Characterization of Murine cDNAs Encoding P-57, a Neural-specific Calmodulin-binding Protein*

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Polyclonal antibodies raised against bovine brain P-57, a neural-specific calmodulin-binding protein, were used to isolate murine cDNAs encoding P-57 from murine brain cDNA libraries in the expression vector λgt 11. Two of the overlapping clones contained an open reading frame encoding a polypeptide of 227 amino acid residues (predicted Mr. 23,635), a 163-nucleotide 5'-untranslated sequence, and a 403-nucleotide 3'-untranslated sequence. Hydrophobicity analysis of the predicted polypeptide indicated the lack of any considerable stretch of hydrophobic residues that may span the membrane. This is consistent with prior data suggesting that P-57 exists in a soluble, as well as a membrane-associated, form. The predicted amino acid composition of P-57 is rather unusual in that it is highly enriched in alanine, glutamic acid, and lysine residues, and relatively enriched with proline residues. This amino acid composition accounts for the very low helical content of the predicted polypeptide. A search of the GenBank and EMBL sequence data banks (GenBank Inc., release 44.0 (August, 1986); European Molecular Biology Library, release 8.0 (April, 1986)) indicated that the P-57 nucleotide sequence shows no significant homology to any reported sequences. RNA blot analysis of brain, heart, liver, and testes RNA revealed that cDNAs detect P-57 transcripts of 1.5 kilobases in brain, but not in other tissues. Genome blot analysis was consistent with P-57 being encoded by a single or small number of genes. These data demonstrate that the accumulation of this novel calmodulin-binding polypeptide in neural tissue is controlled primarily at the level of RNA abundance.

P-57 is a neural specific calmodulin-binding protein, first described by Andreasen et al. (1983) during photoaffinity cross-linking of azido-125I-CaM to crude bovine brain preparations. P-57 cross-linked to the calmodulin derivative with a 1:1 molar ratio in the absence of free calcium but not in its presence. It was subsequently purified to apparent homogeneity by exploiting the differential affinity of the protein for CaM-Sepharose in the absence of calcium than in the presence of calcium, under low ionic strength conditions. P-57 has been detected in bovine brain soluble and solubilized membrane fractions, rat, chick, mouse, and human fetal brain (Cimler et al., 1985). To date, P-57 has not been detected in non-neural tissues.

Based on radioimmuno assay data, P-57 is relatively abundant in many regions of bovine brain. It constitutes 0.5 and 0.35% of the total protein in solubilized caudate and cerebral cortex membranes, respectively (Cimler et al., 1985). At these concentrations, we proposed that P-57 would serve to bind and localize calmodulin to specific sites within the cell at resting cell conditions (low intracellular free calcium levels) and would release calmodulin locally when intracellular calcium levels increase, thereby allowing calmodulin to interact with its target enzymes at a more rapid rate. Recently, we reported that P-57 is a good substrate for protein kinase C (Alexander et al., 1987). The protein kinase C-catalyzed phosphorylation of P-57 serves to lower further the affinity of P-57 for calmodulin and affect the release of calmodulin from P-57. Therefore, the concentration of free calmodulin in brain may be controlled by protein kinase C-catalyzed phosphorylation of P-57 and fluctuations in intracellular free calcium levels.

The protein was originally designated P-57 based on its apparent molecular weight on SDS-polyacrylamide gels; however, subsequent hydrodynamic studies indicated that P-57 has a molecular weight of 25,700 and a Stokes radius of 4.58 nm. Sucrose density gradient sedimentation and fluorescence polarization measurements indicate that P-57 is an elongated molecule with an axial ratio in excess of 15 (Masure et al., 1986). The unusual mobility of P-57 on SDS-polyacrylamide gels is probably due to the existence of an atypical conformation in SDS.

In order to explore any homologies of the primary structure of P-57 to other reported proteins, and to study further its functional domains, we decided to pursue the primary sequence of P-57. We report here the isolation and characterization of murine cDNAs encoding the complete sequence of P-57, using previously characterized polyclonal P-57 antibodies to screen murine brain cDNA libraries constructed in the expression vector λgt 11.

The abbreviations used are: CaM, calmodulin; protein kinase C, the Ca"/phospholipid-dependent enzyme; SDS, sodium dodecyl sulfate.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J02869.

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EXPERIMENTAL PROCEDURES

Materials—All enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim, or New England Biolabs and used as specified by the manufacturers. Radiochemicals (32P)[ATP] and (35S)[c]DNA were obtained from Du Pont-New England Nuclear. Ultrapure acrylamide, bisacrylamide, ammonium persulfate, low melting point agarose, cesium chloride, and phenol were obtained from Bethesda Research Laboratories; agarose from FMC Corp.; ultrapure urea from Schwarz/Mann; and antibiotics from Sigma. All other reagents were of the highest grade available.

Isolation of the cDNA Clones—A mouse whole brain and a mouse cerebellar cDNA library constructed in the expression vector λgt 11 (Young and Davis, 1983) were kindly provided by Dr. James Sikela and Dr. William Hahn at University of Colorado School of Medicine. Approximately 6 × 10^6 plaques from the whole brain cDNA library were screened with polyclonal P-57 antibodies at a dilution of 1:1000 as previously described (Moon et al., 1985). Positive plaques were isolated and rescreened to homogeneity. Phage DNA was isolated from four positive plaques, purified, and digested with EcoRI to isolate the inserts. One of the four identical inserts, designated P57.W1, was isolated from low melting point agarose gel (Moon et al., 1985), ligated into the EcoRI site of pGEM-1 (Promega Biotec, Madison, WI) with T4 DNA ligase, and used to transform Escherichia coli strain RRI (Lebanon, NH), was used on a MacIntosh computer for editing and sequencing and used to screen 10^6 plaques of the cerebellar cDNA library. Nitrocellulose filter lifts were prehybridized and hybridized at 42°C in 50% formamide, 5 × SSC, 1 × Denhardt's solution, and 0.1 mg/ml denatured herring sperm DNA, washed at 42°C in 0.2 × SSC (1 × SSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 0.2% SDS (Maniatis et al., 1982), air-dried, and exposed at −70°C to Kodak XAR-5 x-ray film with intensifying screens. The 32P hybridization probes were prepared by hexanucleotide primer extension of P57.W1 and P57.W1 restriction fragments to a specific activity of 1–3 × 10^8 cpm/μg of cDNA (Feinberg and Vogelstein, 1983, 1984) and used at a concentration of 3 × 10^5 cpm/ml.

We obtained more than 100 positive plaques from 10^6 plaques screened. To increase the probability of obtaining a clone extending 5′ of P57.W1, we used a P-labeled 5′-end restriction fragment of P57.W1 (an Nael fragment present at nucleotides 377–382 in Fig. 2) to rescreen 50 of the 100 putative positives. Forty-eight of the 50 plaques rescreened positive. We then decided to increase further the probability of obtaining full-length clones by screening duplicate filter lifts with 5′- and 3′-end probes, 336 nucleotide Nael fragment (nucleotide 377–382 in Fig. 2) and 120-nucleotide Real fragment (nucleotide 964–967 in Fig. 2) of P57.W1, respectively. Thirty-two of the 48 plaques rescreened positive. Phage DNA from 12 of these putative positive plaques were purified and digested with EcoRI, and the inserts were subcloned into PGE-1. Three cerebellar cDNA clones, designated P57.C7, P57.C8, and P57.C9, were obtained.

RNA and DNA blot Analyses—For the analysis of P-57 transcripts present in murine tissues, poly(A)+ RNA was isolated from murine brains, livers, hearts, and testes using phenol:chloroform:isoamyl alcohol. The RNA was then denatured, electrophoresed on 1.2% agarose gel (Maniatis et al., 1982). This cDNA was then characterized by DNA sequencing and used to screen 10^6 plaques from the whole brain cDNA library. Nitrocellulose filter lifts were prehybridized and hybridized at 42°C in 50% formamide, 5 × SSC, 1 × Denhardt's solution, and 0.1 mg/ml denatured herring sperm DNA, washed at 42°C in 0.2 × SSC (1 × SSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 0.2% SDS (Maniatis et al., 1982), air-dried, and exposed at −70°C to Kodak XAR-5 x-ray film with intensifying screens. The 32P hybridization probes were prepared by hexanucleotide primer extension of P57.W1 and P57.W1 restriction fragments to a specific activity of 1–3 × 10^8 cpm/μg of cDNA (Feinberg and Vogelstein, 1983, 1984) and used at a concentration of 3 × 10^5 cpm/ml.

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RESULTS

Isolation of cDNA Clones—In order to explore structural homologies that P-57 may have in common with other known calmodulin-binding proteins, and in order to explore the molecular basis for the apparent neural-specific expression of P-57, we decided to isolate cDNAs encoding P-57. We used P-57 polyclonal antibodies, previously characterized (Cimler et al., 1985), to screen a murine whole brain cDNA library constructed in the expression vector λgt 11. Four putative P-57 clones were isolated and found to contain a 1-kilobase insert. The EcoRi insert from one clone (P57.W1) was subcloned into pGEM-1 and characterized by restriction endonuclease mapping (Fig. 1) and DNA sequencing (Fig. 2).

P57.W1 encodes a portion of a P-57-like polypeptide, as determined by a comparison of the amino acid sequence encoded by the cDNA with the amino acid sequence of bovine brain P-57 (Wakim et al., 1987) (Fig. 3). Assuming a high homology between the bovine and murine proteins, comparison of the amino terminus of the predicted amino acid sequence of murine P-57 with the bovine brain P-57 amino acid sequence (Fig. 3) indicates that a very small part of the 5′-end of the clone is not present in the P57.W1 cDNA. We then used P57.W1 to screen the murine cerebellar library for P-57 cDNAs extending 5′ of P57.W1. Thirty-two clones were obtained. Phage DNA was purified from 12 randomly selected clones, and the EcoRi inserts were subcloned into pGEM-1. We used both promoter primers (Sp6 and T7) to obtain partial sequences from both strands of three of these cDNAs, designated P57.C7, P57.C8, and P57.C9. These sequences were examined to determine the extent of overlap between these cDNA clones and the whole brain P57.W1 cDNA. In the regions where the cDNA clones overlapped, all sequences were in complete agreement (data not shown). Clone P57.C9 was determined to be shorter than P57.W1 at both the 5′- and the 3′-ends and was, therefore, not characterized any further. Clones P57.C7 and P57.C8 are both about 100 nucleotides shorter than P57.W1 at the 3′-end; however, they are both longer at the 5′-end. P57.C8 is 204 nucleotides longer at the 5′-end than P57.W1.

Nucleotide Sequence and Predicted Amino Acid Sequence—Although no enzymatic activities have been found associated with P-57, and its physical and calmodulin-binding properties are novel, we considered the possibility that it may be a known protein or a fragment from a known protein. One of our main objectives in this study was to search for structural homologies that P-57 may have in common with other known proteins. Elucidating the primary structure of P-57 may enable us to detect certain functional domains that are shared by certain classes of proteins. Clones P57.W1 and P57.C8 contain overlapping nucleotide sequences that encode the full coding sequence of P-57 (Fig. 3). The resulting nucleotide and the predicted amino acid sequences are shown in Fig. 2. The
Fig. 2. Nucleotide sequence and predicted amino acid sequence of mouse brain P-57. Nucleotides are numbered in the 5' to 3' direction. Number 1 was assigned to the first residue of the ATG triplet encoding the initiator methionine; the nucleotides to the 5' side of this triplet are indicated by negative numbers. Nucleotides -163-42 are from clone P57.C8, and nucleotides 43-1086 are from clone P57.W1. The predicted amino acids are presented above each respective triplet, in the standard one-letter code.

Fig. 3. Alignment of amino terminus, carboxyl terminus, and internal sequences from bovine brain P-57-derived protein sequence and predicted amino acid sequence from cDNA data. The numbers corresponding to the amino acids of the murine brain P-57 are presented above the specific residue, and the numbers corresponding to the bovine brain P-57 amino acids (Wakim et al., 1987) are presented below the residue. Non-identical amino acid residues are indicated by darker letters. P57.C8, predicted amino acid sequence of P-57 clone P57.C8; P57.W1, predicted amino acid sequence of P-57 clone P57.W1; P-57bov, derived bovine brain protein sequence (Wakim et al., 1987); *, carboxy terminus.

The amino acid composition of the predicted P-57 sequence is very unusual (Table I). Based on the predicted amino acid sequence of the cDNA clone, murine brain P-57 is highly enriched in alanine, glutamic acid, and lysine residues, and contains no tyrosine or tryptophan residues, and is relatively enriched in proline residues. This amino acid composition of the murine brain P-57 is in very close agreement with the composition obtained from purified bovine brain P-57 (Masure et al., 1986). However, as shown in Table I and Fig. 3, there are some species differences between the bovine and murine brain P-57s. The predicted molecular weight of the mouse brain P-57 is 23,635, including the initiator methionine. This confirms the earlier findings of Masure et al. (1986) that P-57 behaves anomalously on SDS-polyacrylamide gels by migrating with an apparent Mr, of 57,000. The actual molecular weight of the polypeptide (23,635) is much smaller than would be expected from its mobility on SDS-polyacryl-
Comparison of amino acid composition of predicted mouse brain P-57 and purified bovine brain P-57

| Amino acid | Purified bovine brain P-57 | Mouse brain P-57 |
|------------|---------------------------|-----------------|
| Alanine    | 52                        | 46              |
| Arginine   | 6                         | 5               |
| Asparagine | 2                         | 3               |
| Aspartic acid | 19                      | 22              |
| Cysteine   | 2                         | 2               |
| Glutamine  | 9                         | 11              |
| Glutamic acid | 50                        | 34              |
| Glycine    | 16                        | 13              |
| Histidine  | 3                         | 3               |
| Isoleucine | 5                         | 3               |
| Leucine    | 2                         | 2               |
| Lysine     | 30                        | 30              |
| Methionine | 2                         | 2               |
| Phenylalanine | 1                      | 1               |
| Proline    | 18                        | 17              |
| Serine     | 13                        | 14              |
| Threonine  | 17                        | 15              |
| Tryptophan | 0                         | 0               |
| Tyrosine   | 0                         | 0               |
| Valine     | 5                         | 7               |

Total: 241 227

* Based on a molecular weight of 34,900 (Masure et al., 1986).
* Predicted from cDNA data (M, 23,635).

RNA Blot Analysis—Earlier Western blot and radioimmune assay data indicated that P-57 is a neural-specific protein (Cimler et al., 1985). It has been detected in chick brain, rat brain, and in various regions of bovine brain. To date, P-57 has not been detected in any non-neural tissues. One of our objectives for the characterization of P-57 cDNAs was to determine whether the tissue specificity of P-57 was controlled at the level of message abundance. Poly(A)* RNA was prepared from murine brain, heart, liver, and testes and was subjected to agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose, and hybridized to 32P-labeled single-stranded P57.W1 cDNA. As shown in Fig. 4A, 57.01 probes hybridize to a single major transcript of approximately 1.5 kilobases in total (lane 5) as well as poly(A)* (lane 1) RNA from brain only. The probes did not hybridize to any transcripts in poly(A)* RNA prepared from heart (lane 4), liver (lane 2), or testes (lane 3). As an additional control, the RNA blot shown in Fig. 4A was stripped and rehybridized with a murine α-actin clone which has been previously shown to hybridize to both muscle and non-muscle actin mRNAs (Minty et al., 1981). As shown in Fig. 4B, this probe hybridizes to two transcripts in testes (lane 3) and heart (lane 4), the 1650-nucleotide muscle and, to a much lesser extent in heart, the 2100-nucleotide non-muscle actin message only. This demonstrates that the lanes which showed no hybrization to the P-57 probes did indeed contain other messages. These data strongly support the earlier evidence indicating that P-57 is neural-specific (Cimler et al., 1985) and that the accumulation of this poly-peptide in neural tissues is controlled at the level of message abundance.

Genomic DNA Blot Analysis—I n order to determine the number of genes that code for P-57, we examined the hybridization pattern on a genomic blot of mouse liver DNA, probed with radiolabeled P57.W1 cDNA. Fig. 5 shows that the probe hybridizes to two distinct restriction fragments generated by digestion of the genomic DNA with BamHI (lane 1), EcoRI (lane 2), PstI (lane 3), and RsaI (lane 4). The detection of two bands following digestion of genomic DNA with PstI and RsaI is consistent with the presence of a single P-57 gene per haploid genome, since there are two, very closely positioned PstI sites and a single RsaI site within the P57.W1 cDNA. The detection of two bands in genomic DNA following digestion with EcoRI or BamHI, which do not have restriction endonuclease sites within P57.W1, is probably due to the presence of an EcoRI and BamHI site within an intron spanned by the P57.W1 cDNA. Although the data do not rule out the presence of more than one P-57 gene per haploid genome, it is clear that there is not a multigene family encoding proteins related to P-57.

DISCUSSION

P-57 is a highly abundant, neural-specific, calmodulin-binding protein known to interact with CaM in an unusual...
manner. P57 binds CaM in the presence of excess calcium chelators at low ionic strength conditions (dissociation constant, 2.3 \times 10^{-9}). This interaction is disrupted when free calcium levels increase and upon protein kinase C-catalyzed activation and immunological studies have indicated that P-57 is neural-specific and that it is a distinct polypeptide, not the result of proteolytic cleavage of a larger parent protein. In order to determine whether P-57 is related to other known proteins and to examine possible functional domains it may have in common with known classes of proteins, we pursued the primary sequence of the polypeptide.

In the present study we used polyclonal antisera raised against purified bovine brain P-57 to isolate P-57 cDNAs from murine brain cDNA libraries in the expression vector \textit{lambda} 11. Overlapping cDNAs were used to generate the full coding sequence of murine brain P-57 (682 nucleotides), in addition to a 163-nucleotide 5'-untranslated sequence and a 403-nucleotide 3'-untranslated sequence. A search of two nucleotide data banks indicated that P-57 shows no considerable homology to any reported sequences. However, the predicted amino acid sequence shows about 77% identity with bovine brain P-57 protein sequence (Wakim \textit{et al.}, 1987). This is consistent with the cross-reactivity of the polyclonal antiserum raised against the bovine protein with the murine antigen. The P-57 cDNA codes for a polypeptide of 227 amino acids (Mr, 23,635). The bovine brain P-57 protein sequence (Wakim \textit{et al.}, 1987) indicates that the bovine polypeptide is composed of 239 residues (Mr, 24,721). We previously reported that rat brain P-57 migrates at a lower molecular weight than bovine brain P-57 on SDS-polyacrylamide gels (Cimler \textit{et al.}, 1985). Murine brain P-57 migrates with a similar mobility as rat brain (data not shown). This difference in mobility of the rodent and bovine polypeptides is the result of differences in their primary structure.

Analysis of the predicted secondary structure of the murine polypeptide using the criteria established by Chou and Fasman (1978a, 1978b) indicates that P-57 has some potential for \alpha-helix formation (data not shown). Earlier circular dichroism spectroscopy data indicated that bovine P-57 contains only 1% \alpha-helix, 21% \beta-sheet, and 78% random coil (Masure \textit{et al.}, 1986). Even though P-57 has a high content of \alpha-helix former (alanine and glutamic acid residues), the presence of strong \alpha-helix breakers (proline and glycine residues) seems to destabilize \alpha-helix formation. Hydrophobicity analysis of the predicted amino acid sequence indicated that the polypeptide has very little hydrophobic character and that there were no hydrophobic regions that could span the membrane. This is consistent with the fact that P-57 is found in both soluble and membrane fractions during purification procedures (Cimler \textit{et al.}, 1985). It has been proposed by a number of laboratories that calmodulin-binding domains of calmodulin inhibitory peptides and target enzymes have a basic, amphiphilic \alpha-helix in common (Stull \textit{et al.}, 1986, Erickson-Vit lantern and DeGrado, 1987). Examination of the predicted P-57 sequence for putative calmodulin-binding domains indicated that only one region beginning with amino acid 43 (Arg-Gly-His-Ile-Thr-Arg-Lys-Lys-Leu-Lys-Glu-Lys-Lys) would weakly satisfy the proposed criteria for a normal CaM-binding domain. Preliminary evidence does indicate, however, that the peptide Ser-Phe-Arg-Gly-His-Ile-Thr-Arg-Lys-Leu (nucleotides 122–154) binds to CaM-Sepharose with higher affinity in the absence of calcium than in its presence.\footnote{K. A. Alexander, B. T. Wakim, K. A. Walsh, and D. R. Storm, unpublished observations.}

P-57 cDNAs detected a transcript of about 1.5 kilobases in RNA isolated from brain, with no transcripts being detected in RNA isolated from heart, liver, or testes. These results are in agreement with earlier findings indicating that P-57 is neural-specific (Cimler \textit{et al.}, 1985) and demonstrate that this neural specificity of P-57 is controlled at the level of mRNA abundance. We are presently characterizing genomic clones of P-57 to isolate and characterize sequences involved in this neural-specific expression.

Over 50% of rodent brain mRNAs are thought to be expressed in brain only (Bantle and Hahn, 1976; Hastie and Bishop, 1976; Chikaraishi, 1979). To date, very few brain-specific proteins have been characterized. In order to understand the complex function of the brain, it will be necessary to identify and characterize the proteins involved. Milner, Sutcliffe, and co-workers (Milner and Sutcliffe, 1983; Sutcliffe \textit{et al.}, 1983) have utilized recombinant DNA techniques to identify and characterize some of these brain-specific molecules. Milner and Sutcliffe (1983) randomly selected, and classified into one of five classes, 191 cDNA clones prepared from rat brain cytoplasmic poly(A)+ RNA based on their tissue distribution patterns detected by Northern analysis. The P-57 clones isolated in this study fall into the category of class III clones, which are those that hybridize only to brain mRNA. Recently, Tsou \textit{et al.} (1986) reported a composite nucleotide sequence of brain-specific 5'-untranslated ends constructed from sequence data of a heterogeneous class II
family of clones (those that hybridize differentially to the mRNA of brain, liver, and kidney). The 5′-untranslated sequences of the P-57 cDNAs isolated here do not share any significant identity with those reported by Tsou et al. for the class I1 family.

In conclusion, we have isolated cDNAs encoding the neural-specific, calmodulin-binding protein P-57 and determined that its neural specificity appears to be controlled at the level of RNA abundance. The nucleotide sequence encoding P-57 indicates that the protein has a unique amino acid sequence which distinguishes it from all known CaM-binding proteins. Future comparisons of the 5′- and 3′-untranslated nucleotide sequences of P-57 to other known brain-specific nucleotide sequences will aid in identifying specific sequences which may be expressed only in brain.

Addendum—After submission of this manuscript, the cDNA sequence of a rat, neuronal, growth-associated protein “GAP-43” was reported by Karns et al. (Karns, L. R., Ng, S., Freeman, J. A., and Fishman, M. C. (1987) Science 236, 597-600). The predicted amino acid sequence of GAP-43 is so closely related to the one illustrated in Fig. 2 that rat GAP-43 must correspond to bovine and mouse P-57.

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