

Molecular cloning of clathrin assembly protein gene (rCALM) and its differential expression to AP180 in rat brain

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Abbreviations: CCV, clathrin-coated vesicle; CALM, clathrin assembly protein lymphoid myeloid; PLD, phospholipase D; RT-PCR, reverse transcriptase polymerase chain reaction; AP, adaptor protein

Abstract

Binding of clathrin assembly protein to clathrin triskelia induces their assembly into clathrin-coated vesicle (CCV) in neurons. The clathrin assembly protein gene (rCALM) was cloned from rat brain cDNA library. rCALM deduced 69 kD molecule has overall 73% amino acid homology compared with that of AP180 protein. The N-terminal domain, where amino acid sequences are very similar with AP180, harbours binding sites for clathrin and inositides, as well as possible phosphorylation sites, but the proline rich C-terminal domain is different from that of AP180. The mRNA expression of rCALM and AP180 by *in situ* hybridization histochemistry revealed that the rCALM mRNA was more intensely expressed than that of AP180, and the distribution patterns were different from each other. These results suggest that the rCALM mediates the assembly of clathrin in neural and supporting cells of brain, and regulates the clathrin coated-vesicle formation through phosphorylation and inositide metabolism.

Keywords: clathrin-coated vesicle, AP180, CALM, gene cloning, expression

Introduction

Clathrin-coated vesicles (CCV) are involved in pathways of receptor-mediated intracellular transport and transfer of proteins from trans-Golgi network to pre-lysosomal

compartment and recycling of synaptic vesicles (Pearse and Robins, 1990; Keen, 1990). The CCV coat is formed by polymerization of triskelion-shaped clathrin molecules into lattice of clathrin cage, and is catalyzed by the assembly proteins. The major coat protein is a clathrin, which consists of triskelion having three identical 190 kD heavy chains and three 23-27 kD light chains. Coated vesicle also contains one or more of the assembly proteins (Robinson, 1994). The assembly proteins are adaptors, and are believed to link receptors to the clathrin network. The assembly proteins all participate in promoting an assembly of clathrin triskelia into artificial clathrin cage that resemble coated vesicle. To date, six different adaptor proteins (AP) are known: AP-1, AP-2, AP-3, AP4, AP180, CALM, and auxillin. The AP-1, AP-2, AP-3, and AP-4 are tetramer, whereas AP180, CALM, and auxillin are monomer.

AP-1 and AP-2 adaptors mediate clathrin assembly at the trans-Golgi network and plasma membrane, respectively, and play a role in selecting cargo protein in CCVs (Marsh and McMahon, 1999). AP-3 plays a role in trafficking from *trans*-Golgi network to the lysosome (Simpson *et al.*, 1997). The function of AP-4 associated with trans-Golgi network was not characterized precisely (Dell'Angelica *et al.*, 1999). AP180 is well studied because it is monomeric protein and neuron specific (Kondury and Roland, 1988; Zhou *et al.*, 1992). AP180 was first discovered as a specific coat component of clathrincoated vesicles from neural tissue and as a phosphoprotein (Stephan *et al.*, 1990). The native protein was shown to associate with clathrin triskelia on equimolar basis and induces clathrin assembly into a homogenous population of 60-70 nm coats (Zhou *et al.*, 1993). AP180 is phosphorylated at serine residues (Morris *et al.*, 1990). The disassembly of clathrin from the vesicle coat was promoted by the phosphorylation of AP's (Wilde and Brodsky, 1996). Two known primary functional properties of the AP180 are clathrin assembling and high affinity for specific inositol polyphosphates (Morris *et al.*, 1993; Ye *et al.*, 1995; Norris *et al.*, 1995). The CALM gene was cloned from human leukemia cell line, and was shown to be homologous to the monomeric clathrin adaptor of AP180. But the molecular property of the gene was not characterized. To understand the regulation of CCV formation at a molecular level, I have cloned and characterized the clathrin assembly protein gene from rat brain. In this study, the primary structure of CALM gene (rCALM) from the rat brain cDNA libraries and the differential expression between two monomeric adaptor proteins, AP180 and rCALM, in adult rat brain are presented.

Materials and Methods

Materials

Rat brain cDNA library was purchased from Stratagene. T7 sequencing kit was from US Biochemicals. Exo III deletion kit and Wizard miniprep kit were obtained from Promega. Restriction endonucleases were from Boehringer Mannheim. Nitrocellulose transfer membranes (BA85, 0.45 μ m) were from Schleicher and Schuell. Other chemicals were the highest purity available.

Cloning of rCALM gene from rat cDNA library

The rCALM gene was isolated from rat cDNA library using oligonucleotides, 5'-AGC CAG GTT GGC TGT GTA-3', which was designed from the third transmembrane segment of the glutamate receptor 1. About 7.0×10^5 plaques were screened with the 32 P end-labelled oligonucleotide probe. The plaques were transferred onto nitrocellulose membranes, immobilized, and hybridized with hybridization solution containing the probe. The membranes were washed with 0.2x SSC/ 0.1% SDS for 10 min three times at room temperature and followed by at 37°C for 10 min. The signal was visualized by exposure onto X-OMAT film overnight. The positive plaques were picked, and the second screening was performed as described above. The resulting two plaques, G12 and G18, were cultured and their phage DNA s were isolated. The clones were digested with *Eco* RI, shown to have 1.2 and 2.3 kb insert. Restriction mapping was carried out to make deletion mutant for the sequencing.

Sequencing and sequence analysis of cDNA

Basically the deletion mutants were prepared as previously described (Kim *et al.*, 1998). Briefly, the 5'-/3'-overhang DNA was made, and was digested with Exo III nuclease followed by S1 nuclease. The unidirectional deletion DNA was ligated and transformation was carried out. The plasmids DNA from the deletion clones were prepared by Wizard miniprep kit (Promega). The template DNA for the sequencing was prepared by alkali denaturation-neutralization of the double-stranded plasmid. The sequencing was carried out by using the Sequenase v.2.0 sequencing kit (Amersham). The sequencing data from the deletion mutants were analyzed by using the MacVector program from IBI Co.

Reverse transcription (RT) and PCR

Total RNA was isolated from a 3 month-old rat cerebrum by using the acid guanidium phenol method (Chomczynski and Sacchi, 1987). Approximately 5 μ g of total RNA was incubated at 37°C for 60 min in 50 μ l volume containing 10 units of MMLV reverse transcriptase, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 0.5 mM dNTP, 0.1 μ g of random hexamer primers. After incubation, 10 μ l of the first-strand reaction mixture was

amplified in a 50 μ l reaction mixture that included 10 pmoles of upstream primer (ASU: 5'-CAG GGG GAA TAA TGA CTG CACC-3') and of downstream primer (ASD: 5'-TGA AGG ATG TGG CTG TGT AAC C-3'), for 35 cycles (10 sec at 94°C, 10 sec at 50°C, and 0.5 min at 72°C) in Thermocycler Model 9600 (Perkin Elmer). The PCR products were separated on 3% agarose (Agarose : NuSieve = 2 : 1) gel. To verify the alternative splicing, the PCR products were cloned into T-vector and the sequencing was carried out as previously described.

In situ hybridization histochemistry

The hybridization probe for rCALM was prepared from pGEM plasmid containing 615 bp fragment of rCALM cDNA (*Pst* I and *Eco* RI fragment, nucleotide residues 1095-1700) by transcribing with appropriate RNA polymerases using a Riboprobe System (Promega Co.) in the presence of α -[35 S]UTP (1000-1500 Ci/mmol, New England Nuclear). The 645 bp-sized AP180 cDNA (nucleotide residues 2222-2866) was prepared by RT PCR of rat brain cDNA and labelled with [35 S]UTP as above. *In situ* hybridization histochemistry was performed essentially as previously described (Kim *et al.*, 1992). Briefly, frozen rat brain sections (12 μ m thick) were cut, thaw-mounted onto gelatin-coated slides. The sections were fixed in 4% paraformaldehyde, treated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8.0) to reduce nonspecific hybridization due to electro-static force, dehydrated and defatted in ethanol and chloroform, and finally air-dried. The sections were hybridized overnight at 53°C with 5×10^6 cpm of labeled RNA probe per slide. Then the sections were washed in 2x SSC, treated with RNase A (20 mg/ml, Boehringer-Mannheim) for 30 min at 37°C and washed sequentially for 60 min in 2x SSC at 50°C, 60 min in 0.2x SSC at 55°C, and 60 min in 0.2x SSC at 60°C. After drying, the slides were processed for film (β -max Hyperfilm, Amershampharmacia) autoradiography by exposing 4 days.

Results and Discussion

Primary structure of clathrin assembly protein from rat (rCALM)

The clathrin assembly protein gene was found from the rat brain cDNA library by low stringency screening using the oligonucleotide designed from the third transmembrane segment of the glutamate receptor 1. Among 48 cDNA clones, two clones, G12 and G18, were shown to have high homology with N-terminal region of AP180 and CALM when they were searched with blast program of NCBI at NIH. The size of G12 and G18 were 1.2 and 2.3 kb respectively. G18 encompasses a full coding region sequences from putative translation initiation

GGGGTGGCGGACGCTGCAGAG ATG TCT GGC CAG AGC CTG ACG GAC CGA ATC ACC GCG GCC CAG 65	TTA AAA GCA CTA AAG GAA CAG CGT CTA AAA GAA CTT GCA AAG AAA CCG CAT ACC TCT TTA 1085
Met Ser Gly Gln Ser Leu Thr Asp Arg Ile Thr Ala Ala Gln 14	Leu Lys Ala Leu Lys Glu Gln Arg Leu Lys Glu Leu Ala Lys Lys Pro His Thr Ser Leu 354
CAC AGT GTC ACT GGC TCC GCG GTA TCT AAG ACA GTA TGC AAG GCC ACG ACC CAC GAG ATC 125	ACA ACT GCA GCC TCT CCT GTG TCC ACC TCA GCA GGG GGA ATA ATG ACT GCA CCA GCC ATC 1145
His Ser Val Thr Gly Ser Ala Val Ser Lys Thr Val Cys Lys Ala Thr Thr His Glu Ile 34	Thr Thr Ala Ala Ser Pro Val Ser Thr Ser Ala Gly Gly Ile Met Thr Ala Pro Ala Ile 374
ATG GGC CCC AAG AAA AAG CAC CTG GAC TAC TTA ATT CAG TGT ACA AAT GAG ATG AAT GTG 185	GAC ATA TTT TCT ACC CCT AGT TCT TCT AAC AGC ACA TCC AAG CTG CCA AAT GAC CTG CTT 1205
Met Gly Pro Lys Lys Lys His Leu Asp Tyr Leu Ile Gln Cys Thr Asn Glu Met Asn Val 54	Asp Ile Phe Ser Thr Pro Ser Ser Ser Asn Ser Thr Ser Lys Leu Pro Asn Asp Leu Leu 394
AAT ATC CCA CAG TTG GCA GAC AGT TTG TTT GAA AGA ACT ACT AAT AGT AGT TGG GTG GTG 245	GAT TTG CAG CAG CCA ACC TTT CAT CCA TCT GTC CAT GCT ATG TCA GCT GCT CCT CAG GTA 1265
Asn Ile Pro Gln Leu Ala Asp Ser Leu Phe Glu Arg Thr Thr Asn Ser Ser Trp Val Val 74	Asp Leu Gln Gln Pro Thr Phe His Pro Ser Val His Ala Met Ser Ala Ala Pro Gln Val 414
GTC TTC AAA TCA CTC ATT ACA ACT CAT CAT TTG ATG GTG TAT GGA AAC GAG CGT TTC ATT 305	GCA AGT ACA TGG GGA GAT GCT GTT GAT GAT GCT ATT CCA AGC TTA AAT CGT TTC CTC ACA 1325
Val Phe Lys Ser Leu Ile Thr Thr His His Leu Met Val Tyr Gly Asn Glu Arg Phe Ile 94	Ala Ser Thr Trp Gly Asp Ala Val Asp Asp Ala Ile Pro Ser Leu Asn Pro Phe Leu Thr 434
CAG TAT TTG GCT TCA AAG ACA ACA TTG TTT AAC TTA AGC AAC TTT TTG GAT AAA AGT GGA 365	AAA AGT AGT GGT GAT GTT CAC CTT CCT ATT TCT TCA GAT GTA TCC ACT TTT ACT ACT AGG 1385
Gln Tyr Leu Ala Ser Arg Asn Thr Leu Phe Asn Leu Ser Asn Phe Leu Asp Lys Ser Gly 114	Lys Ser Ser Gly Asp Val His Leu Pro Ile Ser Ser Asp Val Ser Thr Phe Thr Thr Arg 454
TTG CAA GGA TAT GAT ATG TCT ACA TTT ATT AGA CGA TAT AGT AGG TAC TCA AAT GAA AAG 425	ACA CCT ACT CAT GAA ATG TTT GTT GGA TTC AGT CCT TCT CCG GTT ACA CAG CCA CAT CCT 1445
Leu Gln Gly Tyr Asp Met Ser Thr Phe Ile Arg Arg Tyr Ser Arg Tyr Leu Asn Glu Lys 134	Thr Pro Thr His Glu Met Phe Val Gly Phe Ser Pro Ser Pro Val Thr Gln Pro His Pro 474
GCA GTT TCA TAC AGA CAA GTT GCA TTC GAT TTC ACA AAA GTG AAG AAG GGA GCT GAT GGA 485	TCA GCT GGC CTT AAT GTT GAC TTT GAA TCT GTT TTT GGA AAT AAG TCT ACG AAT GTT GCT 1505
Ala Val Ser Tyr Arg Gln Val Ala Phe Asp Phe Thr Lys Val Lys Arg Gly Ala Asp Gly 154	Ser Ala Gly Leu Asn Val Asp Phe Glu Ser Val Phe Gly Asn Lys Ser Thr Asn Val Ala 494
GTT ATG AGA ACA ATG AAC ACA GAA AAA CTG TTA AAA ACT GTA CCA ATT ATC CAA AAT CAA 545	GTA GAT TCT GGT GGT GGA CTT CTC AAA ACA CCA GTG GCC TCT CAG AAC CAG AGT CTT CCT 1565
Val Met Arg Thr Met Asn Thr Glu Lys Leu Leu Lys Thr Val Pro Ile Ile Gln Asn Gln 174	Val Asp Ser Gly Gly Gly Leu Leu Lys Pro Thr Val Ala Ser Gln Asn Gln Ser Leu Pro 514
ATG GAT GCA CTT CTT GAT TTT AAT GTT AAT AGT AAT GAA CTT ACA AAT GGG GTA ATA AAT 605	GTT GCC AAA CTT CCG CCT AAC AAA TTA GTG TCT GAT GAC TTG GAT TCA TCT TTA GCC AAC 1625
Met Asp Ala Leu Leu Asp Phe Asn Val Asn Ser Asn Glu Leu Thr Asn Gly Val Ile Asn 194	Val Ala Lys Leu Pro Pro Asn Lys Leu Val Ser Asp Asp Leu Asp Ser Ser Leu Ala Asn 534
GCT GCC TTC ATG CTC TTC TTT AAA GAT GCC ATT AGA CTA TTT GCA GCA TAC AAT GAA GGA 665	CTT GTG GGC AAT CTT GGC ATT GGA AAT GGA ACC ACT AAG AAT GAT GTA AGT TGC AGT CAA 1685
Ala Ala Phe Met Leu Leu Phe Lys Asp Ala Ile Arg Leu Phe Ala Ala Tyr Asn Glu Gly 214	Leu Val Gly Asn Leu Gly Ile Gly Asn Gly Thr Thr Lys Asn Asp Val Ser Cys Ser Gln 554
ATT ATT AAT TTA TTG GAA AAA TAT TTT GAT ATG AAA AAG AAC CAG TGC AAA GAA GGT CTT 725	CCA GGT GAA AAG AAG TTA ACT GGA GGA TCT AAG TGG CAA CCA AAG GTC GCA CCA ACA ACT 1745
Ile Ile Asn Leu Leu Glu Lys Tyr Phe Asp Met Lys Lys Asn Gln Cys Lys Glu Gly Leu 234	Pro Gly Glu Lys Lys Leu Thr Gly Gly Ser Asn Trp Gln Pro Lys Val Ala Pro Thr Thr 574
GAC ATC TAT AAG AAG TTT TTG ACT AGG ATG ACA AGA ATC TCA GAG TTT CTT AAA GTT GCA 785	GCC TGG AGT GCT GCA ACA ATG GCA CCC CCT GTA ATG GCC TAT CCT GCT ACT ACA CCA ACG 1805
Asp Ile Tyr Lys Lys Phe Leu Thr Arg Met Thr Arg Ile Ser Glu Phe Leu Lys Val Ala 254	Ala Trp Ser Ala Ala Thr Met Ala Pro Val Met Ala Tyr Pro Ala Thr Thr Pro Thr 594
GAG CAA GTT GGA ATT GAC AGA GGA GAT ATT CCA GAT CTT TCA CAG GCC CCC AGC AGT CTT 845	GGC ATG ATA GGA TAT GGA ATT CCT CCT CAG ATG GGA AGT GTA CCT GTA ATG ACA CAG CCA 1865
Glu Gln Val Gly Ile Asp Arg Gly Asp Ile Pro Asp Leu Ser Gln Ala Pro Ser Ser Leu 274	Gly Met Ile Gly Tyr Gly Ile Pro Pro Gln Met Gly Ser Val Pro Val Met Thr Gln Pro 614
CTT GAT GCT TTA GAA CAA CAT TTA GCT TCC TTG GAA GGG AAG AAA ATT AAA GAT TCC ACA 905	ACC TTA ATA TAC AGC CAG CCT GTC ATG AGA CCG CCA AAC CCC TTT GGC CCT GTA CCA GGA 1925
Leu Asp Ala Leu Glu Gln His Leu Ala Ser Leu Glu Gly Lys Lys Ile Lys Asp Ser Thr 294	Thr Leu Ile Tyr Ser Gln Pro Val Met Arg Pro Pro Asn Pro Phe Gly Pro Val Pro Gly 634
GCT GCA AGC AGG GCT ACA ACA CTT TCC AAT GCA GTC TCT TCT TTG GCA AGC ACT GGC CTA 965	GCA CAG ATA CAG TTT ATG TAA CTAGATGGAAGAGAATGGAATTACTCCAAGAATAGAAGTGCACAGGTGGCG 1997
Ala Ala Ser Arg Ala Thr Thr Leu Ser Asn Ala Val Ser Ser Leu Ala Ser Thr Gly Leu 314	Ala Gln Ile Gln Phe Met *** 640
TCT CTG ACC AAA GTG GAT GAA AGG GAA AAG CAG GCA GCA TTA GAG GAA GAA CAG GCT CGA 1025	ACTCCTTACTCCAGCAAAATCCAACCTGCTGCTCTAAGACTCTTCC 2048
Ser Leu Thr Lys Val Asp Glu Arg Glu Lys Gln Ala Ala Leu Glu Glu Glu Gln Ala Arg 334	

Figure 1. Complete nucleotide and deduced amino acid sequence of the rat CALM. Numbers refer to nucleotide and amino acid positions. Beneath the nucleotide sequence is the deduced amino acid sequence coded for by the open reading frame between nucleotide 24 and 2048. The alternatively spliced region is shown as bold. The arrows indicate the primers (ASU and ASD) to verify the alternatively splicing sequences. The possible phosphorylation sites are: casein kinase II (S-X-X-D/E, shown as open circles), cAMP-dependent protein kinase A (R-R-X-S, closed circle), protein kinase C (S/T-X-R/K, open square), Ca²⁺/calmodulin-dependent protein kinase (R-X-X-S/T, closed square). The above sequence has been deposited in the GenBank database under the accession number AF041373 (short form) and AF041374 (long form).

methionine (ATG) at position 30 through termination codon (TAA) located at position 1946 (Figure 1). The 3'-untranslated region (3'-UTR) terminates with a polyA tail preceded by an AAUAAA polyadenylation signal. Analysis of the deduced amino acid sequences of the rCALM did not reveal a signal sequences, or any regions of extensive hydrophobicity. G18 contains a full open reading frame encoding the clathrin assembly protein (short form of rCALM) with 597 amino acids. Because the nucleotide sequence of rCALM shows 129 bp-short comparing to that of CALM, PCR was carried out to determine whether alternative splicing exists in rCALM. The rCALM with 640 amino acids was shown to have an alternative splicing variant with 129 nucleotide insert at the middle of the transcript (long form of rCALM). Estimated molecular masses of short form and long form of rCALM are 63,792 and 69,280, respectively.

The primary structure of the rCALM, shows a remarkable homology to the murine and rat clathrin assembly protein AP180 (Zhou *et al.*, 1992; Morris *et al.*, 1993;

Zhou *et al.*, 1993) (Figure 2). The homology ranges from 97% to 28% in different portions of the protein. The most striking homology of more than 95% is found between the first 289 amino acids of rCALM and that of AP180. The rCALM protein was 256 amino acid short compared to AP180, the middle part of which is missing in rCALM. The amino acid sequences of the C-terminal half of the rCALM is different from that of AP180. The overall pI values of the rCALM and AP180 were 8.7 and 4.6, respectively. The missing 250 amino acids which correspond to the acidic middle region of AP180 caused the pI value of rCALM alkaline. AP180 is interrupted by acidic and proline rich middle domains.

The distribution of charged amino acids, proline and alanine suggest a two-domain structure of rCALM in contrast to the three-domain structure of AP180. The N-terminal region of 340 amino acids is highly charged and predominantly basic. Twenty five percent of the amino acids in the region is charged, 14% of which is basic amino acids. Especially the first 289 residues are

rCALM MSGQSLTDR I TAAQHSVTGS AVSKTVCKAT THEIMGPKKK HLDYL IQCTN 50	rCALM PHTSLTTAAS .P.VSTSAGG 367
hCALM MSGQSLTDR I TAAQHSVTGS AVSKTVCKAT THEIMGPKKK HLDYL IQCTN 50	hCALM PHTSLTTAAS .P.VSTSAGG 367
AP180 MSGQtLDR I aAAQySVTGS AVaraVCKAT THEvMGPKKK HLDYL IQaTn 50	AP180 tttattsaaa attaaappal difgdIfdsa Pe. .va. AAS kPdv. .Aps 583
rCALM EMNVNIPQLA DSLFERTTNS SWVVVFKSL I TTHLMVYGN ERF IQYLASR 100	rCALM IMTAPAIDIF STP. .SSSN STSKLPNDLL DLQQTFFHPS VHAMSAAPQV 414
hCALM EMNVNIPQLA DSLFERTTNS SWVVVFKSL I TTHLMVYGN ERF IQYLASR 100	hCALM IMTAPAIDIF STP. .SSSN STSKLPNDLL DLQQTFFHPS VhPMStAsQV 414
AP180 EtNVNIPQm A DtLFEraTNS SWVVVFKaLv TTHLMVhGN ERF IQYLASR 100	AP180 l. difgtDaF SsPrgaSpv peSsLtaDLL S VdAf. AAPsp 622
rCALM NTLFNL SNFL DKSGLQGYDM STF IRRYSRY LNEKAVSYRQ VAFDFTKVKR 150	rCALM ASTWG---. .----DAVD. DAIPSLNPFL TKSSGDVHL. PISSDVSTFT 452
hCALM NTLFNL SNFL DKSGLQGYDM STF IRRYSRY LNEKAVSYRQ VAFDFTKVKR 150	hCALM ASTWGdpf. .satvDAVD. DAIPSLNPFL TKSSGDVHL. sISSDVSTFT 459
AP180 NTLFNL SNFL DKSgshGYDM STF IRRYSRY LNEKaFSYRQ mAFDFarVkk 150	AP180 ASTas paka essgviDifg DA. Fg ... SSasetq PapqaVSsss 662
rCALM GADGVMRTMN TEKLLKTVPI IQNQMDALLD FNVNSNELTN GVINAAMFL 210	rCALM TRTPTHEMFV G. F. SPS. .P VTQPHPSAGL . .NVDFESVF GNK. STNVAV 495
hCALM GADGVMRTMN TEKLLKTVPI IQNQMDALLD FNVNSNELTN GVINAAMFL 210	hCALM TRTPTHEMFV G. F. tps. .P VaQPHPSAGL . .NVDFESVF GNK. STNViV 502
AP180 GADGVMRTmV peKLLKsmPI IQgQiDALLE FdVhpNELTN GVINAAMFL 210	AP180 asadI lagFg GsFmaPSttP VT. P. aqnnL qpN. .FEaaf GttptST. sss 707
rCALM FKDAIRLFAA YNEGI INLLE KYFDMKKNQC KEGLDIYKf LTRMTRISEF 250	rCALM VTQPHPSAGL . .NVDFESVF GNK. STNVAV DSG-----GG LLKPTVASQN 510
hCALM FKDAIRLFAA YHEGI INLLE KYFDMKKNQC KEGLDIYKf LTRMTRISEF 250	hCALM VaQPHPSAGL . .NVDFESVF GNK. STNViV DSGgfdelGG LLKPTVASQN 522
AP180 FKDI kLFac YndGvINLLE KfFeMKKgQC KdnLeIYKrF LTRMTRvSEF 250	AP180 VT. P. aqnnL qpN. .FEaaf GttptST. sss sS. .fdpsgd LLmPTmApag 726
rCALM LKVAEQVGID RGDIPDLSQA PSSLLDALEQ HLASLEGKKI KDSTAASRAT 300	rCALM QSLPVAKLPP NK. L VSDDLSSLA NLVGNLGI GN GTT. KN. DVS 551
hCALM LKVAEQVGID RGDIPDLSQA PSSLLDALEQ HLASLEGKKI KDSTAASRAT 300	hCALM QnLPVAKLPP sK. L VSDDLSSLA NLVGNLGI GN GTT. KN. DvN 563
AP180 LKVAdeVGID kGDI PDLtQA PSSLmetLEQ HLatLEGGK. 289	AP180 QpaPsvmvpP spamsasKgL gSD. LDSSLA sLVGNLGI s. GTTsKkgDIq 774
rCALM TLSNAVSSLA STGLSLTKVD EREKQAALEE EQARLKALKE QRLKELAKK. 349	rCALM CSQPGEKKLT GGSNWQPKVA PTTAWSAATM APPVMAYPAT TPT. 594
hCALM TLSNAVSSLA STGLSLTKVD EREKQAALEE EQARLKALKE QRLKELAKK. 349	hCALM wSQPGEKKLT GGSNcePKVA PTTAWNAATM APPVMAYPAT TPT. 606
AP180 pgnnegsgap spisksppat tvtspnstpa ktidtsppvd ifatasaaap 339	AP180 w. naGEKKLT GGaNWQPKVt PaT. WSAgv. .PPqgtvP. .PTsvppga 817
AP180 vssakpsdI IdIqpdfsga ragaapvpp ptggatawgD IlgedsIaal 389	rCALM GM IGYGIPPQ. MGSVPV MTQPTLIYSQ PVMRPPNPF 630
AP180 ssvpseapis dpfapepspp ttttepasas asattavtaa ttevdI fgda 439	hCALM GM IGYGIPPQ. MGSVPV MTQPTLIYSQ PVMRPPNPF 642
AP180 faaspgeapa aseगतapat papvaaI d csgndpfaps egsaeapeI 489	AP180 gapsvgqG. aGYGmPPagt gmtmPqQv Mfa. Q PmRPP. .FG 858
AP180 dI fampppet sapvvtptas tappvpatap spaptavaat aatttaaaaa 539	rCALM P. .VPG.AQI. QFM* 640
	hCALM P. .VsG.AQI. QFM* 652
	AP180 aaaVPGtQI s psptpatqsp kkpakdIa dInIkdfI* 896

Figure 2. Amino acid sequence comparison between the rat CALM and the human CALM and rat AP180. There is high degree of homology (more than 90%) between the genes from amino acids 1 to 289. The interrupted dots show the very different region, and the low degree of identity (around 30%) was shown at the C-terminal region. Differences between the sequences are indicated as small letter. The dashed line shows the missing of the amino acids and asterisk shows the translation-termination of amino acid.

almost identical to the corresponding sequences of AP180 from rat. Functional studies of the AP180 have shown that a clathrin-binding domain resides in the 33 kD N-terminal portion of the protein. This 33 kD region is thought to have a globular structure and consists mainly of helices (Ye and Lafer, 1995). Although this N-terminal domain is able to bind clathrin triskelia, it is unable to assemble them into clathrin cages and bind to preassembled cages. The assembling function resides in the 58 kD C-terminal region of the AP180. The high conservation of N-half amino acid of the rCALM suggests that the protein has similar clathrin binding properties. Another important function of the 33 kD N-terminal region is its high affinity binding of both inositol hexakisphosphate and diphosphoinositol pentakisphosphate (Norris *et al.*, 1995; Ye *et al.*, 1995). Binding of these ligands inhibits the ability of AP180 to assemble clathrin into cages (Ye *et al.*, 1995). The binding site for the inositide was suggested to be KKK at N-terminal side, that is well conserved in rCALM and AP180. Thus, inositol polyphosphates and phosphatidyl inositides may regulate clathrin-coated vesicle assembly and/or disassembly by means of the monomeric clathrin assembly proteins,

rCALM and AP180. Prasad (1995) reported that myelin basic protein would be a novel clathrin assembly protein. The protein has 23% of charged amino acids, and 30 out of 38 charged amino acids are basic. The highly charged and basic character of N-half of the rCALM is similar with that of myelin basic protein.

Analysis of deduced amino acid composition revealed an unusually large number of proline residues (45 prolines /640 residues). Most (36 out of 45) of the prolines are localized at the C-terminal half of the rCALM, where the proline levels approach 12.5%, while the proline composition is just 1.6% at the N-terminal half. In the case of AP180, which has three domain structures, the middle and C-terminal domain is rich in proline (15%). There are multiple PXXP amino acid sequences, which is a known ligand for SH3 domain. Amphiphysin and other proteins containing SH3 domains would participate in the regulation of the clathrin cage formation. The polyproline could be the ligand for the WW domain. Although the typical sequence of XPPXY was not found in the rCALM, there are XPPX sequences that could bind WW domain. Considering the role of WW domain containing protein, such as dystrophin, in the

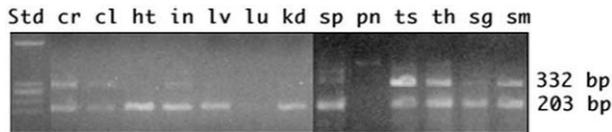


Figure 3. Differential expression of the clathrin assembly protein transcript in various tissues of rat. The oligonucleotides used for PCR are indicated by arrows in Figure 1. The alternative splicing sites were amplified from the mRNA of adult rat tissues by using RT-PCR as described in 'Materials and Methods', and analyzed on 3% agarose (Agarose: NuSieve = 2:1) gel. The left lane indicates Φ X174 Hae III size marker. The sizes of the upper and lower band are 332 bp and 203 bp respectively. The tissues from the left are: cr, cerebrum, cl; cerebellum, ht; heart, in; intestine, lv; liver, lu; lung, kd; kidney, sp; spleen, pn; pancreas, ts; testis, th; thymus, sg; salivary gland, sm; skeletal muscle.

interaction between cytoskeleton and membrane structure (Sudol, 1996), the rCALM and AP180 are important in the transport of vesicle through the bridge-formation between cytoskeleton and CCV. The possible function of the marked different region of the C-terminal half could be an interaction site(s) with proteins in the plasma membrane, possibly contributing to the regulation of the endocytotic activity and receptor turnover of the cell.

There are eight possible phosphorylation sites; three casein protein kinase II and protein kinase C, one cAMP-dependent protein kinase A, and one Ca^{2+} /calmodulin kinase. Except one protein kinase C site, most of the kinase substrate sites are located on the N-terminal half of the protein. where prolines and alanines are rare. The AP180 protein was known as a neuron-specific phosphoprotein (Keen and Black, 1986). High degree of homology of rCALM with AP180 suggested that the rCALM would be phosphorylated *in vivo*. The major role of AP180 would be the formation of uniform-sized vesicle in the nervous system (McMahon, 1999). Considering the ubiquitous expression of the rCALM and inhibitory activity of phosphorylated AP-1 and AP-2 (Wilde and Brodsky, 1996) on the formation of clathrin cage, the phosphorylation of the rCALM would be involved in the regulation of the uniform-sized clathrin cage formation in non-nervous systems.

Alternatively splicing

To determine the differential expression of the alternatively splicing variants, RT-PCR was carried out. The difference between the long and short form of rCALM was 129 nucleotide insertion or deletion by alternatively splicing. The short form was shown as 203 bp-sized PCR product, that was observed in most of the tissues examined (Figure 3). The 332 bp PCR product, the long form of the rCALM, was not observed in heart, lung, and pancreas. The rCALM might have a complementary role in the formation of uniform-sized CCV in neural and non-neural tissues. But there was no functional domains so far known in the alternatively spliced segment. The

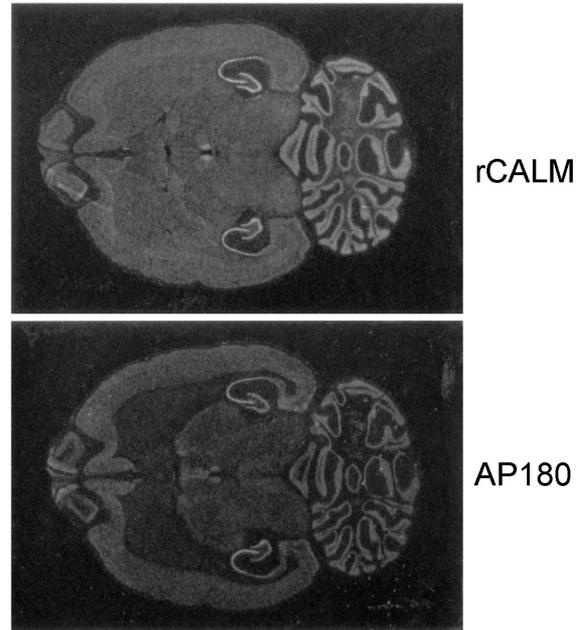


Figure 4. Localization of rCALM and AP180 in adult rat brain by *in situ* hybridization. Both figures show the negative film image of the expression of rCALM (shown upper) and AP180 (lower) in the transverse section of adult rat brain. *In situ* hybridization was carried out as described in 'Materials and Methods'.

exact functional role of the differential expression remains to be studied.

Expression in adult rat brain

The expression pattern of the rCALM gene was determined in adult rat brain by *in situ* hybridization histochemistry. The rCALM riboprobe was 615 bp-sized *Pst* I and *Eco* RI fragment (nucleotide residues 1,095-1700) from G18 cDNA, which encodes a unique sequence compared to that of other types of assembly proteins, in order to avoid a cross reaction with other assembly protein mRNAs. The riboprobe of AP180 was also designed from the unique 3'-end lower one third sequences. To establish the specificity of the labelled probe, control experiments were conducted as follows. First, a control hybridization using a sense probe resulted in autoradiograms virtually devoid of signal. Second, hybridization signal from the tissue was abolished by addition of RNase in the hybridization solution. Figure 4 shows the transverse section of the adult rat brain. The expression of the rCALM mRNA was more intense than that of AP180 and the distribution pattern was different each other. The rCALM mRNA was widely expressed throughout the brain. High levels of rCALM were found on the hippocampus, dentate gyrus, medial habenula nucleus, and cerebellar granule cells. Relatively high levels of expression were observed in olfactory bulb, and cerebral cortex. Moderate densities were in the

caudate-putamen, thalamus, and superior colliculus. The AP180 gene was expressed moderately in cerebral cortex, olfactory bulb, hippocampus, dentate gyrus, and cerebellar cortex, while it was faintly expressed in caudate-putamen. From this different distribution pattern, it may be suggested that these two monomeric clathrin assembly proteins function differently; AP180 would work as a neuronal synaptic vesicle assembly protein, and rCALM might assemble endocytic vesicles in neural and supporting cells in brain.

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