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Crocalbin: a new calcium-binding protein that is also a binding protein for crotoxin, a neurotoxic phospholipase A_2

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Abstract Utilizing Marathon-ready cDNA library and a gene-specific primer corresponding to a partial amino acid sequence determined previously, the complete nucleotide sequence for the cDNA of crocalbin, which binds crotoxin (a phospholipase A_2) and Ca^{2+}, was obtained by polymerase chain reaction. The open reading frame of the cDNA encodes a novel polypeptide of 315 amino acid residues, including a signal sequence of 19 residues. This protein contains six potential Ca^{2+}-binding domains, one *N*-glycosylation site, and a large amount of acidic amino acid residues. The ability to bind Ca^{2+} has been ascertained by calcium overlay experiment. Evidenced by sequence similarity in addition, it is concluded that crocalbin is a new member of the reticulocalbin family of calcium-binding proteins.

Key words: Crocalbin; Calcium-binding protein; Crotoxin

1. Introduction

Synaptic transmission is vulnerable to a variety of neurotoxins. Among these toxins some exhibit phospholipase A_2 (PLA_2, EC 3.1.1.4) activity. These PLA_2 neurotoxins (or neurotoxic PLA_2s) from different sources have been studied extensively in recent years, mostly in terms of structural, pharmacological and electrophysiological aspects. Based on structural and catalytic differences, PLA_2s have been classified into several groups. Most of the proteins are monomeric and most of the PLA_2 chains from different sources are homologous in structural features. PLA_2s play important roles in phospholipid metabolism, host defense and signal transduction. Many of them also exhibit neurotoxicity, myotoxicity, coagulation effects and other biological actions, which may or may not be related to hydrolysis of phospholipids. A small number of the PLA_2 proteins, including crotoxin from the South American rattlesnake *Crotalus durissus terrificus*, affect primarily the presynaptic site to cause ultimate blockade of synaptic transmission by inhibiting the release of neurotransmitters, though most of them also show postsynaptic toxicity and other effects (see [1–12] for recent reviews). Previously we have identified high-affinity binding proteins for crotoxin by photoaffinity labeling and chemical cross-linking techniques [13–15]. Two polypeptides of ~50 kDa and 18 kDa have been purified from the porcine brain by affinity chromatography using an immobilized crotoxin column. Partial amino acid sequence of the 50 kDa polypeptide has also been reported [16–18]. Herein we report the complete cDNA sequence and the deduced amino acid sequence of the 50 kDa polypeptide[1] which is a new member of calcium-binding proteins. We now propose the name crocalbin (for crotoxin and calcium-binding protein) for this protein, which was referred to previously as CBP-50. We pursued the study of crocalbin with the assumption that it may be involved in part in the actions of crotoxin. A similar approach has also been taken for other PLA_2s with or without toxicity [19–28] [29–33]. Complete sequences have been reported for a binding protein for pancreatic PLA_2 and other non-neurotoxic PLA_2s, and for two binding proteins for taipoxin, which is also a PLA_2 neurotoxin [28–32].

2. Materials and methods

2.1. Materials

Calcium-45 was obtained from Amersham, and PVDF membrane ProBlott from Applied Biosystems. Marathon-ready cDNA library of rat brain and Advantage KlenTaq polymerase Mix were purchased from Clontech. Cloning vector pGEM-T, JM-109 cell line, and Wizard plus plasmid purification kit were from Promega. DNA primers were synthesized by a local company.

2.2. Methods

Rapid amplification of cDNA ends (RACE) was employed to generate complete cDNA sequence encoding crocalbin. Marathon-ready cDNAs of rat brain were used as templates in RACE polymerase chain reaction (PCR) [34,35] to obtain the 5′-end cDNA fragment. The PCR reactions were performed by using two primers: Marathon adaptor primer AP1 as the sense primer, and gene-specific oligonucleotide encoding the internal sequence TEGELKSRIKHAQK of crocalbin as the antisense primer. The latter primer is a non-degenerate 42-mer oligonucleotides (5′-CTTCTGGGCGTGCTTGATCCGGCTCTTCAGCTCGCCCTCGGT-3′), designed on the basis of the above amino acid sequence with the third nucleotides of degenerate codons for amino acids to be the nucleotide appeared most frequently in known DNA sequences encoding the proteins of rat brain. The thermal condition in the reaction was subjected to 25 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min). After the cycling, the PCR reaction was allowed to proceed further for 4 min at 72°C. The PCR product was purified on 1.2% agarose gel, and then ligated to pGEM-T vector for transfecting into competent cell, JM109. The white colonies carrying the insert was randomly picked up and then cultured for 6 h. The plasmid was isolated by using Wizard plus reagent kit according to the manufacturer's instructions, and then subjected to automatic DNA sequencing. A 383 bp (not including AP1 sequence) cDNA fragment was obtained.

For the generation of 3′-end cDNA fragment encoding the C-terminal part of crocalbin, 3′-RACE with Marathon-ready cDNAs as templates was utilized with gene-specific oligonucleotide (5′-GGAATGATTGTAGATAAAATAGACACCGATAAAGATGGG-3′) corresponding to bp 297–335 of the 5′ cDNA fragment of crocalbin as the sense primer and AP1 sequence as the antisense primer. The procedure of PCR was performed according to the manufacturer's protocol using the Advantage KlenTaq Polymerase Mix. The thermal cycles in the reaction were as follows: 1 min at 94°C, 5 cycles of 30 s at 94°C and 4 min at 72°C, 5 cycles of 30 s at 94°C and 4 min at 70°C, 25

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[1] The sequence data are available from GenBank/EMBL/DDBJ under accession number AJH001929.

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cycles of 20 s at 94°C and 4 min at 68°C. The PCR products were gel purified, subcloned into pGEM-T vector and sequenced as above. It was observed that the last 87 bp of the 5′-end fragment match totally with the first 87 bp of the second PCR product (3′-end fragment) which contains poly-A tail.

Detection of Ca^{2+} binding by crocalbin was performed by the calcium overlay assay (^{45}Ca autoradiography) as described by Maruyama et al. [36] with modifications. Briefly, after SDS polyacrylamide gel electrophoresis proteins were blotted onto PVDF membrane, and then washed three times with a solution of 60 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole-HCl, pH 6.8 to get rid of the electrode buffer. The membrane was incubated in the same buffer containing 1 mCi/l ^{45}Ca for 45 min at room temperature. Unbound ^{45}Ca was removed by rinsing with 50% methanol. After drying, autoradiography was carried out with X-ray film for 72 h exposure at −80°C.

3. Results

3.1. Cloning and sequencing of crocalbin from the rat brain cDNA

Previously we reported the partial amino acid sequence KPTEKKDRVHHEPQL for the N-terminal part of crocalbin (CBP-50) purified from porcine brain, and IVDKIDADKDGFVTEGELKSRIKHAQK for part of an internal fragment [17,18]. Based on the latter partial sequence, a 42-mer oligonucleotide corresponding to the last 14 amino acid residues of the sequence was used as primer (together with AP1) to amplify Marathon-ready cDNA library of rat brain

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                                                                    TA   2
GAATTCAGCGGCCGCTAAATTCTAGGTGGCCACGGAATCCTGCGGCGTGGAGCTCCGGGGAAAACTCAGTCAACC   77

ATGGACCTGCGTCAGTTTCTTATGTGCCTGTCCCTGTGCACGGCCTTTGCTTTGAGCAAGCCTACAGAAAAGAAG  152
M  D  L  R  Q  F  L  M  C  L  S  L  C  T  A  F  A  L  S  K  P  T  E  K  K    25

GACCGAGTACACCATGAACCTCAGCTCAGCGACAAAGTTCACAACGATGCTCAGAATTTCGACTATGACCATGAT  227
D  R  V  H  H  E  P  Q  L  S  D  K  V  H  N  D  A  Q  N  F  D  Y  D  H  D    50

GCCTTCTTGGGAGCAGAAGAGGCAAAGAGTTTTGGTCAGCTGACACCAGAAGAGAGCAAGGAAAAGCTTGGAATG  302
A  F  L  G  A  E  E  A  K  S  F  G  Q  L  T  P  E  E  S  K  E  K  L  G  M    75

ATTGTAGATAAAATAGACACCGATAAAGATGGGTTTGTGACCGAGGGCGAGCTGAAGAGCCGGATCAAGCACGCC  377
I  V  D  K  I  D  T  D  K  D  G  F  V  T  E  G  E  L  K  S  R  I  K  H  A   100

CAGAAGAAATACATATATGACAATGTTGAAAACCAGTGGCAGGAGTTTGATATGAATCAAGACGGCTTAATCTCC  452
Q  K  K  Y  I  Y  D  N  V  E  N  Q  W  Q  E  F  D  M  N  Q  D  G  L  I  S   125

TGGGATGAGTACAGAAACGTGACTTATGGCACTTACCTGGATGATCCAGACCCTGATGATGGATTTAATTATAAA  527
W  D  E  Y  R  N  V  T  Y  G  T  Y  L  D  D  P  D  P  D  D  G  F  N  Y  K   150

CCGATTATGGTTAGAGATGAGCGGAGGTTCAAAATGGCCGACCAAGATGGAGACCTTATTGCCACAAAGGAGGAG  602
P  I  M  V  R  D  E  R  R  F  K  M  A  D  Q  D  G  D  L  I  A  T  K  E  E   175

TTTACCGCTTTTCTGCACCCTGAGGAATATGACTACATGAAAGACATAGTCCTGCAGGAAACCATGGAGGATATA  677
F  T  A  F  L  H  P  E  E  Y  D  Y  M  K  D  I  V  L  Q  E  T  M  E  D  I   200

GACCAGAATGCTGATGGTTTTATTGATCTAGAAGAGTATATTGGTGACATGTACAGTCATGATGGGAATGCTGAT  752
D  Q  N  A  D  G  F  I  D  L  E  E  Y  I  G  D  M  Y  S  H  D  G  N  A  D   225

GAACCCCAGTGGGTTAAGACAGAGCGGGAGCAGTTCGTTGAGTTTCGAGATAAGAACCGGGATGGAAAGATGGAC  827
E  P  Q  W  V  K  T  E  R  E  Q  F  V  E  F  R  D  K  N  R  D  G  K  M  D   250

AAGGAAGAGACCAAAGACTGGATCCTCCCTTCAGACTATGACCATGCAGAGGCCGAAGCCAGGCATCTCGTCTAT  902
K  E  E  T  K  D  W  I  L  P  S  D  Y  D  H  A  E  A  E  A  R  H  L  V  Y   275

GAGTCCGACCAAGACAAGGATGGCAAGCTCACCAAGGAGGAGATTGTCGACAAGTATGATTTATTTGTGGGCAGC  977
E  S  D  Q  D  K  D  G  K  L  T  K  E  E  I  V  D  K  Y  D  L  F  V  G  S   300

CAGGCCACAGATTTCGGGGAGGCCTTAGTACGACACGATGAGTTCTAAGCTGCAAACAGAGGAGCCTTCATTTCT 1052
Q  A  T  D  F  G  E  A  L  V  R  H  D  E  F  *                             315

TCAAAAGTAATTTATTTTTACAGGTCTGGTTTCACATAAAATTGTTTGCGCTACTGAGACTGTTATTACAAACTT 1127
TTTAAGACGTGAAAAGGCATATCGAGATAGTGAAATCACCCGCCCCCATTCCTCCTCCCTCTGAGGGGCTGGAAG 1202
GAACCCATGCTTCTGAGGAACAACTCTGATTAGTACACTTGTGTCTGTAGGTTTACACTTTGTATAATGTATAAC 1277
ATGGTGTGTTTATTTTTGTATTGTTCTCTAGTTGGGAGTATAATATGAAGGATGGAGATCCTCAACCCACACTTG 1352
TAGGCATACATTAGCCATTTACACTTTCTCAATCCCTTACCACATTTTTTTTTTAATAATTCTCACTTAACTAAT 1427
TTTTTAAAGCCTAAGATCAATAAGAAATGTTCAGGAGAGAAAAAGCAGAAGGAAAGCATGTACTTCGTGATTTAC 1502
GTTCAGAGAGAGAATGCTTCATCTTGCTTGTTGAGAAGTCTCATTTCATGAGTAGCTGTTCAGTTGTCACAGGCC 1577
CAGCCACGGAGCCTGCCATTGTCTGGGCAAGGACAGAGTCCTCCGCTGTAAGACAGCGTCACGCAGCTCCACTTC 1652
ACTCTTCCCCTCAGGACTAGCTGTTTGCTAATTTTGTCAAGCACAGCTGTGGTAGGAAGAATTAGGGCCCAGTGT 1727
CTTGAAAAATCAACCAAGTAGTGTGTATGATGTCTTCACAGGGCTATTTCTAGCTCTTTCTAGAGCTGTTTCTAA 1802
CCAGAAACAGCTGGAAAACAAAAAGAACAAAGTGTATGCAGGGCATGCATCTCATTCTTAGTGAAATCACTACAA 1877
GGACCCATCCCAGCCCCTTTCTAAGTCTTAACCTTGGGTTTTACTGCAGTTTAAATTGATTCTTTTCCCATCATG 1952
ACATTGAAAGTTGCCCTTTAACAGGAAAAATGGTCACCGAATGAGAATTGGGACTCAAGAATAACGAATTTGGGG 2027
CGCCCTTACGTTGAAAGCATTTGAACCTCCCTGATACCGAAGGGGATTCCCCTCCCCGCCTTTTTCTCTTGTAAA 2102
CAGGAAGTAAATAGCATTATTAGTTAAAGCTTGGTTGCAGTGTTCTTATCTTGTGGGCTGGTTTCTAAAACCTCA 2177
TGCTGCTGATTTGACCAGGGCATCCTCATACCTCAGATGCAAACCACTCTTCTACCGGGCCTCTGTTTACCGGAG 2252
CTTTGCCTCAAGGATAGAAGGCTGTACAGAGGGGCTCTTTGGTTTGAGGACCACTGCTCACCCTTCCTGTCATTA 2327
ACCTGTCACACCCCATTTTATCATCTCCCTTTCTCTCTGACACACAAAGGTGGGGTACGTGGGAGGGTCGTGGAT 2402
TATTCTTATTAAAAAACAAAATCATCTGTTGCCAACCCCATTTACCCATCTTTGGTCTCTTACTGATGGGCCTCT 2477
TAAGAATTATTGTATTCCAAGTCTTTAACCCTCATGTTACTAATGTAAATATACATCTGGGCAGTCTTTATTACT 2552
TCCTGTATCTCTGAGTAATACATCAAGCTGGTGCTGGGTGATGGTCATATCTGAACCTAGACCTCCCCGTGGGTC 2627
TTCCACAATCCTGTTGATGTGGGCTGCTTGGTGTGGTAAAAAGCCCAGTCGTGGTGTAACTTAACCTTGGCGATT 2702
GCATCAAGCTTCTTGATAGCAGATACACTCTAAGGTTTTAGCCCCAGTAGAGGTGAAATGAACATCCCTCACTGC 2777
CTTCCCCAGATCCTCAACTCTCCATTGTTAAGGAGACCAGAGATAATTAATGCCACCAACCCTGGCTTAGAAAGG 2852
GTACGTCATACACTGTGTAGCAAGAGGGCATTACAGAGCCTAACGCTGGCGTGAAAATCATGTACTTAGCCAGCA 2927
AGTGAGTCTGCGAGGGTGGCGTAGTCTGGACAGGGTGTTCAGCATCGGGAACTGTGCTCTCAGGTCCATAAGCTC 3002
CACATAGTGTTGGGGTTTGGGTTTGGGTTTCTGGTTGAATTTGAGTATTTGTTCTTTTTTTATAGAGTGTAAACC 3077
AAGTTTTATATTCTGTAATGCAAACAGGTACCTGTCGTTTTTTGAATAAAACTGTTTACATCCAAAAAAAAAAAA 3152
AAAAAAAAAAAAA                                                              3166
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Fig. 1. Nucleotide sequence of crocalbin cDNA and predicted amino acid sequence of the protein. Nucleotides and amino acids are numbered to the right with single-letter codes for the amino acids shown below the nucleotide sequence. The leader signal, the polyadenylation signal and the poly-A tail are underlined. The sequence data are available from GenBank/EMBL/DDBJ under accession number AJ001929.

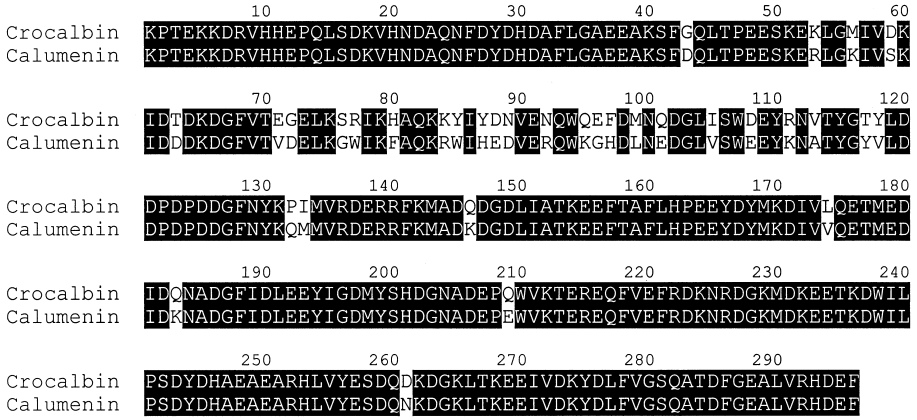

Fig. 2. Sequence alignment of crocalbin with calumenin. The predicted amino acid sequences of mature crocalbin (rat) and of calumenin (mouse) are aligned by the Gene Stream align program. The conserved residues are highlighted.

by PCR with the assumption that this segment of the rat and that of the porcine are highly conserved (they turned out to be identical). After 5′-RACE followed by 3′-RACE, complete nucleotide sequence of the rat cDNA for crocalbin was obtained and the amino acid sequence deduced (Fig. 1). There are two potential initiator methionines within a distance of 7 amino acid residues. The vicinity of the first AUG codon, but not the second, matches perfectly with the consensus sequence for initiator methionine as defined by Kozak [37,38], namely a purine at position −3 and a guanine at position +4. Furthermore, in line with the generalization of signal peptides of 18–20 residues in length for eukaryotic proteins synthesized on rough endoplasmic reticulum (rER) [39], translation starting from the first initiator methionine will give a signal peptide for crocalbin of 19 residues, as inferring from the N-terminal amino acid sequence determined for the mature protein by direct protein sequencing versus the sequence deduced from the cDNA. Therefore, we numbered the first methionine as position +1 in this report. Thus the 3166 bp nucleotide sequence contains an open reading frame of 945 bp, a 77 bp 5′-untranslated region, and a 2144 bp 3′-untranslated region. The signal sequence is made up of mostly hydrophobic residues and conforms to the −3, −1 rule [39] of small, uncharged residues at the −1 and −3 positions, which are Ser and Ala, respectively, for crocalbin, relative to the signal peptidase cleavage site. Thus the mature protein encoded by the crocalbin cDNA is 296 amino acid residues in length, and the predicted molecular mass is 34 861 Da. Judging from the deduced protein sequence, there is only one potential *N*-glycosylation site, which is located at residue 112 of the mature protein (Fig. 2). Only one amino acid residue in the partial sequence determined previously (see above) for the porcine protein is different from the deduced sequence of the rat protein (residue 63 of Fig. 2), indicating that crocalbin is highly conserved and thus deserves further study.

A survey of the GenBank and the SwissProt databases revealed that only the small family of reticulocalbin-type proteins [40] show significant homology to crocalbin. These are calumenin (87.8%), reticulocalbin (58.2%), DNA supercoiling factor (53.1%), yk67a3.5 (46.2%), ERC-55 (38.5%), TCBP-49 (37.7%), and Cab45 (25.1%). Although the score of sequence similarity between crocalbin and calumenin is high, the amino acid residues between 71 and 113 are only 53.5% identical (Fig. 2).

3.2. Crocalbin is a calcium-binding protein of the EF-hand type

Similar to reticulocalbin and many other calcium-binding proteins, the crocalbin sequence also contains domains satisfying the general feature for high-affinity calcium-binding EF-hand domains according to Kretsinger's rule [44]. As shown in Fig. 3, six segments of such domains exist in the crocalbin sequence. The five oxygen-containing residues responsible for the coordination of Ca^{2+} are present in all of the six domains. Although the match of domain III with the consensus sequence is less than perfect, similar deviations also occurs in other calcium-binding proteins, e.g. reticulocalbin, calpain, calumenin, ERC-55, and Cab45 [40–43,45]. Although the central residue in domain III is Leu instead of the conserved Gly, the same replacement occurs in some other Ca^{2+}-binding proteins and was considered to be compatible with Ca^{2+} binding on the basis of secondary structure prediction [40,46,47]. The six calcium-binding domains of crocalbin show significant sequence homology with segments of a large number of calcium-binding proteins, though only the reticulocalbin family of proteins show homology outside the Ca^{2+}-binding domains.

To assess whether crocalbin actually binds Ca^{2+}, the calci-

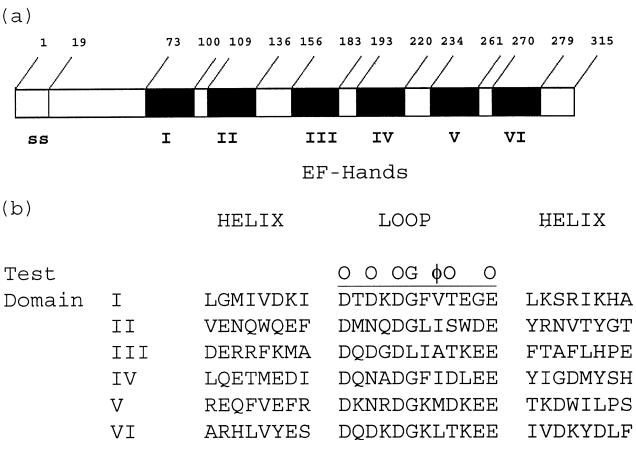

Fig. 3. Alignment of potential calcium-binding domains in crocalbin with EF-hand motif. a: Schematic representation of crocalbin structure. Crocalbin has a signal sequence (ss) and six EF-hand domains. Arabic numbers indicate positions of these landmarks. Roman numbers refer to the EF-hands. b: The protein sequence deduced for crocalbin was analyzed for homologies to the test sequence for EF-hand motif. In the test sequence, ϕ and o represent hydrophobic and oxygen-containing residues, respectively.

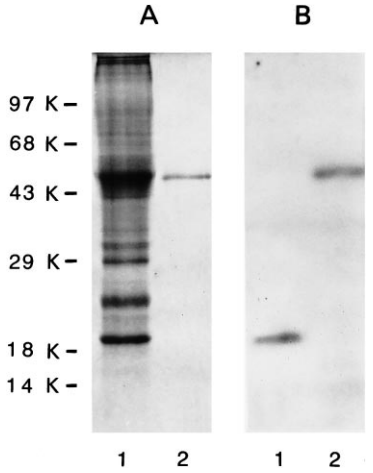

Fig. 4. Calcium overlay assay for Ca^{2+} binding by crocalbin. SDS gel electrophoresis of 75 μg synaptosome (lanes 1 of A and B) and 2.5 μg purified crocalbin (lanes 2 of A and B) was performed with gels containing 12% acrylamide. After electrophoresis, the gels were either stained with Coomassie blue (A) or subjected to Western blotting followed by ^{45}Ca overlay and autoradiography (B).

um-45 overlay (Ca^{2+} autoradiography) assay was employed. The purified crocalbin was subjected to SDS polyacrylamide gel electrophoresis, transferred to PVDF membrane, and then incubated with ^{45}Ca. As shown by the autoradiogram of the membrane, purified crocalbin did indeed bind Ca^{2+}. The band corresponding to crocalbin in the synaptosome preparation showed little if any radioactivity, reflecting that crocalbin is a minor protein in the cell. A protein of 20 kDa appears to be the major calcium-binding protein in the synaptosome preparation (Fig. 4), the identity of which is currently not known.

4. Discussion

In this study, we have shown that crocalbin is a highly conserved new protein, and established that it is a calcium-binding protein with the EF-hand motifs. Previously we reported a molecular mass of ~50 kDa for crocalbin on the basis of SDS gel mobility [16,18], which is considerably higher than that calculated from the deduced amino acid sequence. This behavior has also been observed for reticulocalbin, ERC-55, and Cab45 [40,42,43]. Data are lacking as regards whether other proteins in the reticulocalbin family share this property.

Although TCBP-49 (from rat) is only <40% homologous to crocalbin, it binds to taipoxin, which is also a PLA_2 neurotoxin related to crotoxin. TCBP-49 has been shown to be present in vesicular and reticular structures (presumably inside the lumina), and has been suggested to bind taipoxin after the toxin was internalized into the endomembrane systems by endocytosis following binding of the toxin to membrane acceptors [32]. Crocalbin may behave in similar fashion after crotoxin is internalized. In support of this hypothesis, most of the proteins homologous to crocalbin were shown to be distributed in the endomembrane systems and suggested to play some role in protein synthesis, modification, intracellular transport or Ca^{2+}-dependent regulation of other proteins [40–43], and no data are available for the rest. Members of the reticulocalbin family of calcium-binding proteins contain a HDEL, HDEF or HEEF tetrapeptide sequence at the C-termini, which appears to be (at least partly) the ER or Golgi retention motif. The C-terminal sequence of HDEF for crocalbin also fits in this picture.

Although the homology between crocalbin (from rat) and calumenin (from mouse or human) is high, crocalbin appears not to be the rat counterpart of calumenin, as evidenced by the following observations. Both calumenin and crocalbin are very highly conserved proteins, since calumenin from mouse and that from human [41,48] are almost identical (97.6% sequence identity), and our data showed that the sequence identity between human (unpublished data) and rat crocalbin is also very high (95.3%). Furthermore, although the sequences near both N- and C-termini of crocalbin and calumenin are almost identical, the amino acid residues between 71 and 113 are only 53.5% identical (Fig. 2). Thus this study substantiates our previous claim [18] that crocalbin (CBP-50) is a new member of the yet small family of reticulocalbin-like calcium-binding proteins. It remains to be resolved whether crocalbin and calumenin represent isoforms of similar function or are distinct proteins serving different physiological roles. The calumenin sequence, determined by the signal sequence trap method [41], was reported after the partial sequence of crocalbin was published [16,17]. Purification of most members of the reticulocalbin family has not been reported, whereas crocalbin and TCBP-49 have been highly purified using immobilized crotoxin and taipoxin columns, respectively, useful for further biochemical and other studies.

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