Reticulocalbin, a Novel Endoplasmic Reticulum Resident Ca^{2+}-binding Protein with Multiple EF-hand Motifs and a Carboxyl-terminal HDEL Sequence*

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A novel Ca^{2+}-binding protein, tentatively designated reticulocalbin, has been identified and characterized. Reticulocalbin is a luminal protein of the endoplasmic reticulum with an M_r of 44,000 as revealed by biochemical analysis and immunofluorescence staining. The cDNA of reticulocalbin encodes a protein of 325 amino acids with an amino-terminal signal sequence of 20 amino acids. The protein has six repeats of a domain containing the high affinity Ca^{2+}-binding motif, the EF-hand. Although oxygen-containing amino acids important for the positioning of Ca^{2+} are conserved in all six domains, the conserved glycine residues in the central portion of the EF-hand motif are absent in three of them. Calcium blots showed that recombinant reticulocalbin expressed in bacterial cells binds Ca^{2+}. The protein has the sequence His-Asp-Glu-Leu (HDEL) at its carboxyl terminus. This is similar to the Lys-Asp-Glu-Leu (KDEL) sequence, which serves as a signal to retain the resident proteins in the endoplasmic reticulum of animal cells. A mutant protein lacking the HDEL sequence produced by in vitro mutagenesis has been shown to be secreted into medium in transient expression assays.

Ca^{2+} is involved in the regulation of various cellular activities such as contraction, secretion, and mitogenesis. These events are largely mediated by a family of homologous Ca^{2+}-binding proteins including calmodulin, troponin C, calbindins, and S-100 proteins. These proteins exhibit a common structural motif, the EF-hand (Kretsinger, 1980), which is present in multiple copies (calmodulin and troponin C have four and S-100 proteins have two copies per molecule) and binds Ca^{2+} selectively, with high affinity. Each of these consists of a loop of 12 amino acids that is flanked by two α-helices (Strynadka and James, 1989; Heizman and Hunziker, 1991). Upon Ca^{2+} binding, these proteins undergo a conformational change and consequently interact with their target proteins.

The luminal of the endoplasmic reticulum (ER)† (endoplasm) contains a number of soluble proteins including immunoglobulin heavy chain-binding protein (GRP78) (Munro and Pelham, 1986; Bole et al., 1986), protein disulfide-isomerase (Freedman, 1989; Edman et al., 1985), and GRP94 (endo- plasmin, ERP99) (Mazzarella and Green, 1987; Sorger and Pelham, 1987). Many of these proteins are involved in the initial steps of the maturation of newly synthesized secretory proteins such as folding of nascent polypeptide chains and formation of the correct disulfide bonds. Retention of these resident proteins in the ER is dependent on a carboxyl-terminal signal, which in animal cells is usually Lys-Asp-Glu-Leu (KDEL). The KDEL sequence is recognized by a membrane-bound receptor that continually retrieves the proteins from a later compartment (cis-Golgi cisternae) of the secretory pathway and returns them to the ER (Pelham, 1989, 1990). Although the above-mentioned proteins (immunoglobulin heavy chain-binding protein, protein disulfide-isomerase, and GRP94) reportedly bind Ca^{2+} (Macer and Koch, 1988), none of them have the EF-hand motifs.

Calreticulin, a well-characterized Ca^{2+}-binding protein of the ER and the sarcoplasmic reticulum, binds Ca^{2+} with high affinity, but does not have an EF-hand motif (Smith and Koch, 1989; Fliegel et al., 1989). Calreticulin may be a nonmuscle functional analogue of calsequestrin, a major Ca^{2+}-binding (storage) protein of the skeletal muscle sarcoplasmic reticulum membrane (Milner et al., 1991). So far, there have been no reports of ER resident proteins having EF-hand motifs.

We previously isolated several independent cDNA clones from λgt11 libraries of mouse teratocarcinoma OTT6050 (Ozawa et al., 1988; Furukawa et al., 1990). These clones were isolated by screening the libraries with antibodies against Dolichos biflorus agglutinin-binding glycoproteins. The lectin D. biflorus agglutinin is known to bind specifically with the nonreducing terminal N-acetylglactosamine of carbohydrate chains (Etzler, 1972). Although the majority of the clones showed developmentally regulated expression, others did not, suggesting that the latter are products of housekeeping genes. Sequencing a group of cDNA clones that belong to the latter category and characterizing the protein encoded by these clones revealed that the protein is an ER resident Ca^{2+}-binding protein with multiple EF-hand motifs for which we propose the nomenclature reticulocalbin.

MATERIALS AND METHODS
cDNA Cloning—cDNA clones O1, O9, and O32 were isolated from a λgt11 cDNA library of mouse teratocarcinoma OTT6050 (Ozawa et al., 1988) by screening with antibodies against D. biflorus agglutinin-binding glycoproteins as previously described (Ozawa et al., 1988). Clones M10 and M22 were obtained from a primer extension cDNA library in λgt10 and subcloned into pUC18 and Bluescript KS(+)
Construction of Expression Vectors—To express reticulocalbin in animal cells, the cDNA was cloned into a mammalian expression vector, pCAGGS, which contains an enhancer derived from cytomegalo-
uirus promoter (Niwa et al., 1991). The Bluescript KS(+) vector containing the 1052-bp 5'-fragment of reticulocalbin cDNA in the EcoRI-PstI site was restricted with Smal and HindII. The fragment was isolated and cloned into pCAGGS, which had been digested with EcoRI and filled in with T4 DNA polymerase. The orientation of the cDNA in the vector was confirmed by restriction enzyme digestion. We constructed an expression vector encoding a mutant reticulocalbin lacking the carboxyl-terminal HDEL peptide by polymerase chain reaction (PCR). Oligonucleotides CAGCTCGGA- CGAAGACGG and CCTGCAGTCAATTTTTGGTCAGGTCTTCC were synthesized and used as primers. The former corresponds to the sequence of the 3'-BamHI-PstI fragment, the 5' region was cloned into the SmaI-PstI site of Bluescript KS(+). After confirming the sequence of the PCR product was purified on agarose gel, digested with PstI, and the sequence was confirmed. A 959-bp fragment was purified from pCAGGS and replaced with the authentic fragment and cloned into the cDNA vector as described above.

To express recombinant reticulocalbin in Escherichia coli, the cDNA encoding the mature protein was cloned into the maltose-binding protein fusion vector (pMAL-c) (New England BioLabs, Inc.) with the 5' extension of CAG, which, together with CTG, was inserted into the sequence of the 3'-BamHI-PstI fragment, the 5' fragment was cloned into the SmaI-PstI site of Bluescript KS(+). After confirming the sequence of the 3'-BamHI-PstI fragment, the 5' region was replaced with the authentic fragment and cloned into the pCAGGS vector described above.

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Purification of Recombinant Reticulocalbin—Bacterial cells containing the fusion plasmid were cultured, and the fusion protein was induced and collected according to the manufacturer’s instructions (New England BioLabs, Inc.). The cells were disrupted by sonication in PBS containing 1 mM CaCl2 and 1 mM phenylmethylsulfonyl fluoride and then centrifuged. The supernatant was applied to a column of amylose resin and washed with 5-10 column volumes of the following buffers: PBS containing 10% N,N-dimethylformamide as a control.

Antibodies—Antibodies were raised against D. biflorus agglutinin-binding glycoproteins from teratocarcinoma OTT6050 as described (Ozawa et al., 1982). Monospecific antibodies against reticulocalbin were prepared as controls. The recombinant reticulocalbin of the MBP fusion protein was used as a control. Monospecific antibodies against reticulocalbin were prepared as described above. Some experiments, antibodies eluted from MBP were used as a control.

Immunofluorescence—Cells on coverslips were washed with PBS containing 0.2 mM CaCl2 and 0.2 mM MgCl2 and fixed with 3% formaldehyde in PBS for 15 min at room temperature. After washing with PBS and incubation with 50 mM NH4Cl in PBS, cells were permeabilized by incubating with 0.1% Triton X-100 in PBS for 5 min. Cells were repreincubated with a mixture (1:1) of PBS and Dulbecco’s modified Eagle’s medium with 10% fetal cell serum for 15 min and incubated sequentially with anti-reticulocalbin antibodies and goat anti-rabbit antibodies conjugated with fluorescein isothiocyanate (Jackson Laboratories) in the same solution for 30 min. For double staining, biotinylated ConA and rhodamine-labeled avidin (EY Laboratories) were included in the solutions containing anti-reticu-

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Isolation of Reticulocalbin cDNA Clones—Fig. 1 shows the scheme of isolated reticulocalbin cDNA clones. Clones O1, O9, and O32 were isolated from a cDNA library in λgt11 constructed from mouse teratocarcinoma OTT6050 cDNA by screening with antibodies against D. biflorus agglutinin-binding glycoproteins of the cells. These clones provide the 3'-sequence to the internal EcoRI site at nucleotide 476, but they did not extend further because EcoRI sites were not methylated during construction of the library. Clone O32-8 was obtained by screening a λgt11 library constructed with a cDNA whose internal EcoRI sites were protected with EcoRI methylase, by plaque hybridization using the EcoRI-BamHI fragment of clone O32 as a probe. To isolate clones M10 and M22, we used an oligonucleotide (30-mer) corresponding to nucleotides 625-655 of reticulocalbin cDNA as a primer for construction of another cDNA library. This library in λgt10 was screened by plaque hybridization using the 144-bp EcoRI-NdeI fragment of clone O32 as a probe. Northern hybridization of RNAs from teratocarcinoma OTT6050, embryonal...
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carcinoma cell line F9, and parietal endodermal cell line PYS-2 revealed that the cDNA clones hybridized a major mRNA species of 2.3 kilobase pairs and a minor species of 2.0 kilobase pairs (data not shown).

The composite nucleotide sequence of the cDNA clones and the deduced amino acid sequence of reticulocalbin are presented in Fig. 2. The nucleotide sequence contains 34 bp of the 5'-untranslated region and a 3'-untranslated region of 1016 bp. The coding region specifies a protein of 225 residues with an M₆ of 38,112. The amino acid sequence of the first 20 amino acids of reticulocalbin has the typical features of a secretory leader peptide. There is a positively charged amino-terminal region and a hydrophobic central section followed by two small nonpolar residues with a single residue between them. This latter feature is thought to determine the cleavage point of the signal peptidase, which in this case could be between Ala-20 and Leu-21. Cleavage of this peptide would yield a mature protein of 205 residues with a predicted M₆ of 36,200. The sequence of reticulocalbin does not contain a hydrophobic transmembrane segment, which suggests transfer of the entire protein into the lumen of the ER. There is one consensus site for N-linked glycosylation and a number of serine- or threonine-rich regions that could be O-glycosylated.

Immunoblots of PYS-2 cells with antibodies against reticulocalbin revealed a band of 44 kDa and a faint band of 46 kDa (Fig. 3). The same bands were also detected in mouse fibroblast L cells (data not shown). The 46-kDa species binds to concanavalin A-Sepharose, whereas the 44-kDa species does not (Fig. 3). Neither of the two proteins bound to R. communis agglutinin-agarose, which recognizes terminal or sialylated Galβ1-4GlcNAc sequences (Baenzinger and Fiete, 1979). Therefore, the 46-kDa species may be produced by N-glycosylation (high-mannose type) of the 44-kDa species at residue 47. To confirm that the cDNA codes the 44-kDa protein, the cDNA was cloned into an expression vector (pCAGG/Sneo) and introduced into COS cells. Upon immunoblot analysis, COS cells transfected with the cDNA in a sense orientation showed a 44-kDa band and a faint 46-kDa band, whereas COS cells transfected with the cDNA in an antisense orientation gave faint bands in the region (Fig. 4). We consider that the faint bands (46 and 44 kDa) represent endogenous reticulocalbin in COS cells. Even though the 44-kDa band is present in COS cells transfected with antisense cDNA, the intensity of the band greatly increased in COS cells transfected with sense cDNA. Therefore, we conclude...
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**FIG. 3. Immunoblot analysis of reticulocalbin.** PYS-2 cells were boiled in SDS-PAGE sample buffer (lane 1) or were lysed with Nonidet P-40 and fractionated on ConA-Sepharose or R. communis agglutinin (RCA)-agarose (lanes 2 and 3). Bound materials specifically eluted with the respective haptenic sugars and unbound materials (lanes 2 and 4), and bound materials (lanes 3 and 5) were immunoblotted with anti-reticulocalbin antibodies.

**FIG. 4. Immunoblots of reticulocalbin transiently expressed in COS cells.** PYS-2 (positive control) (lane 1) and COS cells transfected with reticulocalbin cDNA in an expression vector in a sense (lane 2) or antisense (lane 3) orientation were boiled in SDS-PAGE sample buffer and immunoblotted with anti-reticulocalbin antibodies. The antibodies recognized endogenous COS cell reticulocalbin in the immunoblots, but not in immunofluorescence and immunoprecipitation (compare with Figs. 8 and 9); probably the conformational epitopes detectable in these assays are species-specific.

that the cDNA clone has the entire sequence encoding the 44-kDa protein.

**Reticulocalbin Is a Ca"-binding Protein with EF-hand Motifs**—Analysis of the cDNA sequence shows that the predicted protein contains six repeats of ~30 amino acids (Fig. 5). Each repeat has the general feature of a high affinity Ca"-binding EF-hand domain according to Kretsinger's rule (Kretsinger, 1980). Comparing the amino acid sequence of these domains with those required for a perfect EF-hand Ca"-binding site, there is a varying degree of divergence. The 5 oxygen-containing residues important for the coordination of Ca" are present in all the predicted sites. The central glycine is conserved in sites I, IV, and V, whereas in sites II, III, and VI, it is replaced either by glutamic acid or leucine. The replacements appear not to be cloning artifacts because the independent cDNA clones have the same sequence in the region. Furthermore, the sequence of the corresponding region of genomic DNA clones also has the same sequence. The secondary structure prediction (Chou and Fasman, 1978) shows that the replacement of glycine with glutamic acid in sites II and VI causes the formation of an a-helix at the sites instead of a loop structure. Therefore, sites II and VI of reticulocalbin probably no longer bind Ca". Although the glycine is replaced by leucine, site III appears capable of forming the loop structure. The replacement of the normally conserved glycine with lysine has been reported in the EF-hands of the fibrinogen g-chain (Dang et al., 1985) and secreted protein acidic and rich in cysteine (BM-40, osteonectin) (Engel et al., 1987). Therefore, site III seems to bind Ca".

To test whether reticulocalbin actually binds Ca", recombinant reticulocalbin was analyzed by 45Ca" blotting. The cDNA coding mature reticulocalbin was cloned into the MBP vector (pMAL-c), and the recombinant protein was expressed in E. coli as a fusion protein with MBP. After purification by affinity chromatography on a column containing amylose resin, the fusion protein was electrophoresed on a gel, transferred to a nitrocellulose membrane, and incubated with Ca". MBP alone did not bind Ca"; however, the fusion protein did (Fig. 6).

**Reticulocalbin Is a Luminal ER Protein**—After homogenization of PYS-2 cells, reticulocalbin remained in the low speed (5000 x g) supernatant, but sedimented together with crude membranes during high speed (100,000 x g) centrifugation (Fig. 7A). It was solubilized from the membrane by Triton X-100 or by sonication in the absence of detergents (Fig. 7A). Reticulocalbin partitioned into the aqueous phase when cells

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M. Ozawa, unpublished results.
Reticulocalbin is a microsomal protein with a hydrophobic nature. A, PYS-2 cells were homogenized and fractionated by differential centrifugation into nuclear (N lane 1), mitochondrial (Mit lane 2), microsomal (Ms lane 3), and soluble (Sok lane 4) fractions and immunoblotted. Reticulocalbin in the microsomal fraction was solubilized with Triton X-100 (lane 5) or released by sonication (Sonic lane 6). B, cells were extracted with Triton X-114, and the extract (lane 1) was analyzed by phase separation. Reticulocalbin was found in the aqueous (aque.) phase (lane 2), and not in the detergent (deter.) phase (lane 3).

were extracted with Triton X-114 and phase-separated (Fig. 7B).

To localize reticulocalbin, PYS-2 cells were stained with anti-reticulocalbin antibodies by indirect immunofluorescence. When the cells were fixed with formaldehyde and permeabilized with Triton X-100, the intracellular ER regions (but not the cell surface) were stained (Fig. 8A). Fig. 8A shows that reticulocalbin is localized in the perinuclear system of membranes corresponding to that of the ER of PYS-2 cells. Since the ER seemed not to be well developed in PYS-2 cells, we stained transfected COS cells expressing reticulocalbin. Untransfected cells were not detectably stained with the antibody (data not shown). All cells expressing reticulocalbin exhibited prominent staining of the perinuclear region as well as a lattice of fine tubular structures (Fig. 8C), but there was no obvious staining of the Golgi apparatus. This pattern is typical of that obtained for proteins that are retained in the ER (Munro and Pelham, 1987). Furthermore, the structure that contained reticulocalbin also stained with concanavalin A (Fig. 8D), which predominantly stains ER structures (Tartakoff and Vassalli, 1983), confirming that they represented the ER. Because of the bright staining in the perinuclear region, however, it was difficult to get a clearly visible pattern of the tubule network. We overcame this issue using a confocal scanning laser microscope. Fig. 8E shows the distribution of reticulocalbin in the transfected COS cells. The image confirms the results obtained using conventional microscopy and allows detailed examination of the distribution of the protein. There is an intense reticular staining of the ER.

Reticulocalbin has a putative amino-terminal signal peptide, but no hydrophobic transmembrane segment, which is consistent with transfer of the entire protein into the lumen of the ER. To verify that the amino-terminal stretch of hydrophobic amino acids functions as a signal sequence, we carried out the experiments described below. The rationale is as follows. Luminal ER proteins in animal cells are prevented from being secreted by a sorting system that recognizes the carboxyl-terminal sequence KDEL (Munro and Pelham, 1987). Instead of KDEL at the carboxyl terminus, reticulocalbin has the closely related sequence HDEL. Although the HDEL sequence has been reported to be inefficient as an ER retention signal in animal cells because a lysosome fusion protein with the HDEL sequence at the carboxyl terminus was efficiently secreted into the medium (Pelham et al., 1988), recent experiments on liver carboxylesterases have demonstrated that the sequence is functional at least in this case (Robbi and Beaufay, 1991). Therefore, the removal of the carboxyl-terminal HDEL sequence could allow the mutant protein to escape from the retrieval machinery and to be secreted into the medium if the amino-terminal sequence is the signal sequence. For this, a mutant protein specifically lacking the HDEL sequence was constructed. The synthesis and secretion of the wild-type and mutant reticulocalbins were evaluated by transient expression in COS cells (Fig. 9). After labeling with [35S]methionine for 30 min and a chase (3 h) with excess unlabeled methionine, proteins were immuno- precipitated from the cell lysates and from media and then analyzed by SDS-PAGE. Under these conditions, wild-type reticulocalbin was secreted very slowly from the COS cells. Less than 10% of the pulse-labeled protein was found in the medium. Reticulocalbin lacking the HDEL sequence, however, was secreted rapidly from the COS cells (Fig. 9). Denaturation of the fluorographs showed that ~80% of the mutant protein was recovered from the medium. No reticulocalbin was secreted from PYS-2 cells under the same conditions (data not shown). These results show that the amino-terminal

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and mutant reticulocalbin lacking carboxyl-terminal HDEL peptide. COS cells transfected with wild-type (wt) (lanes 1 and 4) or mutant (ΔHDEL) (lanes 2 and 5) reticulocalbin cDNA in an expression vector or the vector without the cDNA (control (con)) (lanes 3 and 6) were labeled with [35S]methionine for 30 min. After a chase for 2 h with excess unlabeled methionine, the cells and media were collected. The cell lysates (lanes 1–3) and media (lanes 4–6) were incubated with anti-reticulocalbin antibodies, and immunocomplexes were analyzed by SDS-PAGE.

![Immunoprecipitation of wild-type reticulocalbin and mutant reticulocalbin lacking carboxyl-terminal HDEL peptide](image)

**Fig. 9.** Immunoprecipitation of wild-type reticulocalbin and mutant reticulocalbin lacking carboxyl-terminal HDEL peptide. COS cells transfected with wild-type (wt) (lanes 1 and 4) or mutant (ΔHDEL) (lanes 2 and 5) reticulocalbin cDNA in an expression vector or the vector without the cDNA (control (con)) (lanes 3 and 6) were labeled with [35S]methionine for 30 min. After a chase for 2 h with excess unlabeled methionine, the cells and media were collected. The cell lysates (lanes 1–3) and media (lanes 4–6) were incubated with anti-reticulocalbin antibodies, and immunocomplexes were analyzed by SDS-PAGE.

**FIG. 10.** Domain structure of reticulocalbin and calbindin D28. The filled boxes represent the sequences corresponding to the loop of the EF-hand structure. The consensus N-glycosylation site is marked by CHO. The evidence presented here demonstrates that reticulocalbin is a luminal Ca²⁺-binding protein residing in the ER. The sequence of the cDNA clone showed that it has a secretory leader peptide that is not present on the mature protein. This leader sequence has functional coat that it causes reticulocalbin to enter the ER, as shown by its glycosylation, and to be secreted into the medium since a mutant reticulocalbin lacking the carboxyl-terminal HDEL sequence was secreted in COS cells. Mature reticulocalbin does not contain a hydrophobic transmembrane sequence, and there is no evidence for its secretion from the cells; it thus appears likely that it accumulates as a soluble protein within the intracellular membrane system. Consistent with this is the finding that reticulocalbin cosediments with crude membranes (microsomes) and can be released from the membranes with detergents or by sonication. Upon phase separation in Triton X-114, reticulocalbin partitioned into the aqueous phase. Furthermore, anti-reticulocalbin antibody stained endogenous reticulocalbin in PYS-2 cells as well as transiently expressed reticulocalbin in COS cells with an ER-like pattern.

**Fig. 10.** Domain structure of reticulocalbin and calbindin D28. The filled boxes represent the sequences corresponding to the loop of the EF-hand structure. The consensus N-glycosylation site is marked by CHO.

hydrophobic amino acids of reticulocalbin function as a signal for the transfer of the entire protein into the lumen of the ER.

**DISCUSSION**

We identified and characterized a novel ER resident Ca²⁺-binding protein called reticulocalbin by cDNA cloning, sequence analysis, and biochemical as well as cell biological studies. The major structural features of reticulocalbin, as deduced from the cDNA sequence, are outlined in Fig. 10. The protein consists of 325 amino acids with a single hydrophobic sequence at the amino terminus that constitutes a leader sequence. At the amino-terminal region of the mature protein, there is a potential N-glycosylation site, which was indeed partially glycosylated. The carboxyl terminus contains a version of the KDEL ER retention signal, HDEL. The rest of the protein consists of the six domains of the EF-hand motif of high affinity Ca²⁺-binding proteins.

The most interesting feature of the sequence of reticulocalbin is the presence of the domains of the EF-hand motif. Therefore, reticulocalbin can be classified into the EF-hand calcium-binding protein superfamily, which includes calmodulin, troponin C, and myosin light chain. All members of this diverse protein family share multiple conserved sequence domains based on a distinct helix-loop-helix structure, the EF-hand (Kretsinger, 1980). The loop constitutes the Ca²⁺-binding site. The proteins of the superfamily identified to date contain from two to eight EF-hands or variants thereof. In some of them, the EF-hands have been duplicated or lost; and in others, the calcium binding properties have been altered or lost entirely (Heizman and Hunziker, 1991).

Although the reticulocalbin sequence has no significant homology to any other proteins except for the EF-hand motifs, the overall structure of reticulocalbin is similar to that of calbindin D28 and calretinin (Rogers, 1989) in that both proteins have six EF-hand motif domains. Calbindin D28 and calretinin have been found at high concentrations in the central and peripheral nervous systems of many species, but their function is presently unknown. Calbindin D28 binds only four Ca²⁺ atoms/mol of protein, and the second and sixth domains may have lost their Ca²⁺ binding capability because some oxygen-containing amino acids in the loop are missing (Hunziker, 1986). Similarly, the second and sixth domains of reticulocalbin seem to have lost their Ca²⁺ binding ability. Reticulocalbin, however, has a long amino-terminal extension as well as a short carboxyl-terminal extension. The latter has an HDEL sequence that could serve as part of the retention signal for the protein in the ER. The amino-terminal extension is composed of a leader sequence, which directs the protein to be translocated into the lumen of the ER, and the amino-terminal region of the mature protein of ~50 amino acids, where a single N-glycosylation site resides.
functional in animal cells (Robbi and Beaufay, 1991). The other possibility, that the removal of HDEL caused altered protein folding, which in turn affected the interaction of reticulocalbin with other ER resident proteins, however, is not formally excluded.

Although our studies revealed valuable information about the structure and localization of reticulocalbin, the function of this protein remains unknown. Its localization in the lumen of the ER and its expression in different types of cells suggest a role in protein synthesis, modification, and intracellular transport. We speculate that reticulocalbin functions in the regulation of Ca\(^{2+}\)-dependent activities in the lumen of the ER or post-ER compartment. One intriguing possibility is that reticulocalbin is involved in the retention mechanism of KDEL-terminated proteins in the ER, in which Ca\(^{2+}\) may be involved (Booth and Koch, 1991). Reticulocalbin may associate with an as yet unidentified protein and regulate its activity by binding Ca\(^{2+}\). That reticulocalbin may perform multiple functions like calmodulin should be considered.

Finally, we believe that the name reticulocalbin is appropriate for the protein because it reflects its intracellular localization in the lumen of the ER (reticuloplasm), established Ca\(^{2+}\) binding properties, and the six calbindin D28-like domains of the EF-hand motifs.

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REFERENCES

Bosnich, G., and Fiete, D. (1979) J. Biol. Chem. 254, 9785–9799

Booth, C., and Koch, G. L. E. (1989) Cell 59, 729–737

Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607

Chow, P. Y., and Pasman, G. M. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. 47, 45–148

Dang, C. V., Ebert, R. F., and Bell, W. R. (1986) J. Biol. Chem. 260, 9713–9719

Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., and Rutter, W. J. (1985) Nature 317, 267–270

Engel, J., Taylor, W., Paulson, M., Sage, H., and Hogan, B. (1987) Biochemistry 26, 6958–6965

Etzler, M. (1972) Methods Enzymol. 28, 340–344

Fliegel, L., Burns, K., MacLennan, D. H., Reithmeier, R. A. F., and Michalak, M. (1989) J. Biol. Chem. 264, 21522–21528

Fliegel, L., Newton, E., Burns, K., and Michalak, M. (1990) J. Biol. Chem. 265, 15496–15502

Freeman, R. B. (1989) Cell 57, 1069–1072

Furukawa, T., Orawa, M., Huang, R.-P., and Muramatsu, T. (1990) J. Biochem. (Tokyo) 103, 297–302

Heizman, C. W., and Hunziker, W. (1991) Trends Biochem. Sci. 16, 98–103

Hunziker, W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7578–7582

Kelly, R. B. (1990) Nature 345, 480–481

Kretzinger, R. H. (1980) Ann. N. Y. Acad. Sci. 358, 14–19

Macer, D. R. J., and Koch, G. L. E. (1988) J. Cell Sci. 91, 61–70

Maruyama, K., Mikawa, T., and Ebashi, S. (1984) J. Biochem. (Tokyo) 95, 511–519

Mazzarella, R. A., and Green, M. (1987) J. Biol. Chem. 262, 8875–8883

Mazzarella, R. A., Srinivasan, M., Haugejorden, S. M., and Green, M. (1990) J. Biol. Chem. 265, 1094–1101

Milner, R. E., Bakes, S., Shemanko, C., Carpenter, M. R., Smillie, L., Vance, J. E., Oupa, M., and Michalak, M. (1991) J. Biol. Chem. 266, 7153–7163

Muro, T., and Pelham, H. R. B. (1988) Cell 54, 291–300

Muro, T., and Pelham, H. R. B. (1987) Cell 48, 899–907

Nawa, H., Yamamura, K., and Miyazaki, J. (1991) Gene (Amst.) 108, 193–200

Ozawa, M., Yonezawa, S., Sato, E., and Muramatsu, T. (1982) Dev. Biol. 91, 351–359

Ozawa, M., Huang, R.-P., Furukawa, T., and Muramatsu, T. (1988) J. Biol. Chem. 263, 3959–3962

Ozawa, M., Barabault, H., and Kemler, R. (1989) EMBO J. 8, 1711–1717

Pelham, H. R. B. (1980) Annu. Rev. Cell Biol. 2, 1–23

Pelham, H. R. B. (1990) Trends Biochem. Sci. 15, 483–486

Peters, M. (1972) J. Cell Biol. 53, 155–165

Peters, M. (1988) J. Biol. Chem. 263, 45–53

Pelham, H. R. B., Hardwick, R. G., and Lewis, M. J. (1988) EMBO J. 7, 1757–1762

Robbi, M., and Beaufay, H. (1991) J. Biol. Chem. 266, 20498–20503

Rogers, J. H. (1989) Neuroscience 31, 71–721

Smith, M. J., and Koch, G. L. E. (1989) EMBO J. 8, 3581–3586

Sorger, P., and Pelham, H. R. B. (1987) J. Biol. Chem. 262, 341–344

Sturzu, N. C. J., and James, M. N. G. (1983) Annu. Rev. Biochem. 52, 951–988

Tanaka, K., and Vassalli, P. (1983) Cell 31, 2347–2348

Zagoures, P., and Rose, J. K. (1989) J. Cell Biol. 109, 2653–2660