Calumenin, a Ca^{2+}-binding Protein Retained in the Endoplasmic Reticulum with a Novel Carboxyl-terminal Sequence, HDEF*

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We have identified and characterized a cDNA encoding a novel Ca^{2+}-binding protein named calumenin from mouse heart by the signal sequence trap method. The deduced amino acid sequence (915 residues) of calumenin contains an amino-terminal signal sequence and six Ca^{2+}-binding (EF-hand) motifs and shows homology with reticulocalbin, Erc-55, and Cab45. These proteins seem to form a new subset of the EF-hand protein family expressed in the lumen of the endoplasmic reticulum (ER) and Golgi apparatus. Purified calumenin had Ca^{2+}-binding ability. The carboxyl-terminal tetrapeptide His-Asp-Glu-Phe was shown to be responsible for retention of calumenin in ER by the retention assay, immunostaining with a confocal laser microscope, and the deglycosylation assay. This is the first report indicating that the Phe residue is included in the ER retention signal. Calumenin is expressed most strongly in heart of adult and 18.5-day embryos. The calumenin gene (Calu) was mapped at the proximal portion of mouse chromosome 7.

The endoplasmic reticulum (ER) is involved in synthesis and modification of secretory and membranous proteins as well as resident proteins in the lumen of the ER, Golgi apparatus, or lysosomes (1). The ER is also known as the major Ca^{2+} storage compartment in eukaryotic cells. By pumping cytosolic Ca^{2+} into the ER lumen, cells keep their cytosolic concentration of free Ca^{2+} at extremely low levels so that they can use Ca^{2+} as an intracellular signal (2). Besides, the ER itself needs luminal Ca^{2+} for its normal functions such as protein folding and protein sorting (3–6). Among many ER-resident proteins, endoplasmic reticulum (GRP94) (7, 8), Bip (GRP78) (9, 10), protein-disulfide isomerase (ERp59) (11, 12), and calreticulin (CRP55) (13) are Ca^{2+}-binding proteins that are associated with Ca^{2+}-dependent folding and maturation of secretory proteins in the ER lumen (14, 15). In addition, two Ca^{2+}-binding ER-resident proteins, reticulocalbin (16) and Erc-55 (17), have been isolated recently. They have multiple EF-hand motifs and constitute a new subset of the EF-hand superfamily, together with a homologous protein in the lumen of the Golgi apparatus, Cab45 (18). However, their physiological functions are still unknown.

ER-resident proteins generally carry a retention signal at their carboxyl terminus. This ER retention signal is first identified to be tetrapeptides, Lys-Asp-Glu-Leu (KDEL) in mammalian cells and His-Asp-Glu-Leu (HDEL) in yeast (19, 20). Soluble ER-resident proteins are trapped by binding to the KDEL receptor expressed in the cis-Golgi and retrieved to the ER (21–23). Further studies have demonstrated that HDEL and several variants of the KDEL sequence can also work as the ER retention signal in mammalian cells (24, 25). Comparison of variants of the KDEL sequence suggests that the replacement of Lys and Asp residues with other amino acid residues does not abolish the ER retention activity. However, the carboxyl-terminal two residues are considered to be critical because the third and fourth positions in all of the ER retention signals are Glu/Asp and Leu/Ile, respectively.

During the embryogenesis, the heart begins to beat already in the 8.5-day mouse embryo, whose heart is still a two-chambered tube with one atrium and one ventricle (26). Beating of cardiac myocytes is maintained by the strict regulation of their cytoplasmic Ca^{2+} concentration. To achieve this regulation, cardiac myocytes develop their specialized ER, called the sarcoplasmic reticulum, as the Ca^{2+} storage compartment and produce the rhythmic Ca^{2+} oscillation between the sarcoplasmic reticulum and the cytosol (27). Molecules involved in this Ca^{2+} oscillation are reported to be present very early in mouse cardiogenesis (28, 29).

Since we are interested in molecules involved in heart embryogenesis, we screened in a signal sequence trap library (30, 31) of mouse embryonic heart to isolate cDNAs encoding amino-terminal hydrophobic signal sequences. We here report cloning and characterization of cDNA encoding calumenin that binds Ca^{2+} and carries a new ER retention signal, HDEF, at the carboxyl terminus. Calumenin is a novel member of the reticulocalbin family, a new subset of the EF-hand superfamily in the ER. Calumenin is most strongly expressed in the heart of adult and 18.5-day embryos.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning—Poly(A) RNA from approximately 100 hearts of 9.5-day postcoitus (dpc) mouse embryos was extracted with TRizol reagent (Life Technologies, Inc.) and Oligotex™-dT30 Super (Roche). cDNA was synthesized from 1.35 μg of poly(A) RNA using Super Script II (Life Technologies, Inc.). First strand cDNA was synthesized with 25 pmol of URPX3 primer: GAG-ACG-GTA-ATA-CCA-TCG-ACA-GTA-GCT-CGA-GXX-XXX-XX-X (where X represents one of the following: A, G, C, or T). After alkali lysis of RNA and the poly(A) tailing procedure, second strand cDNA was synthesized with 25 pmol of ESTN primer: CGG-CCG-GTA-CCG-ACT-AAT-GTA-(T)_{3}-XX. Then cDNA of 400–800 base pairs were fractionated by agarose gel electrophoresis and subjected to polymeerase chain reaction (PCR) using ExTaq (TaKaRa, Japan) and 25 pmol of ESP primer (CCG-CGA-ATT-CTG-ACT-AAC-TGA-TT) and Ad-P1 primer (GAC-GCT-GAA-ATC-GAC-GATT-AGG) under the following conditions for Thermal-Cycler (TaKaRa, Japan): 94 °C for 5 min and then 94 °C for 45 s, 52 °C for 60 s, and 72 °C for 2 min for 25 cycles and 72 °C for 10 min. After cloning cDNA unidirectionally between the EcoRI and XhoI sites of pBluescriptII (Stratagene) vector, screening procedures were performed as described (31). To clone full-length cDNA, the 3′-rapid amplification of cDNA ends

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U81839.

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1 The abbreviations used are: ER, endoplasmic reticulum; Bip, immunoglobulin heavy chain binding protein; dpc, day(s) postcoitus; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

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Fig. 1. Nucleotide and predicted amino acid sequences of C39 cDNA. A, positions of nucleotides (upper row) and amino acids (lower row) are shown at the right. cDNA sequence data is available from GenBank/EMBL/DDBJ under accession number U81829. The polyadenylation signal is boxed. B, schematic view and hydropathy plot of C39 protein. C39 protein has an amino-terminal signal sequence (SS), a predicted N-glycosylation site at position 131, and six EF-hand motifs. Arabic numbers under the schema indicate positions of amino acids at these landmarks. Roman numbers refer to EF-hand motifs. The hydropathy plot calculated by the algorithm of Kyte and Doolittle shows that the C39 protein has a typical amino-terminal signal sequence but no membrane anchor sequence.
For sequencing, amplified cDNA was cloned into pGEM-T vector (Promega).

Sequencing was performed with an automated sequencer (model 373A; Applied Biosystems), and sequence analysis was performed with the computer analysis program, GeneWorks (IntelliGenetics, Inc.). Homology search was performed with BLAST and FASTA, using GenBank and EMBL as DNA data bases and PRF, PIR, and SwissProt as protein data bases. Motif search and localization analysis were performed on line at Prosite.

Expression and Purification of Proteins—To express proteins in mammalian cells, cDNAs were cloned in XbaI site of pEF-BOS expression vector (32), and their sequences were confirmed before assays. cDNAs of FLAG-calumenin (FLAG-C39), FLAG-calumenin-DHDEF, and FLAG-calumenin-rHDEL were constructed with PCR. Flag epitope (8 amino acids; DYKDDDDK) was incorporated 5 amino acids downstream of the putative signal sequence cleavage site. The following primers were used for PCR: FLAG-calumenin primer, AAG-CCT-ACT-AGT-ATG-GAC-GAT-CAT-GAT-TGG-TGC-TGG-TCC-CTG-TGC-AAC-GCC-CCC-TCT-TTG-AGC-AAG-CTC-GCA-GAC-GAT-GAC-GAA-GAG-GAC-GCA-GCA-GCA-GCA-GCA-GCA-GCA-GC; calumenin-DHDEF primer, AAG-CCT-ATG-TGG-AGG-CAT-TGG-GAT-GAT-GAA-GAC-GAA-GAC-GCA-GCA-GCA-GCA-GCA-GCA-GCA-GC; calumenin-HDEF primer, AAG-CCT-ATG-TGG-AGG-CAT-TGG-GAT-GAT-GAA-GAC-GAA-GAC-GCA-GCA-GCA-GCA-GCA-GCA-GCA-GC; calumenin-rHDEL primer, AAG-CCT-ATG-TGG-AGG-CAT-TGG-GAT-GAT-GAA-GAC-GAA-GAC-GCA-GCA-GCA-GCA-GCA-GCA-GCA-GC. Transfection of COS-7 cells was performed using lipofectamine (Life Technologies) according to the manufacturer's protocol. Protein synthesis was stopped before protein purification and immunostaining assay by treating cells with 300 μM cycloheximide for 2 h (34). The filter for Northern blot was prepared as described (35) and hybridized using Quick-Hyb solution (Stratagene).

RESULTS

Cloning and Sequencing of a Novel cDNA C39—4.4 × 10⁵ yeast transformants from 9.5 dpc embryonic heart cDNA library were screened, and 386 positive clones were obtained by the signal sequence trap method described previously (30, 31). Among these, nucleotide sequences of 17 clones were not redundant and 15 clones were homologous to sequences reported in mouse or other mammals and the rest were redundant clones. One of the novel clones (C39) was picked up for further studies because its mRNA was most strongly expressed in heart (see below). The slow fade Antifade kit (Molecular Probes, Inc.) was used to prevent photobleaching. Retention of calumenin in ER was assayed as follows. Cells transfected with FLAG-calumenin and FLAG-calumenin-DHDEF were further incubated for 4 h in serum-free medium. The media were then concentrated using Centricron-30 (Amicon). Cell extracts and concentrated media were analyzed by 12% polyacrylamide gel electrophoresis with SDS followed by Western blotting. The reverse transcriptase-PCR method. After first strand synthesis using random 9-mer, PCR was performed with C39N3 primer (GGA-AGA-TGG-ACA-GGT-CGT-CAT) and C39C1 primer (biotinylated) (AGA-GCTT-GTC-CCA-GGA-GCG-GCA-GA). Transfection of COS-7 cells was performed using lipofectamine (Life Technologies) according to the manufacturer's protocol. Protein synthesis was stopped before protein purification and immunostaining assay by treating cells with 300 μM cycloheximide for 2 h (34). The filter for Northern blot was prepared as described (35) and hybridized using Quick-Hyb solution (Stratagene). The 45Ca²⁺ binding assay was performed as described (36). Membranes of 45Ca²⁺ binding assay and Northern blotting was analyzed using an image analyzer (BAS 2000, Fujifilm). Deglycosylation assay was done as described (38) and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. For Western blotting, ECL Western blotting detection reagents (Amersham Life Science, Inc.) was used. Chromosomal mapping of the calumenin gene was done as described (35, 37).
These proteins appear to form one subfamily of Ca\(^{2+}\)-binding proteins. Homology search with the BLAST and FASTA computer programs showed that the C39 protein does not have any other hydrophilic stretch long enough to anchor the protein in the membrane (Fig. 1B). The C39 protein carries six potential Ca\(^{2+}\)-binding EF-hand motifs, B; a \(^{45}\)Ca\(^{2+}\)-binding assay was performed as described (36). Strong signal was detected in the FLAG-C39 fusion protein but none in the FLAG-bacterial alkalai phosphatase fusion protein (Fig. 3B). The positive band was approximately 57 kDa in size, which was equal to the size of the FLAG-C39 fusion protein detected by Amido Black staining, indicating that EF hands in the C39 protein indeed have Ca\(^{2+}\)-binding ability.

Subcellular Localization of the C39 Protein—To determine whether six EF hands in the C39 protein really have Ca\(^{2+}\)-binding ability, the \(^{45}\)Ca\(^{2+}\)-binding assay was performed as described (36). Strong signal was detected in the FLAG-C39 fusion protein but none in the FLAG-bacterial alkalali phosphatase fusion protein (Fig. 3B). The positive band was approximately 57 kDa in size, which was equal to the size of the FLAG-C39 fusion protein detected by Amido Black staining, indicating that EF hands in the C39 protein indeed have Ca\(^{2+}\)-binding ability.

The C39 Protein Is a Ca\(^{2+}\)-Binding Protein—cDNA sequence showed that the C39 protein does not have any other hydrophobic stretch long enough to anchor the protein in the membrane (Fig. 1B). The C39 protein carries six potential Ca\(^{2+}\)-binding EF-hand motifs and a putative N-glycosylation site. Homology search with the BLAST and FASTA computer programs revealed the presence of many Ca\(^{2+}\)-binding proteins homologous to the C39 protein in the EF-hand region. Among these, reticulocalbin (16) and Erc-55 (17) in the ER and Cab45 (18) in the Golgi apparatus had homology in size and sequence to the C39 protein even outside of the EF-hand region (Fig. 2). These proteins appear to form one subfamily of Ca\(^{2+}\)-binding proteins.

Each EF hand in the C39 protein has the general feature for high affinity Ca\(^{2+}\)-binding according to Kretsinger’s rule (38); and shown to encode a 315-amino acid protein (Fig. 1A). The translation start site was assigned at nucleotide positions 36–38 because of the presence of an NH\(_2\)-terminal signal sequence and the comparison with other family members to be described below.

The HDEF Sequence Is a Novel ER Retention Signal—To examine whether the C-terminal tetrapeptide HDEF of calumenin can serve as a novel intracellular retention signal, we constructed two expression vectors: FLAG-calumenin, a fusion protein of FLAG epitope and calumenin, and FLAG-calumenin-HDEDEF, a fusion protein of the FLAG epitope and calumenin lacking its C-terminal HDEF sequence. FLAG epitopes were incorporated 5 amino acids downstream of the putative signal sequence cleavage site in each construct. COS-7 cells were transfected by these constructs, and concentrated media and cell extracts were analyzed by Western blotting. Most of the FLAG-calumenin-HDEDEF protein was secreted into the medium, while almost all of the FLAG-calumenin protein was

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Ca\(^{2+}\)-binding Protein in the ER with Novel Retention Motif

**DISCUSSION**

A Novel Ca\(^{2+}\)-binding Protein in the ER Lumen—We isolated and characterized a novel Ca\(^{2+}\)-binding protein calumenin from mouse embryonic heart. Calumenin is located in the ER and homologous to previously reported Ca\(^{2+}\)-binding proteins such as reticulocalbin and ErC-55 in the ER (16, 17), and Cab45 (18) in the Golgi apparatus. Since all of these proteins including calumenin have six EF hands and Ca\(^{2+}\)-binding activity, they constitute one subset of the EF-hand superfamily. A data base search also revealed that cDNA (ASCF13) cloned as DNA supercoiling factor of silkworm (43) also belongs to this family. The deduced amino acid sequence of ASCF13 has an amino-terminal signal sequence, six EF hands, and the C-terminal HDEL sequence, which we proved to be the ER retention signal.

Although many ER Ca\(^{2+}\)-binding proteins have been reported so far, their functions are largely not yet well understood. Two possible functions of ER Ca\(^{2+}\)-binding proteins have been suggested. First, calreticulin, one of the Ca\(^{2+}\)-binding proteins in the ER, is reported to regulate the capacity of Ca\(^{2+}\) in the ER (44, 45) as well as to have chaperone function (46, 47). Another luminal Ca\(^{2+}\)-binding protein, calsequestrin, is also reported to regulate Ca\(^{2+}\) flow from the ER (48, 49). These reports suggest that Ca\(^{2+}\) fluxes may be regulated somehow by...

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**Fig. 5.** HDEF works as an intracellular retention signal. A retention assay was performed using COS-7 cells transfected with pEF-BOS-FLAG-calumenin, pEF-BOS-FLAG-calumenin-\(\Delta\)HDEF and pEF-BOS (control). After transfection, cells were washed and chased for a further 4 h in serum-free media. Then media were concentrated and analyzed by Western blotting together with cell extracts. FLAG-calumenin-\(\Delta\)HDEF was secreted (lane 2), while almost all of the FLAG-calumenin was retained in cells (lane 1). Different amounts of proteins in cells may be due to the secretion of FLAG-calumenin-\(\Delta\)HDEF (lanes 4 and 5). The arrowhead indicates the FLAG-calumenin fusion protein.

**Fig. 6.** Calumenin is a glycosylated protein in the ER. Transfected cells were treated with 300 \(\mu\)M cycloheximide for 2 h to stop further protein synthesis. The FLAG-calumenin (HDEF) and FLAG-calumenin-rHDEL (HDEL) proteins were purified and subjected to endoglycosidase H and N-glycosidase F digestion for 1 h at 37 °C. The upper band indicates glycosylated proteins, and the lower band indicates deglycosylated forms. Enz, enzyme.

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expression ubiquitously in all tissues examined. However, the expression of calumenin was strong especially in heart and lung. Compared with expression in adult heart, expression in 18.5-dpc heart was slightly stronger (Fig. 7A). The reverse transcriptase-PCR method showed that calumenin mRNA was already expressed as early as 8.5 dpc (Fig. 7B).

To determine the chromosomal localization of the calumenin gene (Calu),\(^2\) strain distribution patterns of restriction fragment length polymorphisms of the calumenin gene were determined in 24 independent recombinant inbred strains derived from crosses between AKR/J and DBA/2J (AXD) (Table I). Analysis of the distribution pattern revealed the linkage of the calumenin gene with markers located at the proximal region of chromosome 7 (Fig. 8).

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Expression and Chromosomal Localization of the Calumenin Gene—Northern blotting analysis showed that calumenin was...
Ca\textsuperscript{2+}-binding proteins located in the luminal side of the ER. Second, Ca\textsuperscript{2+} in the ER is reported to be necessary for normal functions of the ER such as protein folding and protein sorting (3–6), suggesting the existence of ER-resident molecules whose function is regulated by Ca\textsuperscript{2+}. In fact, Bip, a Ca\textsuperscript{2+}-binding protein in the ER, is reported to function in the Ca\textsuperscript{2+}-dependent manner (50).

Further study is needed to determine whether or not calumenin is involved in either of the two possible functions. Recently, interaction molecules of Erc-55 have been reported: the E6 protein of papilloma virus, which has p53-independent tumorigenic activity (51), and taipoxin, which blocks neuromuscular transmission at the presynaptic site (52). As discussed above, calumenin belongs to the same subset of the EF-hand superfamily as Erc-55. Thus, the search for proteins that interact with calumenin could provide a clue to elucidate biological functions of this subset.

A Novel ER Retention Signal—We report a novel carboxyl-
terminal tetrapeptide HDEF of calumenin as a new ER retention signal to prevent proteins from being secreted and to keep them in the ER. We observed that the HDEF sequence had the intracellular retention activity (Fig. 5). The deglycosylation assay (Fig. 6) and the immunostaining profile (Fig. 4) suggest that the HDEF sequence works as the ER retention signal. The C-terminal tetrapeptide KDEL is the first ER retention signal that the HDEF sequence works as the ER retention signal. The centromere is indicated by a circle. Recombination distances in centimorgans are shown at the left of the chromosome.

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**Fig. 8. Chromosome map surrounding calumenin locus on mouse chromosome 7.** The position of the calumenin locus (designated by Calu) is shown on chromosome 7 based on data from AXD strains (Table I). The centromere is indicated by a circle. Recombination distances in centimorgans are shown at the left of the chromosome.
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