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Identification of the Ca^{2+}-Binding Domains in Reticulocalbin, an Endoplasmic Reticulum Resident Ca^{2+}-Binding Protein with Multiple EF-Hand Motifs[1]

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Reticulocalbin (RCN) is a member of the EF-hand Ca^{2+}-binding protein family and is a luminal protein of the endoplasmic reticulum (ER) with a molecular weight of 44,000 [Ozawa, M. and Muramatsu, T. (1993) *J. Biol. Chem.* 268, 699-705]. Although RCN has six repeats of a domain containing an EF-hand motif, the varying degrees of divergence of the amino acid sequences of these domains from the EF-hand consensus sequences suggested that some domains might have lost their Ca^{2+}-binding capability and adopted new functions. To identify the domains involved in Ca^{2+}-binding, discrete domains of RCN were expressed in *Escherichia coli*, using the glutathione *S*-transferase fusion protein system. $^{45}Ca^{2+}$ blot analysis of the resultant fusion proteins revealed that the first, fourth, fifth, and sixth domains bind Ca^{2+}, however, the second and third ones do not. The fusion proteins containing all six domains, and the first and second domains, respectively, showed Ca^{2+}-dependent increases in their electrophoretic mobilities, suggesting that Ca^{2+} induces a conformational change in reticulocalbin.

Key words: Ca^{2+}-binding protein, EF-hand protein, ER-resident protein, reticulocalbin.

Reticulocalbin (RCN), first described in mouse (*1*), is a luminal protein of the endoplasmic reticulum (ER) with a molecular weight of 44,000, and has six repeats of a domain containing a high affinity EF-hand Ca^{2+}-binding motif (*2, 3*). In addition to the EF-hand domains, RCN has an amino-terminal extension as well as a short carboxyl-terminal extension. The latter contains the sequence, His-Asp-Glu-Leu (HDEL), which serves as a signal retaining the protein in the ER of cells. The amino-terminal extension is composed of a leader peptide, which causes the protein to be located in the lumen of the ER, and the amino-terminal region of the mature protein of ~50 amino acids. Subsequent cloning of human RCN revealed that human and mouse RCN show a high degree of homology (95% identity) in amino acid sequence, indicating that this molecule has been evolutionarily conserved in mammals (*4*). As was found for mouse RCN, human RCN had six repeats of a domain containing an EF-hand motif. Interestingly, the conservation of the amino acid sequence was not restricted to the Ca^{2+}-binding motifs, consistent with the possibility that RCN plays some role(s) besides Ca^{2+}-binding. As was found for the mouse homologue, the human protein has the HDEL sequence at its carboxyl terminus instead of the Lys-Asp-Glu-Leu (KDEL) sequence, which is more common as a signal retaining resident proteins in the ER of animal cells (*5, 6*).

Although previous studies have provided compelling evidence for a role of reticulocalbin as a Ca^{2+}-binding protein, the work also raised a number of questions. For example, of the six EF-hand domains found in reticulocalbin, some may not bind Ca^{2+} as a result of amino acid substitutions. However, because of the highly hydrophilic nature of the sequences of the domains, they could potentially be located on the surface of the protein, and may be involved in some functions such as interaction with luminal or membrane target proteins in the ER. To begin to address these questions, we attempted to identify the domains that bind Ca^{2+} and those that do not. For this, a plasmid vector that allows expression of proteins as fusion proteins with the enzyme, glutathione *S*-transferase (GST), thereby enabling affinity purification under nondenaturing conditions (*7*), was used. This system provides sufficient amounts of specific domains of RCN to identify the domains involved in Ca^{2+}-binding to this protein. We have localized the Ca^{2+}-binding sites to domains I, IV, V, and VI. Importantly, although the second and third domains did bind Ca^{2+}, these two domains show a high degree of sequence identity between mouse and human RCN. This observation should provide important information concerning the structural requirements associated with the function of this novel Ca^{2+}-binding protein.

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Abbreviations: bp, base pair(s); ER, endoplasmic reticulum; GST, glutathione *S*-transferase; IPTG, isopropyl-1-thio-β-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; PBS, Dulbecco's phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RCN, reticulocalbin; SDS, sodium dodecylsulfate.

MATERIALS AND METHODS

Construction of Plasmids Encoding Different Fusion Proteins—For the expression and isolation of recombinant proteins, a GST fusion system including the pGEX-4T plasmid (Pharmacia) was used. This plasmid encodes GST followed by a thrombin cleavage site, unique cloning sites and a stop codon. In this study we have expressed this protein in six domains.

Plasmids containing domains I, II, III, IV, V, and VI of the protein, designated as pGEX-D1, pGEX-D2, pGEX-D3, pGEX-D4, pGEX-D5, and pGEX-D6, respectively, were generated by subcloning of cDNA restriction fragments (see Fig. 1). The *Stu*I-*Bgl*II DNA fragment (nucleotides 198-417) encodes domain I; the *Alu*I-*Eco*RI fragment (nucleotides 324-476) encodes domain II; the *Eco*RI-*Nde*I fragment (nucleotides 324-620) encodes domain III; the *Nde*I-*Bgl*II fragment (nucleotides 620-784) encodes domain IV; the *Acc*II-*Fsp*I fragment (nucleotides 703-856) encodes domain V; and the *Bam*HI-*Pst*I fragment (nucleotides 831-1052) encodes domain VI. The restriction fragments were purified by agarose gel electrophoresis and subcloned into the *Sma*I/*Sal*I site of an appropriate pGEX-4T vector. For this, the 5′ ends of the fragments with the protrusion were blunt-ended with T4 DNA polymerase using a DNA blunting kit (Takara), and the 3′ end of the fragments were linked to an oligonucleotide, CGATACCGTCGAC, after blunting. The oligonucleotide contains the recognition sequence for the *Sal*I restriction enzyme, therefore the addition of the oligonucleotide facilitated uni-directional subcloning of the fragments into the vector. The identity of each construct was assessed by nucleotide sequencing of the constructs using an oligonucleotide (GGGCTGGCAAGCCACGTTTGGTG), which anneals the 5′ regions of the multicloning sites of the pGEX vectors, as a sequencing primer.

Expression and Purification of Recombinant Proteins—GST fusion proteins were expressed in JM109 *Escherichia coli* as a host in LB medium containing 0.2% glucose and 50 μg/ml of ampicillin. Cultures were grown to the mid-log phase ($A_{600}=0.6$), followed by induction of the expression of fusion proteins with isopropyl-1-thio-β-galactopyranoside (IPTG) (final concentration, 0.3 mM) for 2 h. The cells were spun down at 3,500$\times g$ for 20 min, and the cell pellet was frozen at −20°C. After thawing, the pellet was resuspended in 20 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, 5 mM EDTA, 2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF), and then lysed by sonication followed by centrifugation at 10,000$\times g$ for 20 min. The supernatant containing the fusion proteins were used for single-step purification by glutathione-Sepharose 4B (Pharmacia) affinity chromatography. The extracts were applied onto glutathione-Sepharose columns (1 ml bed volume) equilibrated with PBS containing 0.1% Tween 20. After washing with 20 column volumes of the buffer and 10 column volumes of PBS, the fusion proteins were eluted from the affinity column with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM glutathione.

Biochemical Analysis—Immunoblotting was carried out as described previously (*1*). Calcium blotting was performed as described by Maruyama *et al.* (*8*). Alkaline urea gel electrophoresis was carried out as described by Head and Perry (*9*).

RESULTS

Expression and Purification of Recombinant Reticulocalbin and Its Domains—In order to identify specific domains in RCN that are responsible for Ca^{2+}-binding we utilized the fusion protein approach. By cDNA cloning, reticulocalbin has been shown to have six repeats of a domain containing an EF-hand motif. Figure 1 shows schematic representations of reticulocalbin and its domains expressed in *E. coli* as fusion proteins with GST. Above the scheme, its cDNA is represented by a thin line together with a map of the restriction enzymes used in the present study. The following domains were expressed as GST fusion proteins: domain I (D1), domain II (D2), domain III (D3), domain IV (D4), domain V (D5), domain VI (D6), domains I+II (D1+2), domains III+IV (D3+4), domains V+VI (D5+6), and domains I+II+III+IV+V+VI (D1-6).

GST fusion proteins containing domain I (GST-D1) or domain II (GST-D2) were expressed at relatively low levels in the bacterial cells after induction with IPTG as compared with GST fusion proteins without these domains (data not shown). This may reflect a toxic effect of the expressed fusion proteins. Upon lysis of the cells by sonication in the presence of Triton X-100, all the fusion proteins were found to be present in the high speed supernatant fraction. This is in contrast to many proteins expressed in *E. coli*, which are recovered in insoluble inclusion bodies. The solubility of the fusion proteins supports the suggestion that they might be properly folded within *E. coli*. Upon storage, however, the fusion protein D1-6 becomes insoluble and is not suitable for detailed analysis such as a quantitative calcium binding.

The GST fusion proteins purified by one-step affinity chromatography on glutathione-Sepharose 4B migrate as single prominent bands corresponding to the expected molecular weights with some degradation products (Fig. 2), except for the fusion proteins containing domain IV. For example, the GST-D4 fusion protein migrated on SDS-PAGE with a mobility corresponding to that of a polypeptide of 40.8 kDa. This mobility does not correspond to the predicted size of the fusion protein (32.5 kDa; 6.5-kDa

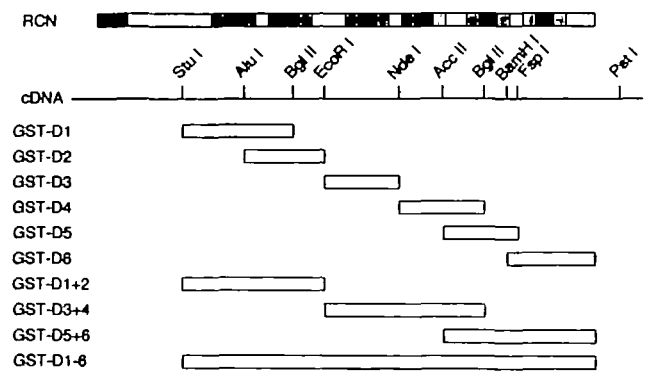

Fig. 1. **Schematic representations of reticulocalbin and its domains expressed in *Escherichia coli* as fusion proteins with GST.** Loops are filled, helices are stippled, and linker regions are unshaded. The signal sequence is hatched. Above the scheme, its cDNA is represented by a thin line together with a map of the restriction enzymes used in the present study.

D4+26-kDa recombinant GST). The anomalous mobility of the fusion proteins containing domain IV is reminiscent of the anomalous mobility of native RCN on SDS-PAGE (44 kDa on Laemmli SDS-PAGE *versus* a molecular weight of 38,112 predicted for the protein from its amino acid sequence) (*1*).

Identification of the Ca^{2+}-Binding Domains in Reticulocalbin—To determine which domains are responsible for the Ca^{2+}-binding of RCN, GST fusion proteins were subjected to $^{45}Ca^{2+}$ blot analysis. As shown in Fig. 3, the GST-D1, GST-D4, GST-D5, GST-D6, GST-D1+2, GST-D3+4, GST-D5+6, and GST-D1-6 all bound $^{45}Ca^{2+}$. These fusion proteins were also stained blue with Stains-all, further confirming their involvement in Ca^{2+} binding to the protein (data not shown). In contrast, fusion proteins GST-D2 and GST-D3 did not bind detectable amounts of $^{45}Ca^{2+}$, and were not stained blue with Stains-all. Among the fusion proteins that bound $^{45}Ca^{2+}$, the fusion proteins containing domain IV (GST-D4, GST-D3+4, and GST-D1-6) gave a stronger $^{45}Ca^{2+}$-binding signal than those without this domain.

Ca^{2+} Induces a Conformational Change in Reticulocalbin—As a preliminary attempt to determine how Ca^{2+} induces a conformational change in reticulocalbin, recombinant reticulocalbin was subjected to SDS-PAGE in the presence of Ca^{2+} or EGTA. As shown in Fig. 4, GST-D1-6 migrates a bit faster in the presence of Ca^{2+} as compared to in its absence. Although fusion proteins GST-D3+4 and GST-D5+6 did not show any significant change in electrophoretic mobility, fusion protein GST-D1+2 did (Fig. 4). The fusion proteins with each of the single domains also showed no change in the electrophoretic mobility (data not

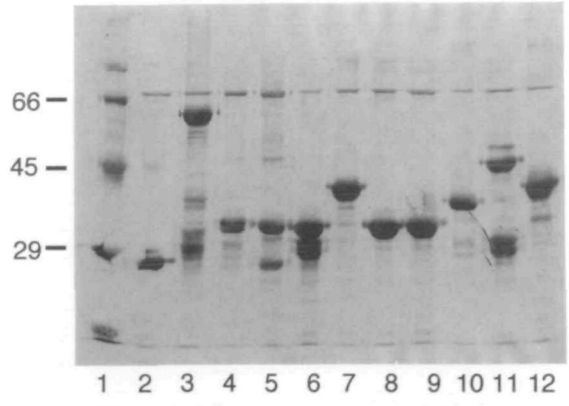

Fig. 2. **SDS-PAGE analysis of the fusion proteins with distinct domains of reticulocalbin.** The GST fusion proteins were purified by one-step affinity chromatography on glutathione–Sepharose 4B, and then subjected to SDS-PAGE. After electrophoresis the proteins were transferred to a nitrocellulose membrane, subjected to $^{45}Ca^{2+}$ blot analysis (shown in Fig. 3), and then stained with Amido Black. 1, Molecular weight markers; 2, GST; 3, GST-D1-6; 4, GST-D1; 5, GST-D2; 6, GST-D3; 7, GST-D4; 8, GST-D5; 9, GST-D6; 10, GST-D1+2; 11, GST-D3+4; 12, GST-D5+6.

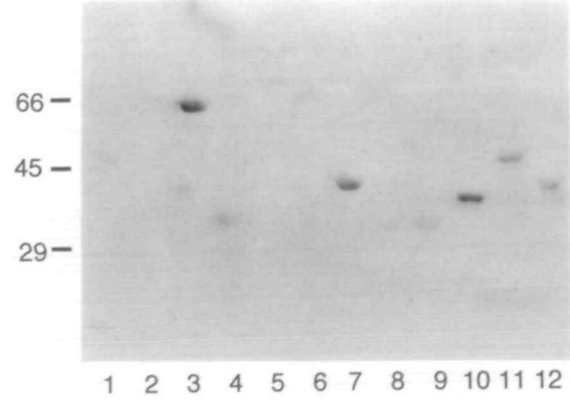

Fig. 3. **Calcium blots of the GST fusion proteins with the distinct domains of reticulocalbin.** The purified fusion proteins were electrophoresed and then transferred to a nitrocellulose membrane. After incubation with $^{45}Ca^{2+}$, the membrane was exposed to X-ray film. The same membrane was stained with Amido Black to detect proteins (shown in Fig. 2). Note that Ca^{2+} did not bind to GST alone.

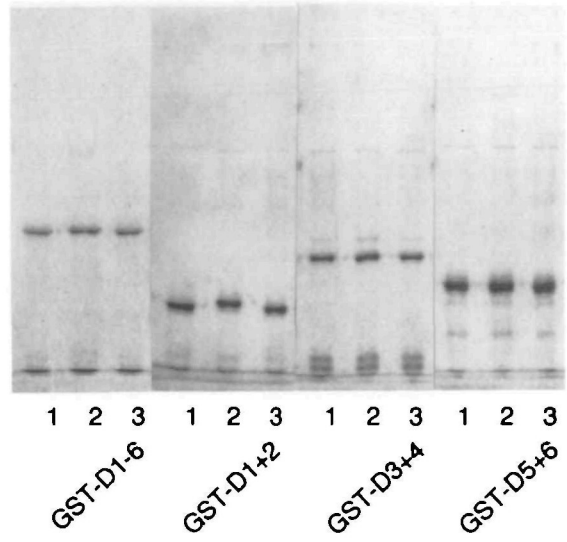

Fig. 4. **SDS-PAGE analysis of the conformational change in the recombinant reticulocalbin induced by Ca^{2+}.** Purified GST-D1-6, GST-D1+2, GST-D3+4, and GST-D5+6 were mixed with stock solutions of $CaCl_2$ (lanes 1 and 3) or EGTA (lane 2) to give a final concentration of 5 mM, and then mixed with an equal volume of 2×SDS sample buffer. After electrophoresis the gels were stained with Coomassie Brilliant Blue.

Test	EL LL L	O O OG LO O	L LL L	Ca^{2+}-binding
Domain I	RLGKIVDRI	DSDGDGLVTTEE	LKLWIKRV	+
II	NVAKVWKDY	DRDKDEKISWEE	YKQATYGY	-
III	RDERRFKAS	DLDGDLTATREE	FTAFLHPE	-
IV	VVLETLEDI	DKNGDGFVDQDE	YIADMFSH	+
V	EREQFNDFR	DLNKDGKLDKDE	IRHWILPQ	+
VI	EARHLVYES	DKNKDEMLTKEE	ILDNWNMF	+

Fig. 5. **Ca^{2+}-binding domains of reticulocalbin.** The protein sequence (*1*) was analyzed for homology to the test sequence for a Ca^{2+}-binding EF-hand motif. The amino acid sequence is shown in a one-letter code. In the test sequence, L represents a hydrophobic residue, and O an oxygen-containing residue. On the right, the results of $^{45}Ca^{2+}$ blot analysis are shown. (+) denotes $^{45}Ca^{2+}$ binding whereas (−) denotes non-binding.

shown). With analysis by electrophoresis on alkaline urea gels (8% polyacrylamide gel in 8-M urea at pH 8.5) (*9*) in the presence of Ca^{2+} or EGTA, however, we could not detect any significant changes in the electrophoretic mobility of the fusion proteins (data not shown).

DISCUSSION

In this report we describe the expression of different domains of RCN in *E. coli* and identified the domains involved in Ca^{2+}-binding. Using the $^{45}Ca^{2+}$ overlay technique we found that domains I, IV, V, and VI bind Ca^{2+}, whereas domains II and III do not. It is possible that the latter two domains might bind Ca^{2+} but not after SDS-PAGE and transfer to a nitrocellulose membrane. The results are, however, consistent, in part, with our previous assumption that domain II may not bind Ca^{2+} because of the replacement of the conserved glycine with glutamic acid in the domain (Fig. 5), which was predicted to cause the formation of an α-helix at the site instead of a loop structure (*10*). For the same reason, we postulated that domain VI may not bind Ca^{2+}. Domain VI, however, bound Ca^{2+}. In domain III, this glycine was replaced by leucine. Since the replacement was thought not to affect the formation of the loop structure, we thought that the domain is capable of binding Ca^{2+}. Furthermore, replacement of the glycine residue with lysine has been reported in the EF-hand domains of the fibrinogen γ-chain (*11*), and secreted protein acidic and rich in cysteine (*12*). Our results, however, showed that domain III did not bind Ca^{2+} under the conditions used.

Our preliminary analysis suggests that reticulocalbin undergoes a conformational change upon Ca^{2+} binding. The fusion protein consisting of the first and second domains also showed a change in conformation, as judged from the change in electrophoretic mobility. Since the conformational change detected as a change in electrophoretic mobility should be a gross one, it will be interesting to determine what kinds of changes in the structure of reticulocalbin are induced by Ca^{2+} using physicochemical methods.

Although reticulocalbin shows similarity to calbindin D28 and calretinin, in that all of these proteins have six EF-hand domains, the former differs from the latter two proteins in several respects. Firstly, reticulocalbin is a luminal protein of the ER, whereas calbindin D28 (*13-15*) and calreticulin (*16, 17*) are cytosolic proteins. Consistent with its different intracellular distribution, reticulocalbin has a long amino-terminal extension as well as a short carboxy-terminal extension. The amino-terminal extension is composed of a leader sequence, which directs translocation of the protein into the lumen of the ER, and the amino-terminal region of the mature protein of ~50 amino acids, whose function is at present unknown. The latter has an HDEL sequence, which serves as a retention signal for the protein in the ER. Besides these structural differences at the protein level, the gene has been found to be encoded on six separate exons (*18*), which is different from in the case of calbindin D28, whose gene is divided into 11 exons (*19*). Furthermore, of the five introns in the reticulocalbin gene, three are mapped at positions different from those in the calbindin gene. The data presented in this paper also provide additional evidence that RCN differs from calbindin D28. Calbindin D28 has been shown to bind four Ca^{2+} atoms/mol of protein, and the second and sixth domains have been assumed to have lost their Ca^{2+} binding ability because some oxygen-containing amino acids in the loop are missing (*14*). In the case of RCN, as shown in this paper, the second and third domains have lost the Ca^{2+} binding ability.

Lastly, a cDNA clone encoding an ER protein termed ERC-55 (55 kDa endoplasmic reticulum calcium-binding protein) has recently been isolated (*20*). The cDNA of ERC-55 is predicted to encode a protein of 317 amino acids, which is similar to the number of amino acids (325 residues) in reticulocalbin. Like reticulocalbin, ERC-55 has six copies of the EF-hand motif. ERC-55 also possesses the carboxyl-terminal HDEL tetrapeptide found in reticulocalbin. Thus, ERC-55 has many of the structural features found in reticulocalbin, although the two proteins show a low degree of homology (35% identity). Therefore it may be interesting to determine whether all the six EF-hand domains have the ability to bind Ca^{2+} or, like RCN, some of them have lost this ability.

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