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MOLECULAR CLONING OF HIPPOCALCIN, A NOVEL CALCIUM-BINDING PROTEIN OF THE RECOVERIN FAMILY EXCLUSIVELY EXPRESSED IN HIPPOCAMPUS*

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SUMMARY: We have isolated a cDNA clone encoding a novel calcium-binding protein of the recoverin family from rat brain cDNA library. This clone (PCB11) has 588 nucleotides in the open reading frame including the termination codon, 174 nucleotides of the 5' leader and 800 nucleotides of the 3' noncoding region. The complete amino acid sequence deduced from the cDNA is composed of 195 residues, has a calculated molecular mass of 22,574 Daltons, and contains three putative calcium-binding domains of the EF-hand structure. The deduced amino acid sequence has a striking sequence homology to those of the retinal recoverin family (recoverin, visinin, P26, 23kD protein, S-modulin) and the brain-derived recoverin family (P23k, 21-kDa CaBP and neurocalcin). Northern blot, *in situ* hybridization, immunoblot and immunohistochemical analyses revealed that the protein is exclusively expressed in pyramidal layer of the hippocampus. The protein was therefore designated hippocalcin.

The most important physiological functions performed by calcium-binding proteins in the cell are to act as receptors for cytosolic calcium and to transduce calcium signals via interaction with target proteins (1). A new family of calcium-binding protein designated as the recoverin family, members of which serve to regulate photo-signal transduction systems, was found in the retina and has been isolated from a variety of vertebrate species (2-8). These proteins activate guanylate cyclase when free calcium decreases to less than 100 nM (2) and/or prolong cyclic GMP phosphodiesterase light-activation when free calcium increases to more than 1 μM (3). The amino acid sequences of these proteins exhibit three putative calcium-binding domains of the EF-hand structure (9) and show more than 60% sequence identities with each other, suggesting that they are members of a new family of calcium-sensitive regulator.

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We have previously isolated a novel calcium-binding protein of molecular mass 23,000 Daltons (P23k) and characterized as a member of the recoverin family (10,11). Recently, structurally related proteins have been isolated from rat (12) and bovine brain (13), indicating that multiple isoproteins exist in the brain and form the brain-derived recoverin family. During cDNA cloning of P23k, we have identified a novel member of this family, which we designated hippocalcin. The present study demonstrates cDNA cloning of this novel calcium-binding protein and selective expression in pyramidal layer of the hippocampus.

MATERIALS AND METHODS

Materials: Restriction endonucleases, T4 DNA polymerase, Taq polymerase and other modifying enzymes were obtained from Takara Shuzo. Oligonucleotides were synthesized on a Model 392A DNA synthesizer (Applied Biosystems). A rat brain λgt 11 cDNA library was purchased from Clontech. [α-^{32}P]dATP (110TBq/mmol) was purchased from Amersham Japan. All other chemicals and reagents were of analytical grades.

Screening of cDNA Library: The sense (5') primer [ATT(CA)TAT(C)GCT(CAG)A AT(C)TTT(C)TTT(C)CC] and the antisense (3') primer [TCT(CAG)CCA(G)TCCAA(G) A(G)TCA(G)TACAT] were synthesized on the basis of two amino acid sequences (IYANFFP and MYDLDGD) from P23k (11). Polymerase chain reaction (PCR) was performed with 1 μg of cDNA reverse-transcribed from rat brain total RNA using a TAITEC thermal cycler and Taq polymerase (14). The resulting PCR products corresponding to the expected size [170 base pairs(bp)] were eluted from an acrylamide gel, blunt-ended with T4 DNA polymerase and subcloned into the *Hinc* II site of the plasmid pBluescript II SK(+) (Stratagene) to yield the plasmid pCBF9. The 196 bp *Xho* I-*Hind* III fragment from pCBF9 was labeled by a multi-priming method and used as a probe. Approximately 1.2 x 10^6 plaques from a rat brain λgt11 cDNA library were screened. Hybridization was carried out at 42°C in a solution containing 5 x SSC, 5 x Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, 0.1% SDS, 20% formamide and the probe for one overnight. After hybridization, the filters were washed with 0.2 x SSC containing 0.1% SDS at 50°C. The filters were then dried and autoradiographed.

DNA Sequencing: The cDNA inserts of the positive clones were subcloned into the *Eco*RI site of pUC118 (Takara Shuzo). The nucleotide sequences were determined by the dideoxy chain termination method (15) with [α-^{32}P]dATP and Sequenase Ver. 2 kit (United States Biochem). The double strand plasmid was first sequenced from both strands using two vector primers which flanked the cDNA insert. The complete sequence was obtained by custom primer-directed DNA sequencing using specific primers complementary to internal cDNA sequence.

Northern Blot Analysis: Total RNA was isolated from various brain regions and tissues of three-month-old male rats (Wistar albino strain) by the guanidinium thiocyanate method (16), electrophoresed on a 0.9% agarose gel after denaturation with glyoxal and dimethyl sulfoxide. The RNA was transferred to nylon membranes (Magnagraph Nylon, MSI) and hybridized for 20 h at 42°C in a solution containing 50% formamide, 5 x SSPE, 250 μg/ml denatured salmon sperm DNA, 5 x Denhardt's solution and 0.1% SDS. A ^{35}S-labeled antisense RNA probe was prepared as described (17). PCR was done between nucleotide residues 39-297 (Fig. 1) using the synthetic oligonucleotide primers to which the *Pst* I site was added. The PCR product was then subcloned into the *Pst* I site of the plasmid pBluescript II SK (+) to yield pCBR22. After cleaving with *Bam* HI, the DNA template was transcribed by using T7 RNA polymerase in the presence of ^{35}S-UTP. The specific activity of the probe was 1.4 x 10^8 c.p.m./μg and 2 x 10^6 c.p.m./ml were added to the hybridization buffer. The filter was washed in 0.1 x SSC containing 0.1% SDS at 50°C. Autoradiography was done at -80°C for 96h with an intensifying screen.

***In Situ* Hybridization Analysis:** Coronal frozen sections were prepared from three-month-old male rats. *In situ* hybridization with the ^{35}S-labeled antisense RNA probe was done essentially as described (18). The sections were dipped in Kodak NTB2 emulsion, followed by exposure for 2 weeks.

Immunoblot and Immunohistochemical Analyses: Anti-hippocalcin antibody was raised in rabbits against the maltose-binding protein (MBP)-hippocalcin fusion protein. The *Pst* I fragment of pCBP11, corresponding to the nucleotide residues 37-1038 (Fig. 1), was

subcloned into the *Pst* I site of E. coli expression vector pMALc (New England Bio Labs.), which was designed to produce fusion protein with MBP and to have a Factor Xa recognition site to release the target protein. The fusion protein was purified by using amylose resin affinity chromatography (19). The epitope-selected antibody was affinity purified using the fusion protein coupled FMP Activated-Cellulofine (Seikagakukogyo). The antibody was then incubated with excess MBP and the immunoreactivity against MBP was removed using amylose resin. Immunoblot and immunohistochemical analyses was done as described previously (10).

RESULTS AND DISCUSSION

cDNA Cloning and Sequencing: Recoverin-like calcium-biding protein (P23k) was previously isolated from rat brain (11). Since the amino terminal end was blocked, the partial amino acid sequence was determined on fragments purified after endoproteinase Lys-C cleavage. Taking advantage of the sequence identity between recoverin (2) and P23k (11), we synthesized two oligonucleotide primer pools based on these two sequences (see Materials and Method), and used them to prime the amplification of a cDNA template reverse-transcribed from rat brain total RNA using the polymerase chain reaction (PCR). The nucleotide sequences of six PCR products were determined. The deduced amino acid sequence of the five PCR products corresponded to that of P23k. The remaining PCR product showed some differences but had a striking sequence homology to P23k, indicating that a novel species of the recoverin family exists in the brain. This sequence was then used to screen a rat brain cDNA library. Two overlapping cDNA clones (CBP3 and CBP11) thus identified were sequenced.

As shown in Fig. 1, the clone CBP11 consisted of 1562 nucleotides including the entire open reading frame of 588 nucleotides, 174 nucleotides of the 5' leader and 800 nucleotides of the 3' noncoding region. The nucleotide sequence surrounding the presumed initiation codon for CBP11 agrees with Kozak's consensus sequence (20), designating residue 1 as the translation initiation site. The clone CBP3, consisting of 1032 bp, is in complete agreement with the partial nucleotide sequences of the clone CBP11 (residues -39 - 994). They showed an open reading frame predicted to encode a protein of 195 amino acids. Molecular mass is calculated to be 22, 574 Daltons and is well accord with that of native protein determined by immunoblot analysis (molecular mass 23, 000 Daltons) using the epitope-selected antibody (Fig. 4-a). Since this protein is exclusively expressed in the hippocampus (see below), we designated it hippocalcin.

As shown in Fig. 2, the primary structure of hippocalcin displays striking homology with those of the retina-derived recoverin family such as recoverin (2,6), visinin (4), and the brain-derived recoverin family such as 21-kDa CaBP (12) and neurocalcin (13). All of these proteins contain three putative calcium-binding domains of the EF-hand structure (14) and a possible amino terminal myristoylation site (GXXXSX) (21). Native forms of these proteins are known to have a blocked amino terminus, indicating that the amino terminal glycine residue of the recoverin family might be myristoylated. Indeed, recoverin was found to be myristoylated at its amino terminus (22). A potential cGMP-dependent protein kinase phosphorylation site (XSRX) (23) is found in hippocalcin in its second EF-hand structure.

Northern Blot and *In Situ* Hybridization Analysis: To study the expression of the hippocalcin gene, we extracted RNA from a variety of rat tissues and brain regions, and analyzed them with a hippocalcin-specific probe (Fig. 3-a). A strong signal at a position corresponding to 2.0 kilobases was detected in the hippocampus. Weak signals were obtained from the cerebral cortex and cerebellum, and faint signals from other brain regions and retina.

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-174        GAGTGTGTGGTCTGGGGCCACCTCTCTCTTTTTGGGTCAAATGAGAGTTTGGGG
-120  TATCTTGTGTCTGAGGGCCCTTCTGGAGTAGTGTCAAGGGACTAAAAGGATGTAACCATT
 -60  GCTCTCTCCTTTCACTTCCTTTGGTTGGGGGGAACCCAGGTGGGACTTGGCTCTTCTGCC
   1  ATGGGCAAGCAGAATAGCAAGCTGCGGCCAGAGATGCTGCAGGACCTGCGAGAGAACACC
      M  G  K  Q  N  S  K  L  R  P  E  M  L  Q  D  L  R  E  N  T   20

  61  GAGTTCTCTGAGCTGGAGCTTCAGGAGTGGTACAAGGGCTTCCTGAAGGACTGCCCGACT
      E  F  S  E  L  E  L  Q  E  W  Y  K  G  F  L  K  D  C  P  T   40

 121  GGCATCCTCAACGTGGATGAGTTCAAGAAGATCTACGCCAACTTCTTCCCCTACGCGATG
      G  I  L  N  V  D  E  F  K  K  I  Y  A  N  F  F  P  Y  A  M   60

 181  CCTCCCAAGTTCGCCGAGCATGTCTTCCGCACTTTTGACACCAACAGCGACGGCACCATC
      P  P  K  F  A  E  H  V  F  R  T  F  D  T  N  S  D  G  T  I   80

 241  GACTTCCGGGAGTTCATCATCGCTCTGAGCGTGACCTCCGGTGGCCGCCTGGAGCAGAAG
      D  F  R  E  F  I  I  A  L  S  V  T  S  G  G  R  L  E  Q  K  100

 301  CTCATGTGGGCCTTCAGCATGTACGACCTGGACGGCAATGGCTACATCAGCCGGGAGGAG
      L  M  W  A  F  S  M  Y  D  L  D  G  N  G  Y  I  S  R  E  E  120

 361  ATGCTAGAAATTGTGCAGGCCATTTACAAGATGGTTTCGTCCGTGATGAAGATGCCTGAG
      M  L  E  I  V  Q  A  I  Y  K  M  V  S  S  V  M  K  M  P  E  140

 421  GATGAGTCTACCCCGGAAAAGAGGACTGAGAAAATCTTCCGCCAAATGGACACAAACAAT
      D  E  S  T  P  E  K  R  T  E  K  I  F  R  Q  M  D  T  N  N  160

 481  GACGGCAAGCTGTCACTGGAGGAGTTCATCCGCGGGGCCAAAAGCGACCCATCGATCGTG
      D  G  K  L  S  L  E  E  F  I  R  G  A  K  S  D  P  S  I  V  180

 541  CGCCTGCTGCAATGCGATCCCAGCAGCGCTTTCCCAGTTCTGAGCTGAGAGGAGCCAGCT
      R  L  L  Q  C  D  P  S  S  A  F  P  V  L  S  *              195
 601  CGCCTCCCTGCCTTCACCGGCCCTCCGGCTCTCAGCTTCCTCTCCCTCGTGTCTATCCAG
 661  GCTGGGGAATCCCCTTCTGGGTCTGCACTGTGGGGGGGCCTCTGGAGAAGCGAACCCTGC
 721  AAGGAAGGGGCTAAGCAAGGCAGCCGTTAGTCCCCACGGCCTAAGAGGCAGAATCTCCCT
 781  TAGTGGGACCGAGATTACCCCCGGGTCAGGTTCTGTATCTTTCCCCAGACAGCTGATCAC
 841  CTTCCACCCACCCACAACCCGAGGCTCTTCCACAACACGGGAAGAGGGGCTAGGTCCTTC
 901  TTGGGTGGTCCTCAGCCTGGAGGGAGGTGGGGATTTGTGCCAAGGTGAAGAGTCTTTTTG
 961  TTTGTTTTGGTGGGGAAGACCGGAGGATTACTGGGCTGTGGGACTTTGAAGAAGTTCCCT
1021  GATCTACTTCCCTGCAGAAGAACACTTCCTGTCCCAGCTCTTGCTGCTGGAGGCATGGTA
1081  AAAAGAAGATCTCCCAGGATTCCAACTGGGGGTCCCCAAACCCAGCCTGCCTCTTTGCAC
1141  TTGGATAACACAGATTTACCCGTCTGTCCTCTGTATTTCTCTCTCCTTCCCCACCTCACA
1201  CACGCACGGGGCCGCGCGACATGCGCGCGCAACACACACACACACACACGCACGATAT
1261  GCACGCACACATGCACAGCACCCGCGCACACAGAGGCCGGCAGACAGATGAGCAGAGCCC
1321  CTCCACAGCGTTCTGTGTGGGAAAGAAGTGCAGCCCCTGCCAAGCCCCATGTCCCTCTGC
1381  ATGCGCCG
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Fig. 1. The nucleotide sequence of cDNA for hippocalcin and the deduced amino acid sequence. The nucleotide and deduced amino acid sequences are numbered beginning with the first nucleotide of the translational initiation codon (left side) and the initiator methionine (right side), respectively. Underline indicates the nucleotide sequence corresponding to the PCR product used for cDNA cloning. The primer sites are double underlined. Three putative calcium-binding domains of the EF-hand structure are boxed. * indicates termination codon.

Under identical hybridization conditions, no signal was detected in liver, kidney and any other tissues tested: gut, lung, spleen, heart and skeletal muscle (data not shown). *In situ* hybridization analysis of coronal sections of rat brain revealed intense signals restricted in pyramidal layers CA1 to CA3 of the hippocampal gyrus (Fig. 3-b). Weak signals were detected in granular layer of the dentate gyrus. Weak and scattering signals could also be detected in layers II to VI of the cerebral cortex. Other brain regions showed no distinct signals.

Immunoblot and Immunohistochemical Analysis: A specific antibody was raised in a rabbit against the maltose-binding protein (MBP)-hippocalcin fusion protein and affinity purified. Native hippocalcin was identified by immunoblot analysis as a protein of about 23,

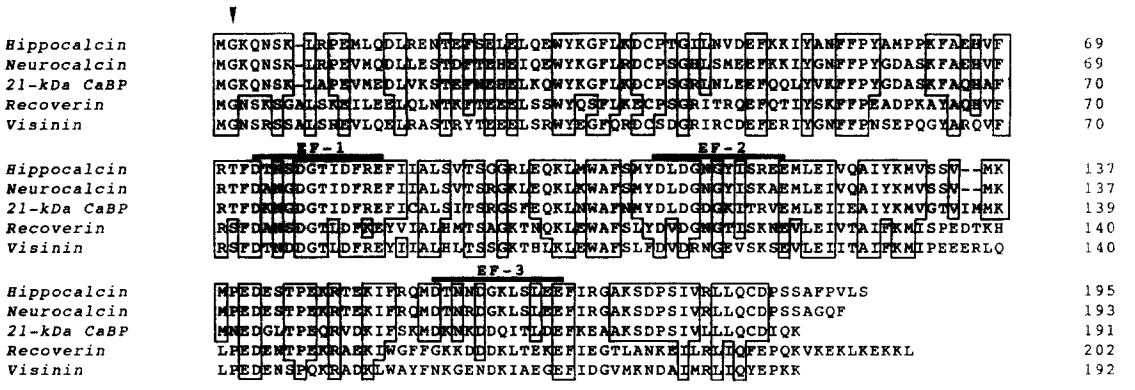

Fig. 2. Alignment of the deduced amino acid sequence of hippocalcin and other members of the recoverin family. Sequence alignment was done using DNASIS software (Hitachi Software Engineering). Amino acid residues are shown in one-letter code and numbered beginning with the initiator methionine. Sets of identical amino acid residues among the recoverin family and three brain-derived members are boxed. Putative calcium-binding domains of the EF-hand structure (15) are indicated by EF-1, 2 and 3, and a possible N-terminal myristylation site (16) is marked with an arrowhead. Potential cGMP-dependent protein kinase phosphorylation sites (17) are underlined. Neurocalcin (12) and 21-kDa CaBP (11) are derived from the brain, and recoverin (3, 7) and visinin (5) are from the retina.

000 Daltons (Fig. 4-a). A prominent protein band was detected in the hippocampus, a weak band in the cerebral cortex and faint bands in other brain regions. No protein band could be detected in the retina and other tissues. Immunohistochemical analysis of coronal sections of rat brain demonstrated the selective expression of hippocalcin in pyramidal layer of the hippocampus (Fig. 4-b) . A small number of immunoreactive cells also lay scattered in granular layer in the dentate gyrus and layers II to VI of the cerebral cortex.

Thus, hippocalcin was identified as a novel member of the recoverin family that is expressed exclusively in pyramidal layer of the hippocampus. The distribution of hippocalcin is very different from that of P23k (24) and neurocalcin (25) which are found in a wide range of brain regions. The physiological functions of the brain-derived recoverin family proteins are

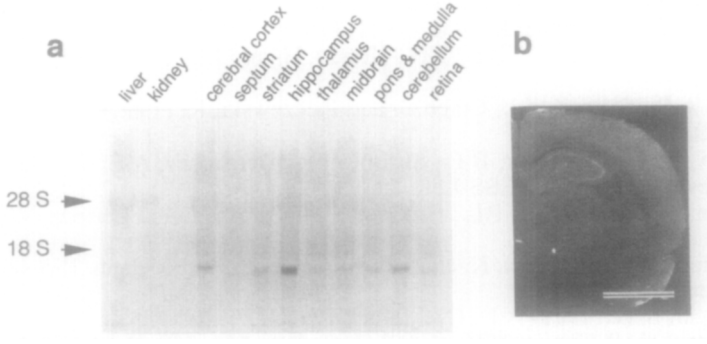

Fig. 3. Northern blot and *in situ* hybridization analyses of hippocalcin mRNA in rat brain and other tissues. a. Autoradiograph of blot hybridization analysis of total RNA (30 μg each) from various brain regions and tissues. Positions of 28S and 18S ribosomal RNAs are indicated on the left side. **b.** Dark-field photomicrograph of *in situ* hybridization of coronal section of rat brain. Scale bar = 5 mm.

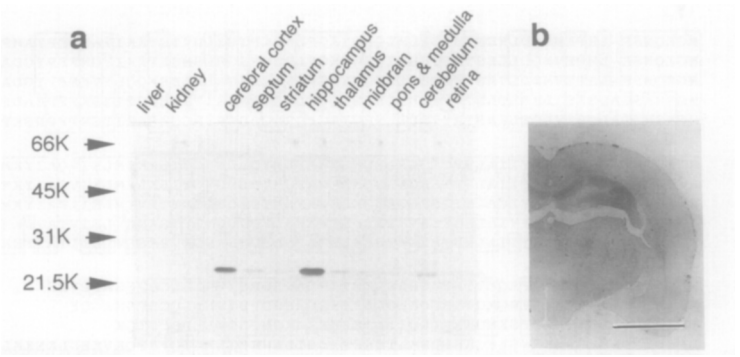

Fig. 4. Immunoblot and immunohistochemical analyses of hippocalcin in rat brain and other tissues. a. Immunoblot analysis of proteins (30 μg each) from various brain regions and tissues using the epitope-selected anti-hippocalcin antibody. **b.** Immunohistochemical analysis of coronal section of rat brain. Scale bars = 5 mm.

not yet known, however, their structural similarity to recoverin (2,6) and S-modulin (8) led us to postulate that the brain-derived recoverin family proteins might have similar functions to those of recoverin and S-modulin, and act as a calcium-sensitive regulator in signal transduction systems in each cell type. Since the activity-dependent change in synaptic efficacy mediated by increased cytosolic calcium entering through the *N*-methyl-D-aspartate receptor channel is known in the hippocampus (26), it seems likely that hippocalcin plays an important role in synaptic plasticity.

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