

Mechanism and Regulation of Cellular Zinc Transport

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Zinc is an essential cofactor for the activity and folding of up to ten percent of mammalian proteins and can modulate the function of many others. Because of the pleiotropic effects of zinc on every aspect of cell physiology, deficits of cellular zinc content, resulting from zinc deficiency or excessive rise in its cellular concentration, can have catastrophic consequences and are linked to major patho-physiologies including diabetes and stroke. Thus, the concentration of cellular zinc requires establishment of discrete, active cellular gradients. The cellular distribution of zinc into organelles is precisely managed to provide the zinc concentration required by each cell compartment. The complexity of zinc homeostasis is reflected by the surprisingly large variety and number of zinc homeostatic proteins found in virtually every cell compartment. Given their ubiquity and importance, it is surprising that many aspects of the function, regulation, and crosstalk by which zinc transporters operate are poorly understood. In this mini-review, we will focus on the mechanisms and players required for generating physiologically appropriate zinc gradients across the plasma membrane and vesicular compartments. We will also highlight some of the unsolved issues regarding their role in cellular zinc homeostasis.

Online address: <http://www.molmed.org>

doi: 10.2119/2007-00037.Sekler

INTRODUCTION

The uniqueness of zinc, in contrast to other abundant transition metals in the body, e.g., Fe³⁺ and Cu²⁺, is that it lacks redox activity. It has been suggested that early in evolution, Fe³⁺ fulfilled the roles of Zn²⁺ as a cofactor in domains that were predecessors to Zn-fingers (1). The emergence of oxygen and oxidative phosphorylation led to the rapid extinction of this form of heavy metal biochemistry. Importantly, between three and ten percent of all proteins in mammalian genomes are considered to bind zinc (2). In these proteins, zinc is essential for folding, conformational change, or activity. It is, therefore, not surprising that zinc deficiency has catastrophic consequences, reflected in, among other syndromes, erosion of the gastrointestinal tract, skin lesions, cardiac failure, and malformations of brain and the male reproductive system (3,4). Zinc, particularly the "free" or loosely-bound form of

the ion, is nevertheless profoundly toxic to mammalian cells (5). Numerous studies have shown that a rise in cellular free Zn²⁺ resulting from permeation of synaptic zinc or from release of this ion from intracellular stores in neurons (6–9), is a key factor in neuronal death following seizure or an ischemic episode (10,11). Dysregulation of free zinc has also been implicated in the formation of β -amyloid plaques associated with Alzheimer's disease (12). Permeation of zinc that is co-released with insulin from pancreatic β -cells is emerging as an important factor in the degeneration of these cells (13–15). These paradoxical aspects of cellular zinc dictate that it be distributed in highly regulated gradients with respect to the plasma membrane and intracellular compartments. It also demands that an adequate supply of this ion be available to the numerous zinc-binding proteins, while preventing its accumulation and the potentially devastating effects it can

readily initiate. The magnitude of the zinc gradient present at the plasma membrane rivals even that of calcium (16). Zinc concentrations in the synaptic cleft, for example, are estimated to rise to micromolar concentrations, while the intracellular free-Zn²⁺ concentration is estimated to be in the picomolar range (17). Similarly steep zinc gradients are also formed within the cells, with millimolar concentrations of Zn²⁺ existing in the synaptic and secretory vesicles (18–20). The complexity and importance of zinc homeostasis is reflected by the large number of proteins that are potentially dedicated to Zn²⁺ transport and buffering (Figure 1), among them, at least ten members of the ZnT (Zn²⁺ Transporter) family (21), 15 members of the ZIP (i.e., Zn²⁺-regulated metal transporter, Iron-regulated metal transporter-like protein) family (22), and 3 distinct isoforms of metallothionein (23).

ACTIVE ZINC TRANSPORT ACROSS THE CELL MEMBRANE

Ion gradients are generated by two main mechanisms: 1) A primary pump, utilizing the energy of ATP-hydrolysis; or 2) a secondary active mechanism that

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Submitted April 23, 2007; Accepted for publication May 30, 2007.

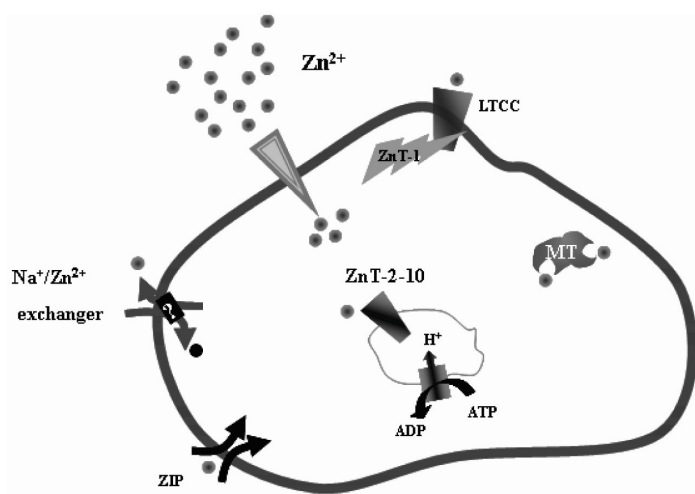


Figure 1. A schematic representation on the mechanisms of zinc homeostasis in mammalian cells. Zinc is distributed at large transmembrane and vesicular gradients. These are generated by the orchestrated activity of multiple zinc transporters and regulators of zinc transport. For the sake of simplicity, the ZnT2-10 have been illustrated on a single compartment although they may be found on multiple organelles.

uses an ion gradient, such as Na^+ , for generating Zn^{2+} gradients. A Zn^{2+} pump has been demonstrated in bacteria, where several forms of p-type ATPases have been shown to catalyze active Zn^{2+} transport (24). Recently, a similar ATPase, which transports Zn^{2+} and Cd^{2+} and to a lesser extent other heavy metals, has been discovered in *Arabidopsis* (25,26). Surprisingly, there is still no evidence for a Zn^{2+} pump in either yeast or mammalian cells, though a Cu pump has been identified that is linked to heavy metal ion transport (27).

A Na^+ -dependent secondary active mechanism has, however, been suggested to facilitate formation of the transmembrane Zn^{2+} gradient in neurons. Early studies suggested that the neuronal $\text{Na}^+/\text{Ca}^{2+}$ exchanger mediates Zn^{2+} extrusion (28), but more recent findings seem to support the existence of a distinct $\text{Na}^+/\text{Zn}^{2+}$ exchanger. These studies have indicated that a putative $\text{Na}^+/\text{Zn}^{2+}$ exchanger, probably a member of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger superfamily, operates with a stoichiometry of $3\text{Na}^+/1\text{Zn}^{2+}$, promoting Zn^{2+} efflux against a 500-fold transmembrane gradient (29). This mechanism is pharmacologically and molecu-

larly distinct from the classical $\text{Na}^+/\text{Ca}^{2+}$ exchangers. Whether this exchanger is the principle plasma membrane extruder of Zn^{2+} or is accompanied by an as yet unidentified Zn^{2+} pump, is an open and intriguing question.

THE ROLE OF ZnT PROTEINS IN CELLULAR ZINC HOMEOSTASIS

To date, human CDF, also known as SLC30, genes, code for ten zinc transporters (ZnTs), i.e., ZnT-1-10 (30). The expression and cellular distribution of the ZnTs is highly regulated by changes in zinc (31–33). The rapid changes occurring in extracellular Zn^{2+} , and the existence of numerous pathways for permeation of this ion, suggest an important role for the ZnT proteins in physiology and pathology (Table 1), though relatively little is known about their mechanism of activity or their regulation. Studies of yeast CDF proteins and the bacterial ZitB protein, which is a distantly related homologue of the ZnT proteins, have suggested mechanisms by which these proteins control Zn^{2+} transport (34,35). Functional analyses of their bacterial and yeast homologues have suggested that these proteins might

catalyze $\text{H}^+/\text{Zn}^{2+}$ exchange (35). If a similar mechanism underlies the activity of mammalian Zn^{2+} transporters, it may have important physiological implications. For instance, metabolic acidosis or a change in pH triggered by inflammation, might attenuate ZnT-dependent Zn^{2+} transport. Glial cells, normally more resistant to zinc toxicity than neurons, are indeed rendered more susceptible by reduction in pHi (36), possibly as a result of lower ZnT-dependent transport.

Although the ZnTs are increasingly recognized as critical players in cellular zinc homeostasis, for the sake of brevity, only a few examples will be discussed.

ZnT-1

ZnT-1, a ubiquitously expressed member of the SLC30 zinc transporter family, is found on the plasma membrane of neurons (37) and glia cells (38,39). In the mouse brain, ZnT-1 is, in general, localized in regions rich in synaptic Zn^{2+} (though cerebellar Purkinje cells are also intensely ZnT-1-immunoreactive) and its expression is developmentally regulated in correlation with the appearance of the synaptic Zn^{2+} (39,40). ZnT-1 has been shown to reduce Zn^{2+} toxicity in neurons and glial cells (37,38,41). Expression of this protein is highly regulated by Zn^{2+} , via the transcription factor MTF-1 (32), and priming of glial cells with non-toxic Zn^{2+} exposures promotes ZnT-1 expression (38). Thus, sublethal exposure to Zn^{2+} might induce ZnT-1 (and perhaps MT) expression to counteract subsequent (toxic) rises in $[\text{Zn}^{2+}]_i$. However, it must be emphasized that the mechanism by which ZnT-1 maintains low $[\text{Zn}^{2+}]_i$ is more complex than previously thought.

Initially, it was widely accepted that ZnT-1 reduces $[\text{Zn}^{2+}]_i$ by acting as a Zn^{2+} extruder (37,41). However, changes in Zn^{2+} effected by ZnT-1 appear to occur without concomitant changes in the concentration of counter ions, such as K^+ , Na^+ , Ca^{2+} , or protons. Nor are changes in cytoplasmic Zn^{2+} affected by depletion of intracellular ATP. This indicates that as a proposed mechanism of ion transport, zinc extrusion fits poorly with the well-

Table 1. ZnT proteins- expression and physiological roles.

	Expression pattern	Cellular distribution	Disease model	Altered expression phenotype	Ref
ZnT-1	Ubiquitous	Plasma membrane		<i>Overexpression</i> : reduced (Zn^{2+}) _i and enhanced resistance against Zn^{2+} toxicity. <i>KO</i> : Lethal at embryonic stage.	(37,42,43,71)
ZnT-2	small intestine, kidney, placenta, pancreas, testis, seminal vesicles, and mammary gland	Vesicles, lysosomes		<i>Overexpression</i> : enhanced lysosomal and vesicular Zn^{2+} accumulation	(72,73)
ZnT-3	brain	Synaptic vesicles (Glutamatergic and GABAergic)		<i>KO</i> : synaptic Zn^{2+} deficiency, enhanced susceptibility to seizure, loss of gender specific Alzheimer's disease plaque formation in a mouse model, decreased susceptibility to amyloid angiopathy	(49,74)
ZnT-4	mammary gland, brain, small intestine and mast cells	Intracellular compartments	Lethal milk syndrome in mice, Asthma (mice), Alzheimer's disease		(75-79)
ZnT-5	Pancreatic β -cells, intestine, heart brain, liver, kidney	Insulin secretory vesicles, Golgi Spliced isoform: plasma membrane Complexed with ZnT-6		<i>KO</i> : poor growth, osteopenia, male specific fatal arrhythmias. Essential for folding and secretion of Zn^{2+} -binding enzymes.	(53-55,59,80)
ZnT-6	liver, brain, and small intestine	Complexed with ZnT-5	Alzheimer's disease (mice)		(53,57,79)
ZnT-7	small intestine, liver, retina, spleen, kidney, and lung	Golgi		Essential for folding and secretion of Zn^{2+} -binding enzymes.	(54,81,82)
ZnT-8	Pancreatic β -cells	Insulin secretory vesicles	Polymorphism marker in diabetes type II	<i>Overexpression</i> : enhances glucose dependent insulin secretion	(60-62)

described modus operandi of traditional transporters. Recent data support the idea that ZnT-1 can affect Zn^{2+} homeostasis through regulating L-type calcium channels (LTCC) (38,42,43). We have found that heterologous expression of ZnT-1 in HEK-293 cells was followed by a decrease in Zn^{2+} accumulation but not by an apparent increase in zinc efflux (43). Notably, the expression of ZnT-1 reduced the Zn^{2+} influx mediated through the L-type Ca^{2+} channels (Figure 2A). Moreover, expression of ZnT-1 in cells endogenously expressing LTCC, as well as heterologous co-expression of ZnT-1 and LTCC, resulted in reduction of [Zn^{2+}]_i following opening of the LTCC

(43). We have determined the physiological implication of this mode of activation in astroglia (38). Induction of ZnT-1 expression mediated by preincubation with zinc or by heterologous expression of ZnT-1 was followed by attenuated influx of zinc via the L-type Ca^{2+} channels. Importantly, heterologous expression of ZnT-1 dramatically reduced astroglia cell death mediated by zinc influx through the latter channels. Our results also indicate that by regulating the activity of the L-type Ca^{2+} channel ZnT-1 confers resistance against toxic rise in Zn^{2+} _i in neurons (Figure 2B). Because ZnT-1 regulates L-type calcium channels, it is conceivable that it also will regulate permeation of

other cations that permeate through this pathway, e.g., calcium and cadmium. Recently, we have demonstrated that silencing of endogenous ZnT-1 by siRNA results in enhanced calcium and cadmium influx into neurons (42). The enhanced Cd^{2+} influx was followed by an increase in neuronal Cd^{2+} toxicity. The enhanced Ca^{2+} influx, on the other hand, was linked to an increase in synaptic transmission. Our results therefore indicate that regulation of the L-type Ca^{2+} channel, mediated by ZnT-1, has a more diverse role in cation homeostasis than previously thought. An open and intriguing question is how ZnT-1 affects the activity of the L-type Ca^{2+} channel

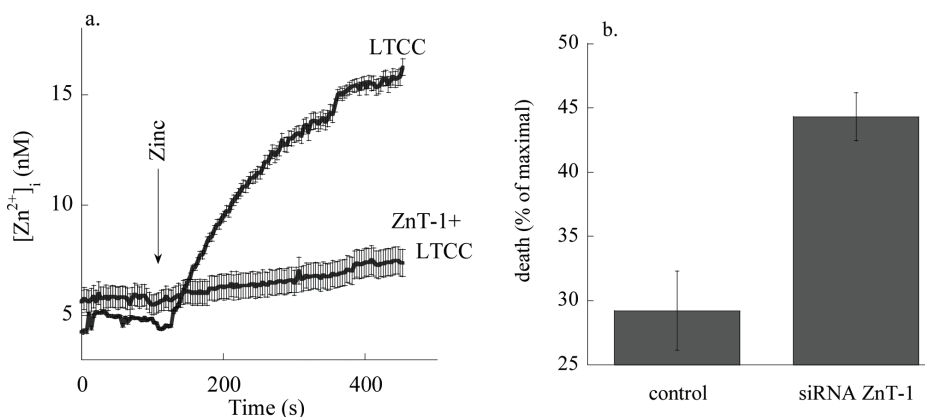


Figure 2. Expression of ZnT-1 is followed by attenuation of Zn²⁺ influx and toxicity. A. HEK-293 cells were co-transfected with ZnT-1 and LTCC plasmids, or with LTCC only. Cells were loaded with Fura-2 (5 μM), which is also a highly sensitive Zn²⁺ dye, and imaged while superfusing with a high K⁺ Ringer's solution (50mM) thereby opening the LTCC in the presence of Zn²⁺ (200 μM). B. Neurons were transfected with a ZnT-1 siRNA construct or control siRNA. Zn²⁺ (200 μM) was applied to a neuronal culture in the presence of high K⁺ Ringer's (ten minutes). Cell death was determined 24 h later using LDH.

(42). We have found that ZnT-1 does not modulate the expression of the L-type Ca²⁺ channel. Furthermore, direct interaction between ZnT-1 and the L-type calcium channel has not been established. It may be that interaction of ZnT-1 with the LTCC, or one of its accessory subunits, is too labile to be monitored using co-immunoprecipitation analysis. Alternatively, this may indicate that ZnT-1 indirectly modulates the activity of the L-type Ca²⁺ channel. The demonstration that ZnT-1 is regulated by the activity of Raf-1 suggests a possible pathway for such interaction. Therefore, further studies will be required to elucidate this intriguing and open question.

ZnT-3

Possibly the most extensively-studied ZnT, ZnT-3 is localized to the membranes of Zn²⁺-containing vesicles in glutamatergic synaptic boutons (44). Support for the idea that this transporter is essential for uploading Zn²⁺ into synaptic vesicles comes from ZnT-3 knockout mice, which lack chelatable Zn²⁺ throughout the brain. While early studies on this mutant failed to discern a distinct phenotype (45,46), later studies, focusing on brain disorders, such as stroke, epileptic

seizure, and Alzheimer's disease, have demonstrated a role for synaptic Zn²⁺, e.g., increasing susceptibility to seizures (47–49). An intriguing question emerging from this work is whether synaptic Zn²⁺ participates directly in fundamental processes of synaptic transmission such as LTP. Two recent studies (50,51) show that LTP in the cortico-amygdala pathway and hippocampus, respectively, does require Zn²⁺ release, while others (e.g.16,52) have indicated that LTP is not required. Although this issue is still unresolved, the ZnT-3 KO model continues to provide an excellent tool to examine these questions.

ZnT-5 and ZnT-6

ZnT-5 and ZnT-6, vesicular ZnTs found on Golgi and ER, are thought to functionally interact (53,54). Deletion of the ZnT-5 gene leads to abnormal bone development, weight loss, and lethal, male-specific, cardiac arrhythmias (55). Interestingly, one of the hallmarks of zinc deficiency is cardiac dysfunction (56). Several studies have indicated that ectopically expressed ZnT-5 or ZnT-6 are capable of independently transporting Zn²⁺, while others suggest that the activity of the Zn²⁺-dependent protein, TNAP (tissue-nonspecific alkaline

phosphatase), requires their hetero-oligomerization (53–55,57,58). While this links ZnT5/6 and vesicular Zn²⁺, the precise mechanism involved and their regulation remain poorly understood. Studies on yeast and bacterial ZnT homologues, considered together with the distribution of mammalian ZnTs in acidic cellular compartments, suggest that intracellular ZnTs are linked to both, Zn²⁺ and H⁺ transport. Results from our group indicate that ZnT-5 is capable of independently mediating Zn²⁺ transport, but coexpression of ZnT-5 and ZnT-6 accelerates its rate. We have further demonstrated that Zn²⁺ transport mediated by ZnT-5 is linked to changes in Golgi pH (Ohana et al. in preparation).

Localization of splice variants of ZnT-5 at various stations in cells (59) suggests a more versatile role in intracellular zinc homeostasis than was previously envisioned.

ZnT-8

ZnT-8 is exclusively expressed in pancreatic β-cells (60). Zinc is essential for the proper processing and packaging of insulin into secretory vesicles. Indeed, recent studies indicate that heterologous expression of ZnT-8 results in enhanced insulin accumulation and secretion (61). Furthermore, it has been shown recently that a polymorphism of ZnT-8 in humans is linked genetically to susceptibility to Type II diabetes (62). It should be noted that other ZnT family members, among them ZnT-5 and ZnT-6, are also found on secretory vesicles, influencing insulin production and secretion. Thus, the question is whether a functional interaction occurs between these Zn²⁺ transporters, and what their specific role in insulin production and secretion is.

ADDITIONAL QUESTIONS AND CHALLENGES FOR FUTURE RESEARCH

As seen by the brief discussion above, the characterization of multiple ZnT proteins has not yet led to elucidation of a mechanism of action for most of them. Many fundamental questions remain about their activity and regulation, as

well as their interaction with other ion transporters. Of particular interest is the question of how these proteins are capable of transporting zinc against the vanishingly low intracellular concentration existing in mammalian cells. One possible mechanism is direct buffering of Zn^{2+} by the ZnTs. Alternatively, the transporters may interact with metallothioneins (MT) which will transfer zinc to the ZnTs. A similar mechanism has been shown for the shuttling of Fe^{3+} and Cu^{2+} (63). Finally, release of zinc from metallothioneins, in response to specific intracellular signals (e.g., NO) or ischemia, may also provide free Zn^{2+} to the ZnTs.

Another question regards the existence of functional crosstalk between the Zn^{2+} transporters and other ion transport mechanisms, particularly proteins involved in maintaining cellular pH homeostasis, such as the Na^+/H^+ exchangers. This is especially relevant considering the putative mechanism of H^+/Zn^{2+} exchange, which may underlie the activity of the intracellular ZnTs.

The structural organization of the ZnTs also is poorly understood at this time. Hetero-oligomerization, has been suggested to underlie the activity of at least some of the transporters. Is this phenomenon exclusive to ZnT5 and ZnT6, or is it shared by other ZnT members? Such interactions would increase significantly the functional repertoire of zinc transport proteins. The functional interaction between ZnT-3 and the vesicular glutamate transporter, Vglut1, would support such a mechanism (64).

Finally, at the dawn of the era of proteomics and cellular networking, is it possible to begin drawing the network map of the zinc transport proteins? Powerful tools to address these issues, e.g., fluorescent zinc sensors targeted to specific organelles, have been developed and are being constantly refined (65–69). The advantage of such an approach has been demonstrated previously for H^+ and Ca^{2+} using GFP-based chameleon and pHluorin constructs. Based on seminal studies performed on yeast and mammalian cells, the feasibility of studying zinc in

organelles using such an approach is promising, (65,70). The results will have an impact far beyond cellular zinc homeostasis.

ACKNOWLEDGMENTS

Thanks to the many members in the zinc community for their invaluable insight and discussions. This work was supported in part by Binational Science Foundation Grant 2001101 (to M. H.), Israel Science Foundation Grant 456/02.1 (to I. S.), German Israel Foundation (GIF, project nr. I-588-99.1/1998 to I.S.), and Israel Science Foundation equipment Grant 456/02.2 (to I. S.).

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