells, and its effect on cellular zinc content and insulin secretion. In human pancreatic islet cells, ZnT-8 was exclusively expressed in insulin-producing beta cells, and

colocalized with insulin in these cells. ZnT-8 overexpression stimulated zinc accumulation and increased total intracellular zinc in insulin-secreting INS-1E cells. Furthermore, ZnT-8-overexpressing cells display enhanced glucose-stimulated insulin secretion compared with control cells, only for a high glucose challenge, i.e. >10 mM glucose. Altogether, these data strongly suggest that the zinc transporter ZnT-8 is a key protein for both zinc accumulation and regulation of insulin secretion in pancreatic beta cells.

Key words: Langerhans islets, Beta cell, Insulin, Insulin secretion, Zinc transport, Zinc

Introduction

Insulin, a pancreatic hormone produced by the beta cells of the islets of Langerhans, tightly regulates glucose homeostasis by promoting glucose uptake from plasma by peripheral tissues, i.e. muscle and fat cells, and inhibiting hepatic glucose production (DeFronzo et al., 1992). Insulin is stored inside pancreatic beta cell secretory vesicles, in which six insulin molecules form solid hexamers with two Zn²⁺ ions. This crystalline structure is osmotically stable at pH 5.5 until secretion (Dodson and Steiner, 1998; Emdin et al., 1980). Typically, following postprandial hyperglycemia, the glucose metabolism in beta cells induces an increase in the ATP/ADP ratio, which triggers closure of ATP-sensitive potassium channels (K_{ATP}). The resulting depolarization of the plasma membrane then provokes influx of extracellular Ca²⁺, activation of insulin-containing vesicle exocytosis and thus secretion of insulin (Deeney et al., 2000; Rutter, 2001). The secreted insulin is then carried through the circulation to peripheral tissues, where insulin receptor activation stimulates glucose uptake and utilization and/or storage as glycogen, reducing blood glucose (Bjornholm and Zierath, 2005; DeFronzo et al., 1985). Thus, insufficient insulin production and/or defective glucose absorption in peripheral tissues can

lead to hyperglycemia, which, in some cases can be compensated by hyperinsulinemia (Kahn, 1998). Development of type 2 diabetes is characterized by a particular breakdown of this system: hyperinsulinemia compensates for the resistance to insulin in peripheral tissues that is found in the early prediabetic state. The subsequent development of hyperglycemia results from the failure of beta cells to secrete enough insulin for effective compensation (Kahn, 1998).

Zinc is an important component for cell survival and participates in structure and function of many proteins (Vallee and Falchuk, 1993). It is necessary in fundamental cellular mechanisms such as DNA replication, metabolic enzyme activity and cellular protection against apoptosis and oxidative stress (Chimienti et al., 2003). Moreover, zinc has a specific role in some specialized cells, such as pancreatic beta cells in which the zinc content is among the highest in the body (Clifford and MacDonald, 2000). In these cells, zinc is necessary to form zinc-insulin crystals in secretion vesicles (Dodson and Steiner, 1998). Analyses of islets from ⁶⁵Zn-treated rats have shown that one-third of the total beta cell zinc content is present within insulin granules. Extra-granular zinc may act as a reservoir for granular zinc and may regulate insulin synthesis, storage, and secretion (Figlewicz et al.,

1984). The zinc/insulin ratio in secretory granules might indicate that a certain level of zinc ions is needed to initialize the crystallization of zinc-insulin hexamers (Sondergaard et al., 2003). Furthermore, after glucose stimulation, large amounts of zinc are secreted locally in the extracellular matrix together with insulin (Qian et al., 2000). Recent studies have suggested that this co-secreted zinc plays a role in islet cell paracrine and/or autocrine communication: it activates K_{ATP} channels (Franklin et al., 2005), thereby exerting a negative control on the secretory process (Bloc et al., 2000), and regulating glucagon secretion by alpha cells (Franklin et al., 2005; Ishihara et al., 2003). Zinc may also contribute to beta cell death by a paracrine mechanism (Kim et al., 2000). To control these zinc-related mechanisms, a tight regulation of zinc homeostasis and localization is ensured by zinc transporters; two families have been described. The SLC39 family of proteins (also named ZIP) allow the intracellular uptake of zinc, whereas the SLC30 family (ZnT) ensures zinc efflux from the cytosol to the extracellular matrix or intracellular organelles (for a review, see Kambe et al., 2004). Among the ZnT family of proteins, we previously identified ZnT-8, which was detected solely in pancreatic islets (Chimienti et al., 2004). In this study, we show that ZnT-8 protein is specifically expressed in human islet beta cells and we further characterize this transporter, including the determination of its membrane topology and zinc accumulation ability in beta cells. We show that overexpression of ZnT-8 in the rat insulinoma INS-1E cells significantly protected cells against zinc-depletion-induced cell death and enhanced glucose-stimulated insulin secretion only in high-glucose conditions. Converging evidence supports the hypothesis that ZnT-8 is a major component of the insulin synthesis/secretion pathway and may thereby constitute a novel pharmacological target for the treatment of diabetes.

Results

Tissue specificity of *ZNT8* gene expression

We have previously shown that *ZNT8* mRNA was specifically expressed in human islets (Chimienti et al., 2004), and identified the ortholog of this transcript in other mammal species, including mouse (Seve et al., 2004). In this work we used expression microarrays to assess the tissue specificity of *ZNT8* mRNA expression in the mouse. As shown in Fig. 1, a strong signal was observed in pancreatic islets, whereas no signal was observed in other tissues tested, including exocrine pancreas. This confirmed in mouse the islet-specific expression of *ZNT8* found in human at the mRNA level.

In agreement with the mouse data, expression of the ZnT-8 protein in human islets was demonstrated with an antibody directed against human ZnT-8 (Fig. 2A, lane 4). A negative control was performed on HeLa-EGFP expressing cells, because HeLa cells were shown not to express *ZNT8* mRNA (Chimienti et al., 2004). No signal was observed in the negative control, indicating that no other ZnTs expressed in HeLa cells were recognized by our antibody. A positive control was performed on HeLa-ZnT-8-EGFP-expressing cells. We observed a band at the expected molecular weight for the fusion protein. However aggregates were often noticeable at high molecular weights. This may indicate post-translational modifications or aggregation of the protein, a phenomenon often observed with membrane proteins (Sagne et al., 1996), and already observed for ZnT-4 (Michalczyk et al., 2002).

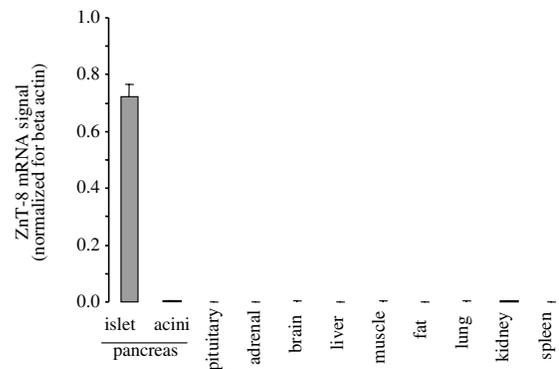


Fig. 1. Expression of *ZNT8* mRNA in mouse tissues. *ZNT8* mRNA expression was assessed by expression microarrays (Affymetrix) using RNA extracted ex vivo from mouse pancreatic islets, pancreatic acini, pituitary, brain, adrenal gland, liver, skeletal muscle, epididymal adipose tissue, lung, kidney and spleen.

ZNT8 mRNA is expressed in INS-1E cells (Chimienti et al., 2004). However the rat isoform of the protein was not recognized (Fig. 2A, lane 3), indicating a good species-specificity of the antibody despite high sequence homology. In human pancreatic islet extracts, we observed one band corresponding to ZnT-8. We also demonstrated by immunohistochemistry using human islet cytospins, that ZnT-8 protein is expressed in some islet cells (Fig. 2B).

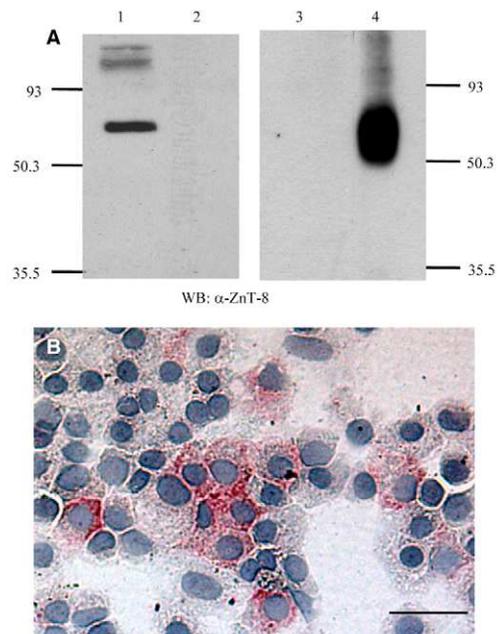


Fig. 2. Expression of ZnT-8 in human islets at the protein level. (A) Western blot with anti-human ZnT-8 antibody showing the presence of the protein in total extracts of human pancreas. Lane 1, HeLa ZnT-8-EGFP (positive control); lane 2, HeLa-EGFP (negative control); lane 3, rat insulinoma INS-1E cells; lane 4, human pancreatic islet extracts. (B) The ZnT-8 protein is detected (red) by immunohistochemistry of pancreatic islet cells. Nuclei were counterstained with Carrazi's hematoxylin. Bar, 30 μ m.

Double immunostaining and confocal microscopy analysis of human islet cytopspins revealed that insulin localization (green) completely coincides with that of ZnT-8 (red), thus confirming the colocalization between these two proteins *in vivo* (Fig. 3). A blind observer manually counted the number of insulin-positive, ZnT-8-positive and doubly positive cells as well as total cell number in 40 randomly selected fields. Among the 1015 cells counted, 465 cells were found positive for ZnT-8 and 459 cells were positive for insulin, all of which were also positive for ZnT-8. The insulin⁺/ZnT-8⁺ ratio was found to be 98.7%, indicating that ZnT-8 is exclusively expressed in pancreatic beta cells.

Overexpression of ZnT-8-EGFP in cultured cells

We generated ZnT-8-EGFP and control EGFP-expressing INS-1E stable cell lines and checked the presence of the fusion protein in INS-1E cells by western blot using an anti-ZnT-8 antibody. Fig. 4A shows a typical experiment obtained with total protein extracts in ZnT-8-EGFP-expressing or control INS-1E-EGFP cells. We observed one band at the expected molecular weight for INS-1E cells expressing ZnT-8-EGFP, which was not present in EGFP-expressing cells. Specificity of immunoblot procedure was also verified using anti-GFP antiserum (not shown). To precisely localize the fusion protein in INS-1E cells, we used confocal fluorescence microscopy. Cells displayed punctuated staining, consistent with insulin colocalization (Fig. 4B, left). A high magnification image of the same cell (Fig. 4B, right) showed that ZnT-8-EGFP mainly localized to granules in close proximity to the plasma membrane. We also observed slight membrane staining in some adjacent cells (Fig. 3C), suggesting the possible presence of ZnT-8 at the plasma membrane after fusion of the insulin-containing granules. Thus, our cell models stably expressed either the fusion protein ZnT-8-EGFP or EGFP and were used in subsequent experiments.

ZnT-8 membrane topology

To address the plasma membrane topology of ZnT-8, we used antibodies 9A or 9B directed against either amino acids 355-

359 or 34-49 of the human ZnT-8 protein, respectively, for immunolabeling of both permeabilized and non-permeabilized ZnT-8-EGFP-expressing INS-1E cells. As shown in Fig. 5A, the ZnT-8-EGFP signal completely superimposed with anti-ZnT-8 staining in permeabilized cells, whereas non-permeabilized cells displayed only ZnT-8-EGFP fluorescence. Because only extracellular epitopes can be immunolabeled in non-permeabilized cells, this indicates that both the N- and C-termini of ZnT-8 are intracellular when the protein is present at the plasma membrane level (Fig. 5B). The same results have been obtained with HeLa cells (not shown), which do not have a secretory pathway and thus express ZnT-8-EGFP mainly at the plasma membrane level.

Cellular zinc content

Zinc concentration in all buffers and media used was determined. Lysis buffer contained very low amounts of zinc, *i.e.* 1.4 $\mu\text{g/l}$ (20 nM), whereas normal complete culture medium contained 176 $\mu\text{g/l}$ zinc (2.7 μM); a value falling within the range reported by other studies (Sondergaard *et al.*, 2005). Conversely, zinc-supplemented medium contained 6175 $\mu\text{g/l}$ zinc, corresponding to 94 μM Zn²⁺, which is a high but non-toxic dose for INS-1E cells (see below). Therefore, the low zinc content in the lysis buffer is unlikely to interfere with the zinc contained in samples, and the zinc-supplemented media, while non-toxic, contained much more zinc than normal medium. In these conditions, zinc supplementation did not increase total zinc content in control cells (Fig. 6A). By contrast, ZnT-8-expressing cells contained significantly more zinc than control cells in normal conditions (830 \pm 109 μg Zn/g protein for control cells vs 1072 \pm 78 μg Zn/g protein for ZnT-8-expressing cells, $P<0.01$). Contrary to control cells, zinc supplementation of ZnT-8-EGFP-expressing cells further increased total cellular zinc (1334 \pm 80 μg Zn/g protein vs non-supplemented cells, $P<0.05$). Therefore, it appears that ZnT-8 overexpression, rather than zinc supplementation alone, significantly increased zinc accumulation ability and total cellular zinc content in INS-1E cells.

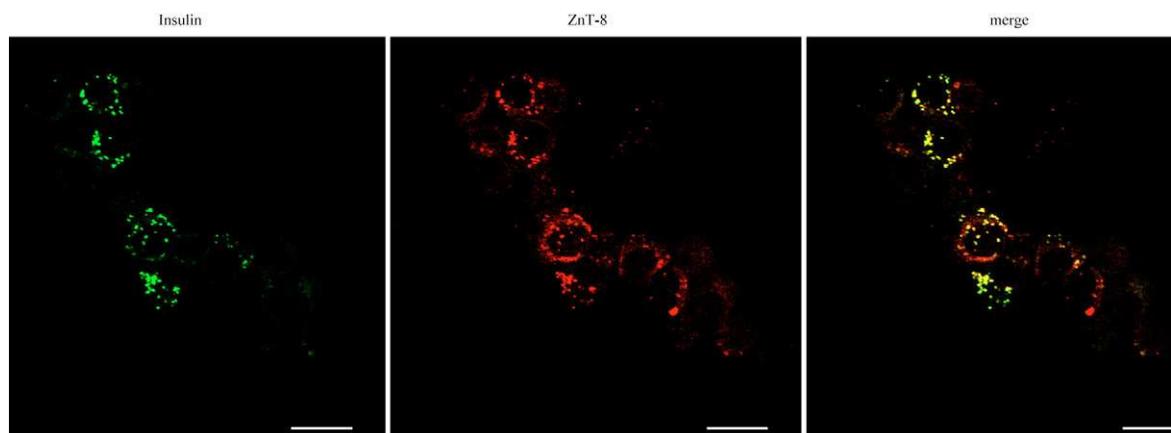


Fig. 3. Colocalization of ZnT-8 and insulin in human islet cells. Analysis of islet cell cytopspins using anti-insulin and anti-ZnT-8 antibodies by confocal fluorescence microscopy. Insulin (green, left) displayed a characteristic punctuate staining. ZnT-8 staining (red, middle) completely coincided with the staining of insulin. Superimposition of the two images demonstrates the colocalization of ZnT-8-GFP and insulin (yellow, right). Bars, 20 μm .

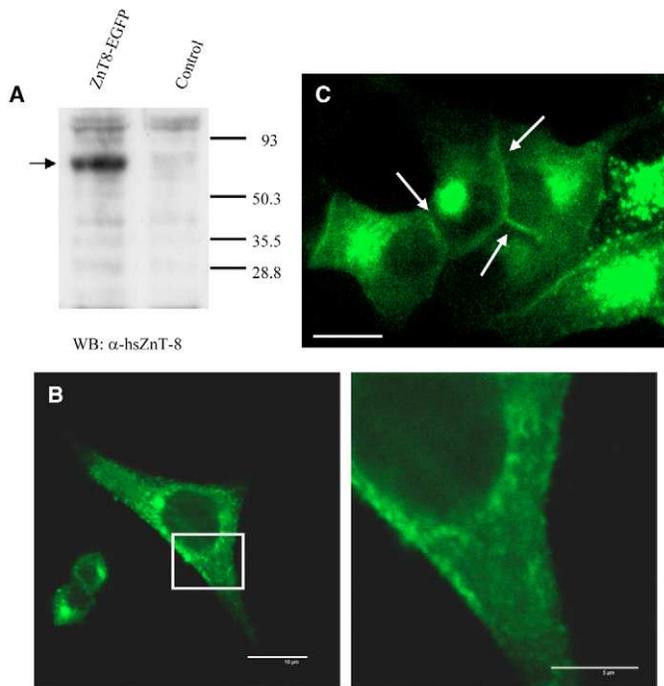


Fig. 4. ZnT-8 overexpression in INS-1E cells. (A) Western blot with anti-human ZnT-8 antibody showing the presence of the fusion protein at the expected size (i.e. 67 kDa) in ZnT-8-expressing cells but not in control EGFP-expressing cells. (B) Confocal fluorescence microscopy of ZnT-8-EGFP-expressing INS-1E cells. A representative cell displaying punctuated staining consistent with insulin colocalization. The panel on the right is a magnified image of the selected area of the cell on the left, showing that ZnT-8-EGFP is mainly localized in granules in close proximity to the plasma membrane. (C) Fluorescence microscopy of ZnT-8-expressing cells. Arrows indicate localization of ZnT-8-EGFP at the plasma membrane. Bars, 10 μm (C, left panel in B); 5 μm (B, right panel).

Protection against zinc depletion by ZnT-8 overexpression

We next further investigated the capability of ZnT-8 to act on zinc metabolism by measuring cell viability after incubation with different concentrations of either zinc sulphate or the zinc chelator TPEN (Fig. 6B,C). There were no differences in zinc toxicity between control and ZnT-8 expressing cells. The calculated LC_{50} (concentration with 50% viability) was $482 \pm 8 \mu\text{M}$ and $510 \pm 8 \mu\text{M}$ zinc sulphate in control and ZnT-8-expressing cells, respectively ($P > 0.1$). We then investigated the effect of zinc depletion and observed that ZnT-8 overexpression conferred resistance to zinc depletion (Fig. 6C). Compared with control cells, which have a LC_{50} for TPEN of $4.59 \pm 0.09 \mu\text{M}$, ZnT-8 expressing cells were protected from the toxicity of the zinc chelator TPEN, with a LC_{50} of $6.66 \pm 0.09 \mu\text{M}$ ($P < 0.0001$). Thus, overexpression of ZnT-8 did not have any effect on zinc toxicity, but significantly protected INS-1E cells from zinc-depletion-induced cell death.

Enhanced insulin secretion by ZnT-8 overexpression

To assess a potential role for ZnT-8 in insulin synthesis and/or secretion, we used INS-1E cells, which were shown to be a stable and valuable beta-cell model for insulin secretion

(Merglen et al., 2004), stably transfected with human ZnT-8-EGFP. Since expression of EGFP alone did not produce any change in glucose-stimulated insulin secretion compared with parental INS-1E cells (data not shown), these cells were used to measure insulin secretion after glucose stimulation. Compared with insulin release in normal conditions, i.e. 5.6 mM glucose, exposure of INS-1E-EGFP cells to 15 or 20 mM glucose resulted, as expected, in a significant increase of insulin secretion with a plateau phase after 15 mM glucose (Fig. 7). Overexpression of ZnT-8 did not lead to a significant increase in insulin release in basal conditions, nor did it significantly change the cellular insulin content ($1.3 \pm 0.1 \mu\text{g}/\text{million}$ cells for control cells vs $1.2 \pm 0.1 \mu\text{g}/\text{million}$ cells for ZnT-8 overexpressing cells, $P > 0.5$), which are values in the range found in the study describing INS-1E cells (Merglen et al., 2004). However, when incubating the cells at high glucose concentrations, insulin secretion in ZnT-8-expressing cells was significantly increased, almost twice as much as control cells. This was not attributable to a change in the storage pool, because data were expressed as percentage of insulin content. Moreover the plateau phase at about 15–20 mM glucose, previously described in INS-1E cells (Merglen et al., 2004), persisted in ZnT-8 expressing cells. Therefore it appears that overexpression of ZnT-8 in INS-1E cells enhanced glucose-induced insulin secretion only in high-glucose conditions.

Discussion

Zinc is concentrated in islets cells and is related to insulin synthesis, storage and secretion (Zalewski et al., 1994). We previously identified the zinc transporter ZnT-8, whose mRNA was solely detected in pancreatic islet cells (Chimienti et al., 2004). In this study we show that *ZNT8* mRNA expression was also restricted to pancreatic islet cells in mouse, and that the ZnT-8 protein is expressed in human islet cells in vivo. Moreover, in human pancreas, ZnT-8 was detected by confocal microscopy only in islet insulin-producing beta cells, and colocalized with insulin in these cells. ZnT-5 has been hypothesized to play a role in transporting zinc into secretory granules (Kambe et al., 2002). However, its expression seems ubiquitous (Seve et al., 2004) and functional studies suggest that this transporter rather participates in zinc loading to apo-proteins in the Golgi apparatus (Suzuki et al., 2005). Indeed, ZnT-8 seems to be the only ZnT expressed exclusively in pancreatic beta cells and thus may be of prime importance for insulin secretory pathway.

In INS-1E cells, ZnT-8 has been shown by confocal microscopy to be present in granules that are in close proximity to the plasma membrane and, to a lesser extent, at the plasma membrane level. To date, predictions of the membrane topology of the ZnT family of proteins were only done in silico. Moreover, most of the programs used predicted the ZnT proteins to have six transmembrane domains, however, some algorithms underline the putative presence of a seventh transmembrane domain (Hirokawa et al., 1998). Therefore four models were possible for ZnT-8 membrane topology: two models with six helices, the N- and C-termini domains facing either the extracellular or the cytosolic side. In the other two models, the presence of seven transmembrane domains implies that N- and C-termini are not located at the same side of plasma membrane. Our experimental data are in accordance with most of predicted models and consistent with the zinc export

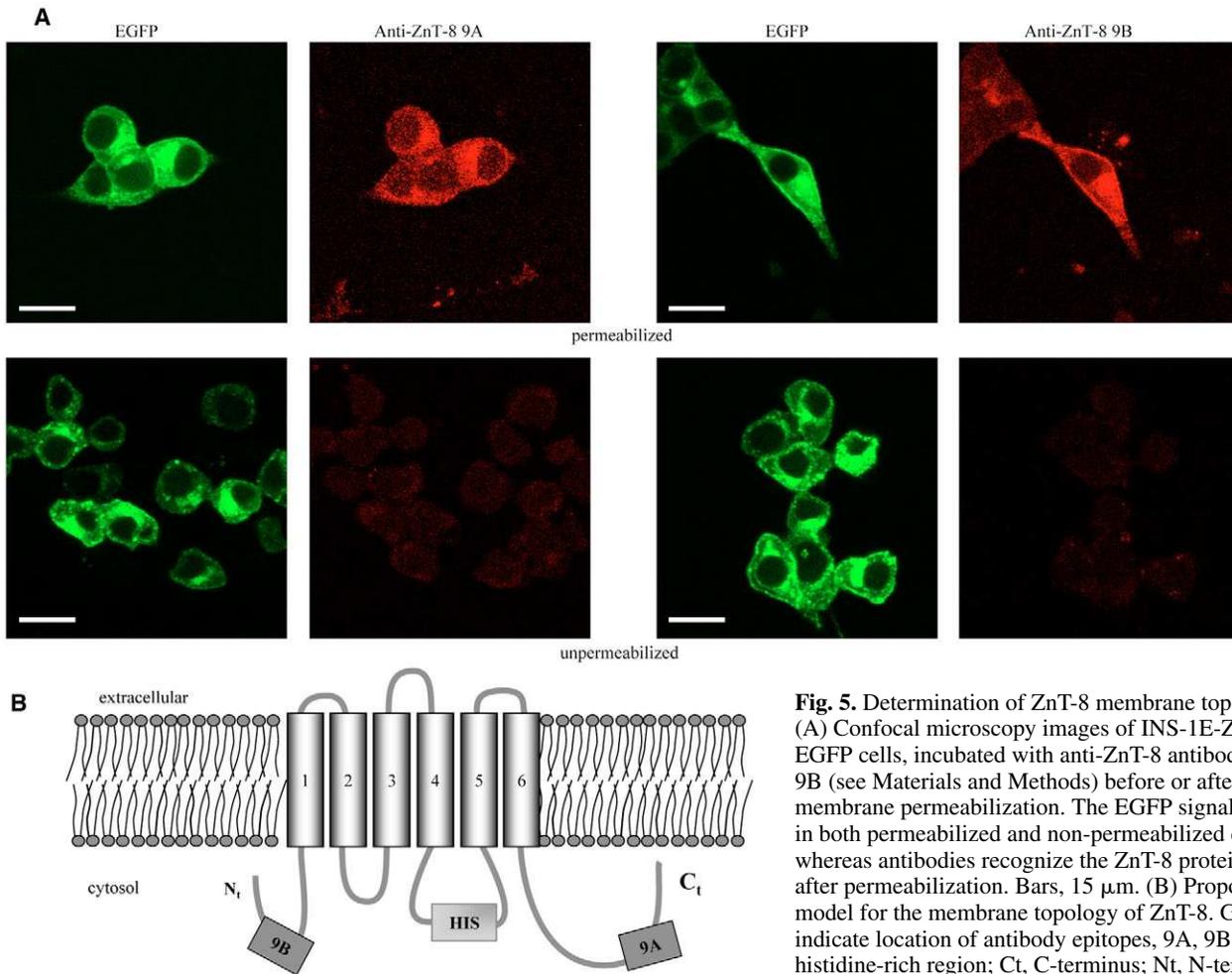


Fig. 5. Determination of ZnT-8 membrane topology. (A) Confocal microscopy images of INS-1E-ZnT-8-EGFP cells, incubated with anti-ZnT-8 antibodies 9A or 9B (see Materials and Methods) before or after membrane permeabilization. The EGFP signal is present in both permeabilized and non-permeabilized cells, whereas antibodies recognize the ZnT-8 protein only after permeabilization. Bars, 15 μ m. (B) Proposed model for the membrane topology of ZnT-8. Gray boxes indicate location of antibody epitopes, 9A, 9B. HIS, histidine-rich region; Ct, C-terminus; Nt, N-terminus.

function of the ZnT family, and with the function of ZIP family, which are zinc uptake transporters whose extremities are located outside the cell (Mathews et al., 2005). Furthermore, this membrane topology of zinc export transporter is also consistent with a role for ZnT-8 in exporting zinc from the cytoplasm to insulin-containing vesicles.

In agreement with previous findings in HeLa cells (Chimienti et al., 2004), ZnT-8 acts as a zinc transporter in INS-1E cells, because its overexpression augments total cellular zinc. Surprisingly, zinc supplementation did not increase total zinc in control cells. This reflects the fact that beta cells, as is the case with prostate cells (Costello et al., 2004), already contain very high amounts of zinc compared with other cell types. However, zinc at high concentrations can be toxic for cells, and may even actively participate in islet cell death under certain conditions (Kim et al., 2000). Indeed, the LC_{50} for zinc was about 490 μ M in INS-1E cells. This is much higher than other cell types, including HeLa cells, which have a LC_{50} for zinc of 142 μ M (Devergnas et al., 2004). It has been suggested that beta cells need to contain sufficient amounts of zinc for proper function and formation of insulin hexamers (Sondergaard et al., 2003). Therefore, added extracellular zinc may not be able to get in the cells, or may be extruded from cells. However, ZnT-8 overexpression enhanced the capacity of the cell to store zinc, demonstrating that the zinc

transport/storage capacity, rather than extracellular zinc status, is important for zinc accumulation in beta cells. As a consequence, zinc toxicity is not modified by ZnT-8 overexpression. However, because they store more zinc, ZnT-8-expressing cells were protected from zinc depletion-induced cell death compared with control cells.

In diabetes, hypozincemia is a common feature (Garg et al., 1994; Roussel et al., 2003). Zinc supplementation can significantly inhibit the development of type 1 diabetes (Ho et al., 2001), and markedly improve the hyperglycemia of streptozotocin-induced diabetic mice (Chen et al., 2000). Zinc has also demonstrated protective effects in type 2 models of diabetes (for a review, see Taylor, 2005). Zinc supplementation has been found to be effective for reducing fasting hyperglycemia and hyperinsulinemia, and reducing weight gain in young *db/db* mice (Simon and Taylor, 2001). Beneficial antioxidant effects of zinc supplementation have been found in people with type 2 diabetes (Anderson et al., 2001). Excessive apoptosis of pancreatic beta cells has been associated with diabetes (for a review, see Chandra et al., 2001). Zinc depletion by itself is a well-known inducer of apoptosis (Chimienti et al., 2001), but can also promote oxidative stress-induced apoptosis (Baynes, 1991), thereby participating in decreased in beta cell mass. In addition, some studies suggest that cells with deprived zinc stores are less able to defend themselves against oxidative

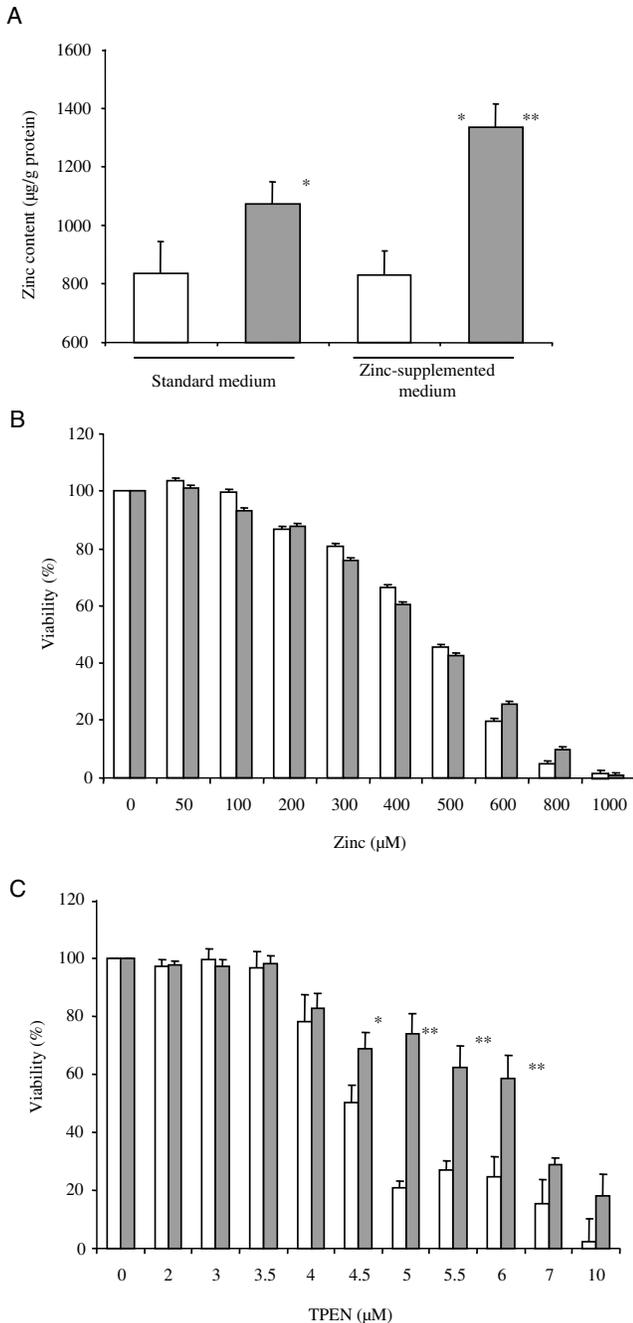


Fig. 6. Effect of extracellular zinc concentration on zinc content and cell viability. (A) Zinc concentrations in cells were measured by electrothermal atomic absorption spectrophotometry in either normal or zinc-supplemented medium. Values are expressed as zinc concentration normalized for protein content. * $P < 0.01$ vs control; ** $P < 0.05$ vs non-supplemented cells. White bars, control cells; gray bars, INS-1E-ZnT8-EGFP cells. (B) After incubation with different concentrations of zinc sulphate, the cell viability was determined by MTT assay. Values are the mean \pm s.e.m. of at least ten independent experiments. White bars, control cells; gray bars, INS-1E-ZnT8-EGFP cells. (C) After incubation with different concentrations of the zinc chelator TPEN, the cell viability was determined by MTT assay. Values are the mean \pm s.e.m. of at least ten independent experiments. * $P < 0.05$ vs control; ** $P < 0.005$ vs control. White bars, control cells; gray bars, INS-1E-ZnT8-EGFP cells.

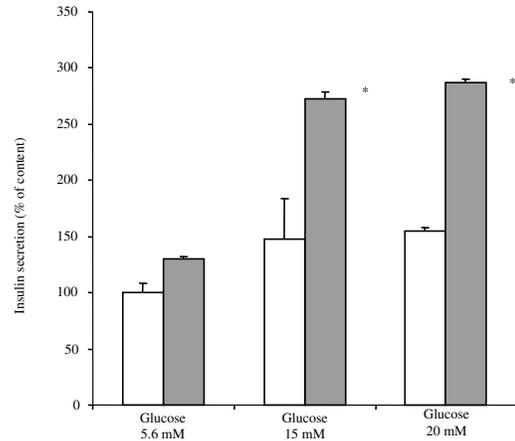


Fig. 7. Influence of ZnT-8 on glucose-induced insulin secretion. INS-1E-EGFP and INS-1E-ZnT8-EGFP cells were used to assess insulin secretion in response to glucose. After incubation in glucose-free buffer (see Materials and Methods), cells were exposed to different concentrations of glucose for 30 minutes. Intracellular and secreted insulin were measured by ELISA. White bars, control cells; gray bars, INS-1E-ZnT8-EGFP cells. Values are the mean \pm s.e.m. of at least five experiments, * $P < 0.01$ vs control, ** $P < 0.005$ vs control.

injuries, underlining the antioxidant properties of zinc (Chausmer, 1998). Therefore, enhancing the capacity of beta cells to store zinc may help to protect the pancreas against zinc depletion and/or oxidative stress frequently observed in diabetes.

Forty years ago, Quarterman et al. demonstrated that zinc-deprived rats had an impaired insulin secretion response to glucose stimulation (Quarterman et al., 1966), and other studies indicated decreased islet cell insulin content in zinc-deficient states (Engelbart and Kief, 1970). In this study, total insulin was not significantly increased in ZnT-8-expressing cells despite higher zinc content, nor was insulin secretion in basal glucose conditions. However, in stimulating glucose conditions, corresponding to hyperglycemia, overexpression of ZnT-8 significantly increased insulin secretion, to almost twice that of control cells, and the plateau phase observed after 15 mM glucose in INS-1E cells was still present. Thus, the increase in insulin secretion induced by ZnT-8 overexpression is glucose dependent. Altogether these results strongly suggest that ZnT-8 enhances zinc storage in insulin granules and is directly implicated in the insulin secretion pathway. Zinc transporters are a growing field of study, and such a role for ZnT-8 is not surprising in the light of the importance of the function of ZnT-3 in zinc-rich vesicles in the brain (for a review, see Frederickson et al., 2005).

In summary we demonstrate that the ZnT-8 protein is expressed only in pancreatic beta cells in vivo. In cultured cells, overexpression of ZnT-8 augmented total cellular zinc content, thus protecting cells from zinc depletion and enhanced insulin secretion under hyperglycemic conditions, suggesting that ZnT-8 has a major role in the insulin secretion pathway. Triggering ZnT-8 expression and/or activity may be an interesting approach in the treatment of type 2 diabetes, in which the zinc depletion frequently observed is likely to participate in beta-cell mass decrease.

Materials and Methods

Chemicals

All chemicals were reagent grade from Sigma (St Quentin-Fallavier, France) or Merck (Grenoble, France).

ZNT8 mRNA expression analysis in mouse

The tissue distribution of mRNA encoding ZnT-8 in the mouse was studied in male C56Bl6 mice (8-12 weeks of age). Total RNA was extracted *ex vivo* from mouse pancreatic islets, pancreatic acini, pituitary, brain, adrenal gland, liver, skeletal muscle, epididymal adipose tissue, lung, kidney and spleen using TRIzol Reagent according to the manufacturer's protocol (Gibco BRL, Carlsbad, CA, USA), followed by a cleanup procedure with RNeasy columns (Qiagen, Cologne, Germany). In addition, total RNA from fresh collagenase-isolated mouse pancreatic islets and pancreatic acini, hand-selected under a dissecting microscope in ice-cold media, was extracted using the Absolutely RNA microprep from Stratagene. The total RNA quantity and quality was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the 2100 Bioanalyzer (Agilent, Waldbronn, Germany), respectively. Total RNA profiles of all tested samples were similar with sharp 18S and 28S rRNA peaks on a flat baseline. RNA quantification was performed using Affymetrix mouse 430 2.0 expression microarrays (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was labeled overnight and fragmented for 35 minutes at 94°C; concentration and quality of labeled cRNA was measured, respectively, with the NanoDrop ND-1000 and Bioanalyzer 2100. Fragmented cRNA was hybridized onto the 430 2.0 arrays for 16 hours at 45°C, followed by washing and staining in the Fluidics Station (Affymetrix) and Scanning using a 3000 GeneScanner. Raw data were analyzed using GCOS software. Signal intensities were scaled using the global scaling method taking 150 as target intensity value. For all tissues, three independent biological replicates were studied; islets and pituitaries were studied with $n=5$ per condition.

Cell culture methods

Aliquots of the INS-1E cell line were a gift from Dr P. Maechler (Geneva, Switzerland). INS-1E cells were grown at 37°C in a 5% CO₂-enriched atmosphere in RPMI 1640 medium (Invitrogen, France) including 10% heat-inactivated fetal bovine serum (Invitrogen, France), 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and routinely split at a 1:5 ratio.

HeLa epithelial cells (ATCC number CCL-2) were grown at 37°C, in a 5% CO₂-enriched atmosphere, in Opti-MEM medium (Modified Eagle's Medium, Invitrogen) supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmid construction and generation of stable cell lines

The cloning of pZnT-8-EGFP has been described elsewhere (Chimienti et al., 2004). ZnT-8-EGFP- and control EGFP-expressing INS-1E stable cell lines were generated by transfecting pZnT-8-EGFP or pEGFP into INS-1E cells using Lipofectamine Plus (Invitrogen, France). The stable cell lines were selected by culturing the cells in the medium containing G418 (350 μg/ml) and cloned using a cell sorter. Selected clones were maintained with G418 (200 μg/ml). After amplification, the expression of the fusion protein was controlled by fluorescence microscopy; size and specificity of the fusion product were verified by western blot.

Western blot analysis

Samples were resolved by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with the specified antibody followed by an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Western blot signal was developed according to standard procedures using ECL (GE Healthcare).

ZnT-8 localization in human islets

Human pancreases were harvested from non-diabetic, non-obese brain-dead donors ($n=3$) in accordance with French regulations and after approval of Lille 2 University ethical committee. Pancreatic islets were isolated after ductal distension of the pancreases as described (Lukowiak et al., 2001). Human islets were washed with PBS and centrifuged (200 g). Cell dissociation was achieved using Splitase® (Autogen Bioclear, UK) for 3 minutes, and stopped by addition of culture medium. Cytospins were made with cell suspension and fixed in 1% paraformaldehyde. Immunocytochemistry was performed on islet cell cytopins.

Cells or cytopins were permeabilized in PBS containing 0.1% Triton X-100 and then blocked in 2% BSA in PBS for 1 hour. Primary antibodies [i.e. rabbit anti-ZnT-8 (1:10 dilution) combined or not with mouse anti-insulin (1:2000 dilution)] diluted in fresh blocking buffer were then added. After washing, cells were either revealed with Phallocyanine Red (Histomark Red, Kirkegaard and Perry Laboratories, Gaithersburg, MD) and nuclei were counterstained with Carazzi's hematoxylin for colorimetric studies or incubated with sheep anti-mouse IgG-FITC conjugate and goat anti-rabbit-Cy3 secondary antibodies (GE Healthcare) for fluorescence studies. Coverslips were mounted onto glass slides using FluorSave

(Calbiochem, La Jolla, CA) and photographed using a Leica confocal fluorescence microscope.

Membrane topology of ZnT-8

Immunolabeling of cultured cells for determination of membrane topology was done essentially as described previously (Mathews et al., 2005). Briefly, pZnT-8-EGFP expressing INS-1E cells were seeded onto glass coverslips and allowed to attach for 72 hours before fixation in 4% formaldehyde in PBS. After washing with 0.25% NH₄Cl in PBS, the cells were permeabilized in PBS containing 0.1% Triton X-100. Cells were then blocked in 2% BSA in PBS for 1 hour, followed by the addition of anti-ZnT-8 antibodies 9A or 9B, directed against amino acids 355-359 or 34-49 of the human ZnT-8 protein, respectively. Cells were washed in blocking buffer then incubated in donkey-anti-rabbit IgG-Cy3 conjugate, diluted in blocking buffer at 1:300. Cells were washed in PBS followed by a final wash in ddH₂O. For immunolabeling without permeabilization, cells were incubated with primary antibody in PBS containing 0.1% bovine serum albumin for 30 minutes before fixation. Cells were then washed in PBS, and fixation and secondary antibody labeling were conducted as with permeabilized cells.

Importance of ZnT-8 for intracellular zinc content

For intracellular zinc determination assays, INS-1E-EGFP- and INS-1E-ZnT-8-EGFP-expressing cells were supplemented with 90 μM ZnSO₄ or not until 80% confluency. Cells were then trypsinized and washed three times in Ca/Mg-free PBS. The subsequent pellet was resuspended in 900 μl lysis buffer (1% Triton X-100 in 10 mM Tris-HCl pH 7.4) and cells were lysed by three cycles of freeze-thawing. Zinc concentrations were determined on total extracts by electrothermal atomic absorption spectrophotometry (Perkin-Elmer, USA) and normalized for protein content (BCA assay).

Zinc and zinc-depletion induced cell death

Cells were plated in 96-well plates and allowed to attach for 24 hours. Cells were then treated by the indicated dose of either zinc sulphate or the zinc chelator N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) for 24 hours at 37°C. 24 hours after treatment, cellular viability in the presence or absence of experimental agents was determined using the MTT assay (Carmichael et al., 1987). Briefly, following experimental treatment, a final concentration of 0.5 μg/ml MTT was added to each well, and the plate was incubated in the dark for 3 hours at 37°C. The medium was then removed, and the coloured reaction product was solubilized in 100 μl DMSO. Absorbance was measured at 570 nm using a Vmax plate reader (Labsystems Multiskan RC). The percentage viability was calculated as follows: percentage specific viability = $\frac{(A-B)/(C-B)}{A} \times 100$ where $A=OD_{570}$ of the treated sample, $B=OD_{570}$ of the medium, and $C=OD_{570}$ of the control (phosphate buffer saline (PBS)-treated cells). The values were expressed as percentage viability relative to vehicle-treated control cultures.

Insulin secretion

Insulin secretion was assessed on INS-1E-EGFP- and INS-1E-ZnT-8-EGFP-expressing cells. The secretory responses to glucose were tested as described before (Merglen et al., 2004). Briefly, cells were maintained for 2 hours in glucose-free culture medium. The cells were then washed twice and preincubated for 30 minutes at 37°C in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH: 135 mM NaCl, 3.6 mM KCl, 5 mM NaHCO₃, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂, 1.5 mM CaCl₂, and 10 mM HEPES, pH 7.4). BSA (0.1%) was added as an insulin carrier. Cells were subsequently washed with glucose-free KRBH and then incubated for 30 minutes at 37°C in KRBH containing different concentrations of glucose. The reaction was stopped by placing the plates on ice; supernatants were collected to determine insulin secretion and cells were used for intracellular insulin content following acid-ethanol extraction. Insulin was measured by ELISA (Linco) according to the manufacturer's instructions.

Statistics

All data were expressed as mean ± s.e.m. Statistical analysis was by two-tailed, unpaired Student's *t*-test, and differences were considered significant when $P < 0.05$.

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