Identification of the Zn^{2+} binding region in calreticulin

Shairaz Baksh[a], Cornelia Spamer[b], Claus Heilmann[b], Marek Michalak[a,*]

[a]MRC Group in Molecular Biology of Membrane and the Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2S2, Canada
[b]Department of Gastroenterology, University of Freiburg, D-7800 Freiburg, Germany

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Abstract **Calreticulin binds Zn^{2+} with the relatively high affinity/low capacity. To determine the location of the Zn^{2+} binding site in calreticulin different domains of the protein were expressed in *E. coli*, using the glutathione *S*-transferase fusion protein system, and their Zn^{2+}-dependent interaction with Zn^{2+}-IDA-agarose were determined. Three distinct domains were used in this study: the N + P-domain (the first 290 residues); the N-domain (residues 1–182) and the proline-rich P-domain (residues 180–273). The N + P-domain bound to the Zn^{2+}-IDA-agarose and were eluted with an increasing concentration of imidazole. The N-domain also bound $^{65}Zn^{2+}$ as measured by the overlay method. The P-domain did not interact with the Zn^{2+}-IDA-agarose and it did not bind any detectable amount of Zn^{2+}. Chemical modification of calreticulin with diethyl pyrocarbonate indicated that five out of seven histidines were protected in the presence of Zn^{2+} but they were modified by diethyl pyrocarbonate in the absence of Zn^{2+} suggesting that these residues may be involved in Zn^{2+} binding to calreticulin. We conclude that Zn^{2+} binding sites in calreticulin are localized to the N-domain of the protein, region that is not involved in Ca^{2+} binding to calreticulin.**

Key words: Calreticulin; Zinc binding; Endoplasmic reticulum

1. Introduction

It is well established that Zn^{2+} plays an important regulatory role in intracellular signal transduction as an activator of transcription factors and in several key enzymes involved in animal metabolism [1,2]. Several Ca^{2+} binding proteins including S100, calmodulin and calsequestrin also bind Zn^{2+} suggesting that Zn^{2+} may modulate their structure and function [3,4]. Calreticulin is a Ca^{2+} binding protein found in the lumen of the endoplasmic reticulum, in the nuclear envelope and in the nucleus of some cells [5]. Although calreticulin was first identified as a Ca^{2+} binding protein [6], recent reports indicate that the protein is multifunctional [7,8]. For example, calreticulin modulates steroid-sensitive gene expression [9], interacts with α-subunit of integrin [10], effects adhesion properties of some cells [11], and has a lectin-like activity [12]. Furthermore, calreticulin affects intracellular Ca^{2+} homeostasis [13–15], replication of Rubella virus RNA [16] and has a chaperone activity [17,18]. In addition to Ca^{2+} calreticulin binds other ions including Mg^{2+} and Zn^{2+} [19,20]. Similarly, the protein has also two distinct Ca^{2+} binding sites: a high capacity site (>25 moles Ca^{2+}/mole of protein) and a high affinity site (K_d < 10 μM) [6,19]. These two Ca^{2+} binding sites are found in different structural regions of the molecule. The P-domain of calreticulin (a proline-rich central region of the protein) binds Ca^{2+} with the high affinity and low capacity, whereas the C-domain (acidic, carboxyl-terminal region of calreticulin) binds Ca^{2+} with the low affinity and high capacity [19]. Localization of the Zn^{2+} binding sites in calreticulin is not known.

In the present study we have used the glutathione *S*-transferase (GST) fusion protein system to express distinct domains of calreticulin to identify region(s) of the protein involved in Zn^{2+} binding. Zn^{2+} binding site is located to the N-domain of calreticulin. Importantly, we showed that the P-domain of calreticulin does not bind any detectable amount of Zn^{2+}. DEPC modification of calreticulin indicated that 5 out of 7 histidines found in calreticulin may be involved in Zn^{2+} binding to the protein.

2. Experimental

Recombinant full-length calreticulin, the domains of calreticulin and recombinant GST were expressed in *E. coli* and purified [19]. In this study we have expressed the full length mature calreticulin and several domains of the protein: N-domain (amino acid residues 1 to 182), N + P-domain (amino acid residues 1 to 290 encoding the N-domain and P-domain), the P-domain (amino acid residues 182–273) [19].

Zn^{2+}-IDA (iminodiacetate)-substituted agarose chromatography was carried out as described by Porath and Olin [21]. Zn^{2+}-IDA-agarose column was equilibrated with a solution containing 50 mM NaH_2PO_4, pH 7.0 and 100 mM NaCl and saturated with 10 mM $ZnCl_2$. Prior to the Zn^{2+}-IDA-agarose chromatography, the GST fusion proteins were purified on the glutathione-Sepharose 4B column [19] followed by factor Xa digestion. Factor Xa was used at a 1:50 dilution in a buffer containing 100 mM NaCl, 1 mM $CaCl_2$ and 50 mM Tris, pH 8.0. The reaction was carried out for 18 h at room temperature and was stopped with 2.5 mM PMSF. Digested protein samples were directly loaded onto the Zn^{2+}-IDA-agarose. Proteins were eluted with a linear gradient of 0 to 50 mM imidazole in 50 mM NaH_2PO_4, pH 7.0 followed by SDS-PAGE analysis.

Zn^{2+}-dependent precipitation of calreticulin (2 mg of protein/ml) was carried out in a buffer containing 10 mM MOPS, pH 7.1, 150 mM NaCl and different amounts of $ZnCl_2$. Precipitation was carried out for 16 h followed by centrifugation for 45 min at 100,000 × *g*. For $^{65}Zn^{2+}$ overlay nitrocellulose membranes were incubated in a solution containing 10 mM imidazole, pH 6.8, 5 mM $MgCl_2$, 60 mM KCl in the presence of 100 μM $^{65}Zn^{2+}$. $^{65}Zn^{2+}$ binding proteins were visualized by autoradiography on Kodak X-OMAT AR films.

DEPC modification of histidine residues in calreticulin was carried out at 25°C in a buffer containing 50 mM sodium phosphate, pH 7.8 and 2 μM native or recombinant calreticulin [22]. Aliquots of DEPC were added at the time indicated in the Figure in a 35-fold molar excess with respect to calreticulin. The modification was monitored by recording changes in absorbance at 243 nm. Modifications of histidine were estimated based on a differential extinction coefficient of 3,200 $M^{-1} \cdot cm^{-1}$ at 243 nm for *N*-carbethoxyhistidine.

SDS-PAGE was on 12.5% or 15% polyacrylamide gels as described by Laemmli [23]. After gel electrophoresis, gels were stained with either

*Corresponding author. Department of Biochemistry, University of Alberta, 424 Heritage Medical Research Center, Edmonton, Alberta, Canada, T6G 2S2. Fax: (1) (403) 492 9753.

Abbreviations: GST, glutathione *S*-transferase; IDA, iminodiacetate-substituted agarose; DEPC, diethyl pyrocarbonate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid.

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Coomassie blue or transferred electrophoretically onto nitrocellulose membrane for $^{65}Zn^{2+}$ overlay or immobilon (polyvinylidene difluoride) membrane for NH_2-terminal amino acid sequence analysis [24].

Protein was determined by the method of Bradford [25] using bovine serum albumin as a standard.

3. Results

Calreticulin binds ~14 moles of Zn^{2+}/mole of protein with a K_d of ~300 μM [19]. In this study, we have tested effects of Zn^{2+} on aggregation of calreticulin. Purified protein was incubated in the presence of different concentrations of Zn^{2+} followed by centrifugation and SDS-PAGE of the supernatants and pellets. Calreticulin was identified by its characteristic blue staining with Stains-All [26]. Fig. 1 shows that calreticulin precipitated at approximately 600 μM concentration of Zn^{2+}. In contrast, calsequestrin precipitated at approximately 200 μM Zn^{2+} (data not shown). Calreticulin was precipitated quantitatively at ≥ 2 mM concentration of Zn^{2+} (Fig. 1).

In order to identify specific domains in calreticulin that might be responsible for Zn^{2+} binding we utilized a Zn^{2+}-IDA-agarose chromatography and fusion protein approaches. Two regions of calreticulin were expressed as GST fusion proteins and purified: the GST-N + P-domain (amino acid residues 1 to 290), GST-N-domain (amino acid residues 1–182), and GST-P-domain (amino acid residues 182–273) (Fig. 2) [19]. The C-domain of calreticulin was not used in these studies. Prior to Zn^{2+}-IDA-agarose chromatography the fusion proteins were purified on the glutathione-Sepharose 4B followed by digestion with factor Xa.

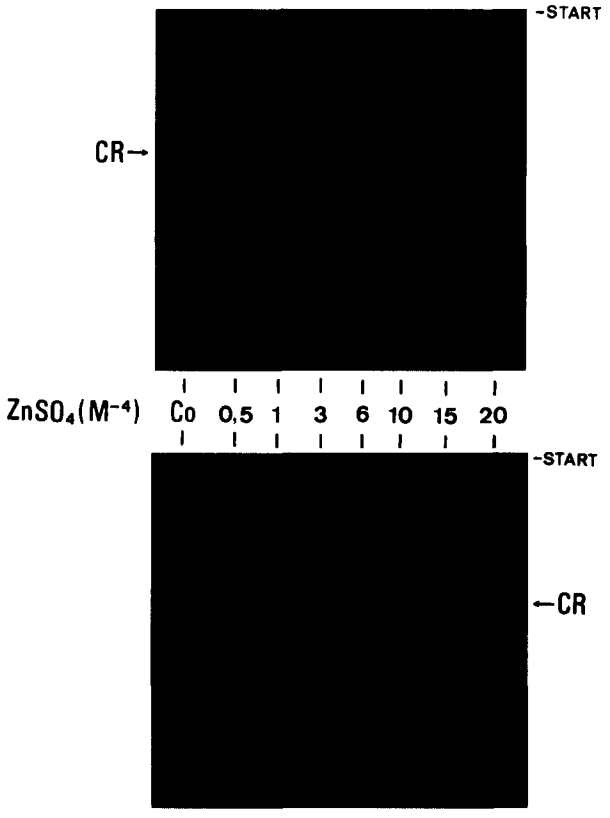

Fig. 1. Zn^{2+}-Dependent Precipitation of Calreticulin. Purified calreticulin was incubated in the presence of increasing concentration of Zn^{2+} followed by centrifugation. Calreticulin was identified in the supernatants (upper panel) and the pellets (lower panel) by blue staining with Stains-All. Co, control; CR, calreticulin.

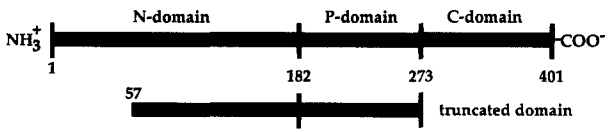

Fig. 2. Schematic representation of calreticulin domains. Three distinct domains of calreticulin were used in this study: the N-domain (amino acid residues 1–182); the proline-rich P-domain (amino acid residues 182–273) and the N + P-domain (the first 290 amino acid residues). Truncated domain, truncated N + P-domain (see text for details).

Fig. 3 shows interaction of the N + P-domain with the Zn^{2+}-IDA-agarose. Under the conditions used in this study digestion of the N + P-domain with factor Xa resulted in appearance of three major protein bands of molecular weight of 39,000, 31,000 and 26,000 (Fig. 3, lanes 2 and 3). A 26-kDa protein band corresponded to the recombinant GST. The identity of the 39- and 31-kDa protein bands was established by NH_2-terminal amino acid sequence analysis. The NH_2-terminal amino acid sequence of the 39-kDa protein band was E-P-A-I-Y-K-. This sequence is identical to the NH_2-terminal amino acid sequence of calreticulin [5] and identifies this protein band as the full length N + P-domain (Fig. 3, double arrow head). The NH_2-terminal amino acid sequence of the 31-kDa protein band was F-Y-A-L-S-A-. This sequence corresponds to amino acid 57 to 62 in the mature calreticulin [5] and is referred to as the truncated N + P-domain (Fig. 3, arrow head). It is not clear why factor Xa digestion of the N + P-domain produced the truncated N + P-domain. However, this may be due to a nonspecific cleavage by the factor Xa.

To identify if the N + P-domain binds Zn^{2+} a mixture of the N + P-domain, truncated N + P-domain and GST (Fig. 3, lanes 2 and 3) was separated by Zn^{2+}-IDA-agarose chromatography. Zn^{2+} binding proteins interact with Zn^{2+}-IDA-agarose and are eluted from the column with an increasing concentration of imidazole [21]. The 'flow-through' fractions from Zn^{2+}-IDA-agarose contain proteins that either do not bind Zn^{2+} or interact very weakly with Zn^{2+}. All three proteins (N + P-domain, truncated N + P-domain and GST) bound to the Zn^{2+}-IDA-agarose and were eluted with imidazole (Fig. 3, Bound fractions). The full length N + P-domain and the GST were the major proteins found in the 'bound' fractions. The major protein found in the 'flow-through' fractions (~70%) was the truncated N + P-domain suggesting that this domain (missing the NH_2-terminal 56 amino acid residues) interacted only weakly with the Zn^{2+}-IDA-agarose (Fig. 3, flow-through fractions, arrow head). Zn^{2+} binding to the N + P-domain was further investigated by $^{65}Zn^{2+}$ overlay. Fig. 4 shows that the GST-N-domain, the N-domain and the truncated N-domain all bound $^{65}Zn^{2+}$. We concluded that the N-domain of calreticulin binds Zn^{2+}. and that the NH_2-terminal portion of the N-domain may play an important role in Zn^{2+} binding to this region of calreticulin.

In order to establish a role of the P-domain in Zn^{2+} binding to calreticulin the GST-P-domain fusion protein was digested with factor Xa, purified by the glutathione-Sepharose 4B chromatography and subjected to Zn^{2+}-IDA-agarose chroma-

Fig. 3. Zn^{2+}-IDA Agarose Chromatography of N + P-domain of Calreticulin. N + P domain of calreticulin was expressed in *E. coli* and purified [19]. The protein was subjected to digestion with Factor Xa followed by Zn^{2+}-IDA-agarose chromatography as described in section 2. Unbound material ('flow-through' fractions), bound and eluted fractions ('bound' fractions) were separated in SDS-PAGE followed by staining with Coomassie blue. Lane 1, purified recombinant GST; lane 2 and 3, purified, factor Xa digested GST-N + P-domain fusion protein (sample loaded onto the Zn^{2+}-IDA-agarose); S, low molecular Bio-Rad protein standards (97-, 66-, 42-, 31-, 21-kDa). Double arrow head, full length N + P-domain. Arrow head, truncated N + P-domain. Small arrow indicate the position of GST.

tography. Fig. 5 shows that the P-domain of calreticulin did not bind to the Zn^{2+}-IDA-agarose and was found in the 'flow-through' fractions (Fig. 5, arrow head). The identity of the P-domain protein band in the 'flow-through' fractions was confirmed by the NH_2-terminal amino acid sequence analysis (data not shown). As expected the 26-kDa GST was found in the bound and eluted fractions (Fig. 5, small arrow). Based on the Zn^{2+}-IDA-agarose chromatography experiments we concluded that Zn^{2+} binding site of calreticulin are located to the N-domains but not to the P-domain of the protein.

The role of histidines in Zn^{2+} binding to calreticulin was investigated by DEPC modification of the protein. DEPC modifies histidine and to a much lesser extent tyrosine residues in proteins and converts them to *N*-carbethoxyhistidine and *O*-carbethoxytyrosine, respectively. *N*-Carbethoxyhistidine and *O*-carbethoxytyrosine are characterized by absorbance at 243 nm and 278 nm, respectively. No tyrosine residues were modified in the protein as there was no change of absorbance at 278 nm over the time course (data not shown). Fig. 6A shows the modification of calreticulin with DEPC as followed by the increase of the absorbance at 243 nm. Calreticulin contains 7 highly conserved histidine residues [8]. Based on the absorbance spectra in Fig. 5A it was estimated that 7 moles of histidine per mole of protein reacted with DEPC in the absence of Zn^{2+} but only two histidines reacted in the presence of Zn^{2+} (Fig. 6B). We concluded that Zn^{2+} protected 5 histidines residues from DEPC modification and that these 5 histidines became accessible to DEPC in the absence of Zn^{2+}.

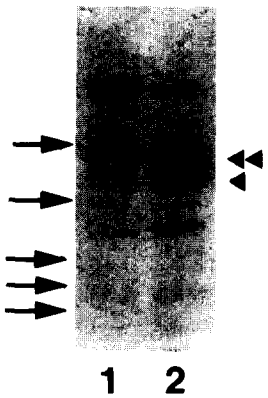

Fig. 4. $^{65}Zn^{2+}$ to the N-domain of Calreticulin. The N-domain of calreticulin was expressed in *E. coli*, purified and digested with factor Xa as described in section 2. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with 100 μM $^{65}Zn^{2+}$. Lane 1, purified GST-N-domain fusion protein; lane 2, N-domain digested with factor Xa. The protein bands were identified by N-terminal amino acid sequence analysis [24]. Double arrow head, GST. Arrow head, N-domain. The postion of molecular weight protein standards (29-, 20-, 14-, 6-, 3.5-kDa) is indicated by the small arrows.

4. Discussion

In this study we have expressed calreticulin and calreticulin domains in *E. coli* and identified a region in the protein responsible for Zn^{2+} binding. Native calreticulin binds Zn^{2+} with a relatively high affinity [21]. In order to define the location of Zn^{2+} binding sites in calreticulin we expressed different domains of the protein and followed by Zn^{2+}-IDA-agarose chromatography and $^{65}Zn^{2+}$ overlay, we have localized the high affinity Zn^{2+} binding site to the NH_2-terminal region of calreticulin (N-domain). A central P-domain of calreticulin did not bind any Zn^{2+} and did not interact with Zn^{2+}-IDA-agarose indicating that this domain is not involved in Zn^{2+} binding to the protein. The C-domain of calreticulin, a negatively charged region which binds Ca^{2+} with high capacity [19], was not used in these studies. This region of the protein bound nonspecifically a relatively large quantity of Zn^{2+} with a very low affinity (data not shown) suggesting that it is not physiologically relevant. GST fusion protein system is a useful system for analysis of ion binding to proteins and their domains. GST fusion protein approach was also utilized to identify and characterize Zn^{2+} binding to Raf-1 kinase [27] and to LIM homodomain protein Isl-2 [28].

One of the most important findings of this work is identification of a relatively high affinity Zn^{2+} binding site to the N-domain of calreticulin, the region of the protein that is not involved in Ca^{2+} binding [19]. We showed that the N + P-domain interacted with Zn^{2+}-IDA-agarose and that the N-domain

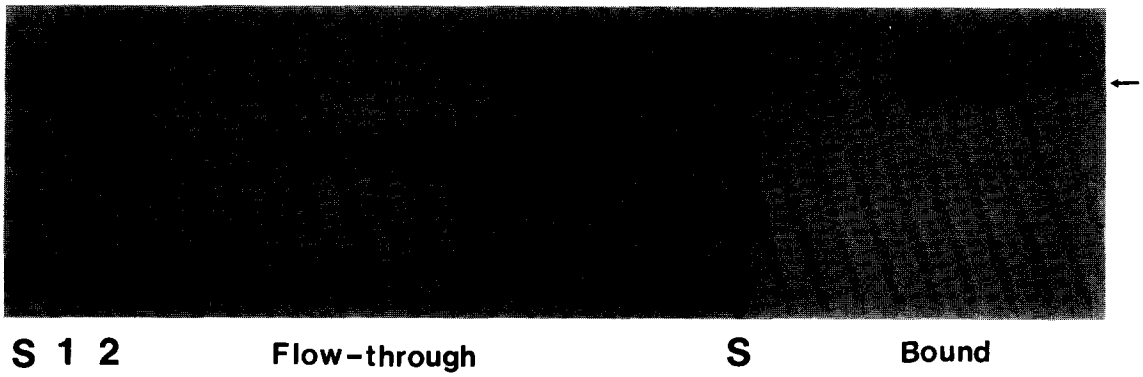

Fig. 5. Zn^{2+}-IDA Agarose Chromatography of P-domain of Calreticulin. The P domain of calreticulin was expressed in *E. coli*, purified and digested with factor Xa [18]. The digested protein was used for Zn^{2+}-IDA-agarose chromatography as described in section 2. 'Flow-through' fractions (unbound material) and 'bound' fractions (bound and eluted fractions) were analyzed by SDS-PAGE. Lane 1, purified recombinant GST; lane 2 and 3, purified, factor Xa digested GST-P-domain fusion protein (sample loaded onto the Zn^{2+}-IDA-agarose); S, molecular weight protein standards (29-, 20-, 14-, 6-, 3.5-kDa). Arrow head, P-domain. Small arrows indicate the position of GST.

bound $^{65}Zn^{2+}$ under overlay conditions. The N-domain of calreticulin does not have any consensus amino acid sequences such as 'Zn^{2+}-fingers' known to be associated with Zn^{2+} binding sites in proteins [1,2,29] making it difficult to predict precisely localization of the Zn^{2+} binding site(s) in the protein. It is well documented that Zn^{2+} is bound by multiple cysteine and histidine residues [1,2,29]. All three cysteines and 5 out of 7 histidines found in the mature protein are located in the N-domain of calreticulin [5]. Importantly, these amino acid residues are all conserved in calreticulin ranging from higher plants to the human protein [5]. The remaining two histidines are found in the P-domain of the protein, a region which does not bind Zn^{2+}. Two out of three cysteines found in calreticulin form a disulfide bridge (Cys^{120} and Cys^{146}) [30] and, therefore, they are unlikely involved in Zn^{2+} binding to the protein. Therefore, histidine residues in the N-domain may play an important role in Zn^{2+} binding to calreticulin. This is documented by two independent observations. First, the truncated N + P-domain (missing His^{25}) does not bind very well to the Zn^{2+}-IDA-agarose as compared to the full length N + P-domain suggesting that the His^{25} may be involved in Zn^{2+} binding to this domain. Second, histidine titration experiments documented that 5 out of 7 histidine residues found in calreticulin are protected from DEPC modification in the presence of Zn^{2+} indicating that these residues may potentially be involved in Zn^{2+} binding to the protein.

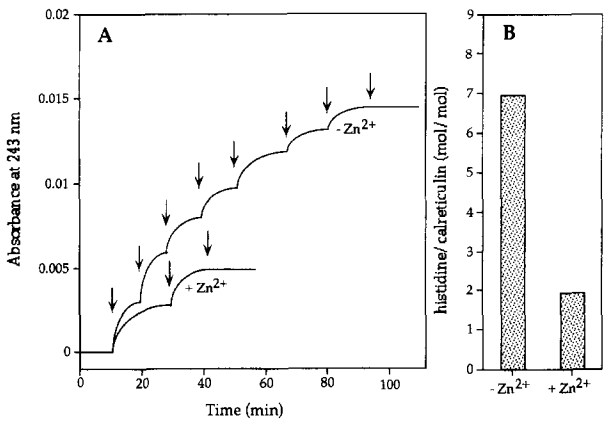

Fig. 6. DEPC Modification of Histidines in Calreticulin. DEPC titration of calreticulin was carried out as described in section 2. In A, the increase of absorbance at 243 nm indicates the progressive formation of *N*-carbethoxyhistidine. Successive additions of DEPC are indicated by arrows. In B, numbers of the DEPC modified histidines in calreticulin in the absence and in the presence of Zn^{2+}.

The N-domain of calreticulin is one of the most interesting regions of the protein. The amino acid sequence of the N-domain is unique to calreticulin and it is highly conserved among all calreticulins cloned so far [8]. This region of calreticulin interacts with the DNA binding domain of the glucocorticoid receptor leading to the modulation of the receptor function [9]. The N-domain of calreticulin may also interact with the cytoplasmic region of the α-subunit of integrin and modulate adhesion-dependent cell signaling [11]. Zn^{2+} may play an important role in the control of these protein-protein interactions.

Little is known about the physiological function of Zn^{2+}-binding proteins. Although Zn^{2+} is under homeostatic control, the intracellular distribution of free Zn^{2+} and its possible role in cellular functions is not well established. Increased intracellular concentrations of Zn^{2+} are found in the nucleus, in the synaptic vesicles [31], in the sarcoplasmic reticulum membrane subfractions [21] and in secretory granules of some cells [32]. These locations are also known to contain calreticulin [5]. Zn^{2+} plays an important role in the regulation of gene expression, action of some metaloenzymes [1,2], and the capsid formation of human immunodeficiency virus (HIV) [33]. Zn^{2+} has also been shown to block Ca^{2+} influx in some cells [34] and induce apoptosis in peripheral blood lymphocytes [35]. Interestingly expression of calreticulin is significantly induced in the stimulated peripheral blood lymphocytes [36]. Therefore, it is essential that Zn^{2+} homeostasis is under a precise control at the right place and at the right time. Calreticulin may play an important role in the regulation of the intracellular levels of Zn^{2+} and Ca^{2+}.

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Council Scientist and a Senior Scholar of the Alberta Heritage Foundation for Medical Research.

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