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Molecular Cloning of Novel Clathrin Assembly Protein Gene from Rat Brain

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= Abstract =

Binding of clathrin assembly protein to clathrin triskelia induces their assembly into clathrin-coated vesicle in neurons. The clathrin binding protein is a neuronal-specific, synapse associated protein that is expressed nonuniformly in rat brain. We isolated two cDNAs, encoding the novel clathrin assembly protein, which has a 73% amino acid homology compared with that of AP180 protein when translated into amino acids. The deduced molecular weight is 64kD. The N-terminal domain harbouring clathrin binding site is very similar to that of AP180, and the C-terminal domain is much more different with that of AP180, which suggests that the novel protein mediates the assembly of clathrin and its regulatory role in the release of secretory vesicle.

KEY WORDS : Clathrin assembly protein · Clathrin-coated vesicle · Gene cloning · AP180 · AP-3.

Introduction

Clathrin-coated vesicles(CCV) are involved in pathways of receptor-mediated intracellular transport[1]. The protein coats of CCV have been well characterized. The major coat protein is a clathrin, which consists of triskelion having three identical heavy chains and three light chains. Coated vesicle also contains one or more of the assembly proteins : AP-1, AP-2, AP-3, or auxillin[2]. The assembly proteins are adaptors and are believed to link receptors to the clathrin network. The assembly proteins all share the property that they promote to assemble clathrin triskelia into homogenous population of clathrin cage in solutiuon. The AP-1 and AP-2 are tetramer, whereas AP3 and auxillin are monomer and have so far been described only in neural tissues[3][4]. Apart from their effects on the polymerization of clathrin, the function of auxilin and AP180 are unknown.

AP-3 is well studied because AP-3 is simple system and synapse specific[5]. The molar concentration of AP 180 in brain coated vesicle preperation is about half of the plasma membrane adaptor and three times that of the Golgi adaptor and auxilin. In bovine brain only 30% of AP180 are associated with membranes, and the remaining forms a cytosolic pool. AP-3 was independently discovered, and known as AP180, F1-20, and pp155[5][6][7]. AP-3 is a neuronal specific phosphoprotein and glycoprotein, which is unusually acidic, and migrates anormalously with Mr of 155-185kDa, although its molecular weight appeared to be shown 112 to 124kDa, as determined by other techniques[5][8]. AP180 has a very low extinction coefficient, it is poorly stained by Coomasssie blue and is very sensitive to proteolytic attack. AP180 is phosphorylated at serine

residues *in vivo* and *in vitro*. The native protein was shown to be a monomer that interacts with clathrin triskelia with a stoikiometry of one[9] and thereby induces clathrin assembly into a homogenous population of 60-70nm coats. AP180 is about four times more active in inducing clathrine assembly than adaptor complexes or auxilin. Two primary functional activities are clathrin assembly protein and high affinity receptor for specific inositol polyphosphates[10][11][12]. To understand the complex function of CCV at a molecular level, we have cloned and characterized the novel clathrin assembly protein gene from rat brain. Here we report the primary structure of AP180 deduced from sequencing rat brain cDNA clones.

Materials and Methods

1. Materials

Rat brain cDNA libray was purchased from Stratagene. T7 sequencing kit was from US Biochemicals. ExoIII deletion kit and Wizard miniprep kit for plasmid purification were obtained from Promega. Restriction endonucleases were from Boerhinger Manheim. Nitrocellulose transfer membranes(BA85, 0.45 μm) were from Schleicher and Schuell. Other chemicals were the highest purity available.

2. Cloning of AP180 gene from rat cDNA library

AP180 gene was isolated from rat cDNA library using oligonucleotides which is from glutamate receptors, 5'-AGC CAG GTT GGC TGT GTA-3'. About 7.0×10^5 plaques were screened with the oligonucleotide probe which was end-labelled with γ-^{32}P-ATP. The plaques were transferred onto nitrocellulose paper, immobilized, and hybridized with hybridization solution containing the probe. The membrane was washed with 0.2x SSC/0.1% SDS for 10min three times at RT and followed by at 37℃ for 10min. The signal was visualized by exposure onto X-OMAT film overnight. The positive plaques was picked, and second screening performed as above. The resulting two plaques, G12 and G18, were cultured and their phage DNAs were isolated. The clones were digested with Eco RI, shown to have 1.1 and 2.3kb insert. Restriction mapping was carried out to make deletion mutants for sequencing.

2. Sequencing and sequence analysis of cDNA

Basically the deletion mutants were preparaed by manufacturer's manual(Promega). The 5'-/3'-overhang DNA was made and digested with Exo III nuclease followed by S1 nuclease. The unidirectional deletion DNA was liagated and transformation was carried out. Resulting plasmids DNA from the deletion clones were prepared by Wizard miniprep kit (Promega). The sequening template DNA was prepared by alkali denaturation-neutralization of the double-stranded plasmid. The sequencing was carried out by using the dideoxy-termination method of Sanger, the Sequenase v.2.0(Amersham). The sequencing data from the deletion mutants were analyzed by using the MacVector program from IBI Co.

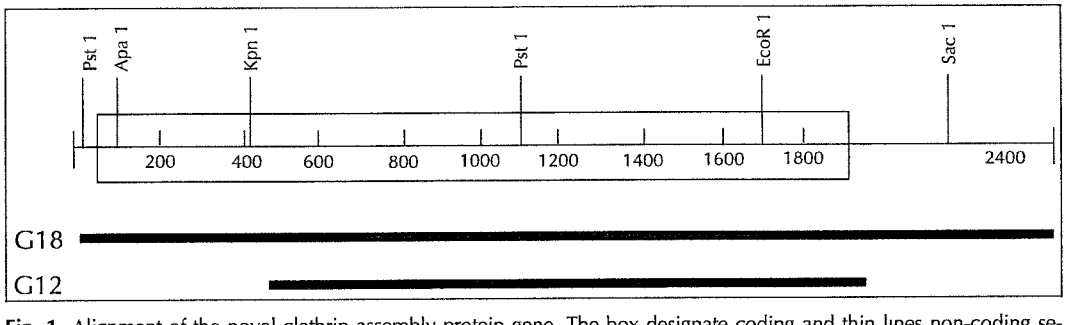

Fig. 1. Alignment of the novel clathrin assembly protein gene. The box designate coding and thin lines non-coding sequence. Both clones were obtained from λZap II expression library by screening with oligonucleotide from glutamate receptor, 5'-agc cag gtt ggc tgt gta-3'. The clone 18 contains an initiation codon and the clone 12 contains nucleotides just before termination codon.

Result and Discussion

The noble clathrin assembly protein gene was found by low stringency screening of glutamate receptor gene using the oligonunleotide probe encoding the common region of the glutamate receptors, nucleotide sequence 1825-1842 from the third transmembrane segment. Among the 48 cDNA clones two clones, G12 and G18, were shown to have high homology with N-terminal region of AP180 and AP-3 when they were searched with blastn program of NCBI at NIH. G12 and G18 were 1.1 and 2.4 kB sized clone respectively(Fig. 1). G18 contains full open reading frame encoding the novel clathrin assembly protein of a molecular mass of 63,792(Fig. 2).

The 597 amino acids show a remarkable homology to the murine and rat clathrin assembly protein AP-3 when translated into amino acids[6)7)10)](Fig. 3). The homology ranges from 18% to 84% in different portions of the protein. The most striking homology of more than 85% is found between first 300 amino acids of the novel protein and AP180. The mouse, rat, and the bovine AP-3 are highly homologous to each other with more than 97% identical amino acids. But there was no significant nucleotide homology to the murine AP-3[7)]. The N-terminal domain harbouring clathrin binding and inositol polyphosphate binding site is very similar with that of AP180[9)]. Amino acid sequences of middle and the C-terminal domain where clathrin cage binds are different from that of AP180.

AP180 was first discovered as a specific coat component of clathrin-coated vesicles from neural tissue[2)]. It binds to the clathrin heavy chain with a stoichiometry of 1 per triskelion[2)], and therby promotes very efficient assembly of clathrin into regular coat structure.

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GAGGGGTGGC GGACGAGCTG CAGAGATGTC TGGCCAGAGC CTGACGGACC GAATCACCGC GGCCCAGCAC AGTGTCACTG GCTCCGCGGT ATCTAAGACA    100
                        M  S  G  Q  S  L  T  D  R  I  T  A  A  Q  H  S  V  T  G  S  A  V  S  K  T>    25
GTATGCAAGG CCACGACCCA CGAGATCATG GGCCCCAAGA AAAAGCACCT GGACTACTTA ATTCAGTGTA CAAATGAGAT GAATGTGAAT ATCCCACAGT    200
 V  C  K  A  T  T  H  E  I  M  G  P  K  K  K  H  L  D  Y  L  I  Q  C  T  N  E  M  N  V  N  I  P  Q>    58
TGGCAGACAG TTTGTTTGAA AGAACTACTA ATAGTAGTTG GGTGGTGGTC TTCAAATCAC TCATTACAAC TCATCATTTG ATGGTGTATG GAAACGAGCG    300
 L  A  D  S  L  F  E  R  T  T  N  S  S  W  V  V  V  F  K  S  L  I  T  T  H  H  L  M  V  Y  G  N  E  R>    92
TTTCATTCAG TATTTGGCTT CAAGAAACAC ATTGTTTAAC TTAAGCAACT TTTTGGATAA AAGTGGATTG CAAGGATATG ATATGTCTAC ATTTATTAGA    400
  F  I  Q  Y  L  A  S  R  N  T  L  F  N  L  S  N  F  L  D  K  S  G  L  Q  G  Y  D  M  S  T  F  I  R>    125
CGATATAGTA GGTACCTAAA TGAAAAGGCA GTTTCATACA GACAAGTTGC ATTCGATTTC ACAAAAGTGA AGAGAGGAGC TGATGGAGTT ATGAGAACAA    500
 R  Y  S  R  Y  L  N  E  K  A  V  S  Y  R  Q  V  A  F  D  F  T  K  V  K  R  G  A  D  G  V  M  R  T>    158
TGAACACAGA AAAACTGTTA AAAACTGTAC CAATTATCCA AAATCAAATG GATGCACTTC TTGATTTTAA TGTTAATAGT AATGAACTTA CAAATGGGGT    600
 M  N  T  E  K  L  L  K  T  V  P  I  I  Q  N  Q  M  D  A  L  L  D  F  N  V  N  S  N  E  L  T  N  G  V>    192
AATAAATGCT GCCTTCATGC TCCTGTTCAA AGATGCCATT AGACTATTTG CAGCATACAA TGAAGGAATT ATTAATTTAT TGGAAAAATA TTTTGATATG    700
  I  N  A  A  F  M  L  L  F  K  D  A  I  R  L  F  A  A  Y  N  E  G  I  I  N  L  L  E  K  Y  F  D  M>    225
AAAAAGAACC AGTGCAAAGA AGGTCTTGAC ATCTATAAGA AGTTTTTGAC TAGGATGACA AGAATCTCAG AGTTTCTGAA AGTTGCAGAG CAAGTTGGAA    800
 K  K  N  Q  C  K  E  G  L  D  I  Y  K  K  F  L  T  R  M  T  R  I  S  E  F  L  K  V  A  E  Q  V  G>    253
TTGACAGAGG AGATATTCCA GATCTTTCAC AGGCCCCCAG CAGTCTTCTT GATGCTTTAG AACAACATTT AGCTTCCTTG GAAGGGAAGA AAATCAAAGA    900
I  D  R  G  D  I  P  D  L  S  Q  A  P  S  S  L  L  D  A  L  E  Q  H  L  A  S  L  E  G  K  K  I  K  D>    287
TTCCACAGCT GCAAGCAGGG CTACAACACT TTCCAATGCA GTCTCTTCTT TGGCAAGCAC TGGCCTATCT CTGACCAAAG TGGATGAAAG GGAAAAGCAG   1000
  S  T  A  A  S  R  A  T  T  L  S  N  A  V  S  S  L  A  S  T  G  L  S  L  T  K  V  D  E  R  E  K  Q>    315
GCAGCATTAG AGGAAGAACA GGCTCGATTA AAAGCACTAA AGGAACAGCG TCTAAAAGAA CTTGCAAAGA AACCGCATAC CTCTTTAACA ACTGCAGCCT   1100
 A  A  L  E  E  E  Q  A  R  L  K  A  L  K  E  Q  R  L  K  E  L  A  K  K  P  H  T  S  L  T  T  A  A>    348
CTCCTGTGTC CACCTCAGCA GGGGGAATAA TGACTGCACC AGCCATCGAC ATATTTTCTA CCCCTAGTTC TTCTAACAGC ACATCCAAGC TGCCAAATGA   1200
S  P  V  S  T  S  A  G  G  I  M  T  A  P  A  I  D  I  F  S  T  P  S  S  S  N  S  T  S  K  L  P  N  D>    382
CCTGCTTGAT TTGCAGCAGC CAACCTTTCA TCCATCTGTC CATGCTATGT CAGCTGCTCC TCAGGTAGCA AGTACATGGG GAGGATTCAG TCCTTCTCCG   1300
  L  L  D  L  Q  Q  P  T  F  H  P  S  V  H  A  M  S  A  A  P  Q  V  A  S  T  W  G  G  F  S  P  S  P>    415
GTTACACAGC CACATCCTTC AGCTGGCCTT AATGTTGACT TTGAATCTGT GTTTGGAAAT AAGTCTACGA ATGTTGCTGT AGATTCTGGT GGTGGACTTC   1400
 V  T  Q  P  H  P  S  A  G  L  N  V  D  F  E  S  V  F  G  N  K  S  T  N  V  A  V  D  S  G  G  G  L>    448
TCAAACCAAC AGTGGCCTCT CAGAACCAGA GTCTTCCTGT TGCCAAACTT CCGCCTAACA AATTAGTGTC TGATGACTTG GATTCATCTT TAGCCAACCT   1500
L  K  P  T  V  A  S  Q  N  Q  S  L  P  V  A  K  L  P  P  N  K  L  V  S  D  D  L  D  S  S  L  A  N  L>    482
TGTGGGCAAT CTTGGCATTG GAAATGGAAC CACTAAGAAT GATGTAAGTT GCAGTCAACC AGGTGAAAAG AAGTTAACTG GAGGATCTAA CTGGCAACCA   1600
  V  G  N  L  G  I  G  N  G  T  T  K  N  D  V  S  C  S  Q  P  G  E  K  K  L  T  G  G  S  N  W  Q  P>    515
AAGGTCGCAC CAACAACTGC CTGGAGTGCT GCAACAATGG CACCCCCTGT AATGGCCTAT CCTGCTACTA CACCAACGGG CATGATAGGA TATGGAATTC   1700
 K  V  A  P  T  T  A  W  S  A  A  T  M  A  P  P  V  M  A  Y  P  A  T  T  P  T  G  M  I  G  Y  G  I>    548
CTCCTCAGAT GGGAAGTGTA CCTGTAATGA CACAGCCAAC CTTAATATAC AGCCAGCCTG TCATGAGACC GCCAAACCCC TTTGGCCCTG TACCAGGAGC   1800
P  P  Q  M  G  S  V  P  V  M  T  Q  P  T  L  I  Y  S  Q  P  V  M  R  P  P  N  P  F  G  P  V  P  G  A>    582
ACAGATACAG TTTATGTAAC TAGATGGAAG AGAATGGAAT TACTCCAAGA ATAGAAGTGC ACAGGTGGCG ACTCCTTACT TCCAGCAAAA TCCAAACTGC   1900
  Q  I  Q  F  M>                587
TGTCTCTAAG ACTCTTCCTC C         1921
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Fig. 2. cDNA and the deduced amino acid sequence of the novel clathrin assembly protein. Numbers refer to nucleotide positions. Beneath the nucleotide sequence is the deduced amino acid sequence coded for by the open reading frame between nucleotide 26 and 1816.

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G18       1  MSGQSLTDRI TAAQHSVTGS AVSKTVCKAT THEIMGPKKK HLDYLIQCTN   50
AP180     1  MSGQtLTDRI aAAQySVTGS AVaraVCKAT THEvMGPKKK HLDYLIQaTN   50

G18      51  EMNVNIPQLA DSLFESTTNS SWVVVFKSLI TTHHLMVYGN ERFIQYLASR  100
AP180    51  EtNVNIPQmA DtLFEraTNS SWVVVFKaLv TTHHLMVhGN ERFIQYLASR  100

G18     101  NTLFNLSNFL DKSGLQGYDM STFIRRYSRY LNEKAVSYRQ VAFDFTKVKR  150
AP180   101  NTLFNLSNFL DKSGshGYDM STFIRRYSRY LNEKAfSYRQ mAFDFarVKk  150

G18     151  GADGVMRTMN TEKLLKTVPI IQNQMDALLD FNVNSNELTN GVINAAFMLL  200
AP180   151  GADGVMRTMv pEKLLKsmPI lQgQiDALLe FdVhpNELTN GVINAAFMLL  200

G18     201  FKDAIRLFAA YNEGIINLLE KYFDMKKNQC KEGLDIYKKF LTRMTRISEF  250
AP180   201  FKDlIkLFAc YNdGvINLLE KfFeMKKgQC KdnLeIYKrF LTRMTRvSEF  250

G18     251  LKVAEQVGID RGDIPDLSQA PSSLLDALEQ HLASLEGKKI KDSTAASRAT  300
AP180   251  LKVAdeVGID kGDIPDLtQA PSSLmetLEQ HLatLEGKKp gnnegsgaps  300
             ----------------------------------------------------
             ----------------------------------------------------
G18     401  FHPSVHAMSA APQVASTWGG FSPSPVTQPH PSAGLNVDFE SVFG---NKS  427
AP180   654  gavSsssaSA dllagfggsf maPSttpvtp aqnnLqpnFE aaFGttpstS

G18     448  TNVAVDSGGG LLKPTVASQN QSLPVAKLPP ------NKLV SDDLDSSLAN  481
AP180   707  ssssfDpsGd LLmPTmApsg QpaPVsmvPP spasaskglg SD-LDSSLAs

G18     492  LVGNLGIGNG TT-KN-DVSC SQPGEKKLTG GSNWQPKVAP TTAWSAATMA  529
AP180   757  LVGNLGIs-G TTsKkgDlqw na-GEKKLTG GaNWQPKVtP aT-WSAgv--

G18     540  PPVMAYPATT PT-------- -------GMI GYGIPPQMGS VPVMTQPTLI  564
AP180   802  PPqgtvP--- PTssvppgag apsvgqpG-a GYGmPPagtg mtmMpQqPVM

G18     575  YSQPVMRPPN PFGP--VPGA QIQFM   587
AP180   848  faQPmMRPP- -FGaaaVPGt Qlsps   896
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Fig. 3. Amino acid sequence comparison between the novel clathrin assemby protein and the rat AP180 gene. There is high degree of homology(more than 80%) between the genes from amino acids 1 to 289. The interrupted dash lines show the very different region and the low degree of identity(around 40%) was shown at the C-terminal region. Difference between the rat and human sequences are indicated as small letter.

Clathrin-coated vesicles play a major role in receptor mediated endocytosis as well as in trans-Golgi network vesicle traffic. Functional studies of AP-3 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 consist mainly of alpha helices[9]. 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 ; this latter function resides in the 58-kD C-terminal region of AP-3, which has a lower homology to the novel protein.

Another important function of the 33-kD amino terminal region of AP-3 is its high affinity binding of both inositol hexakisphosphate and diphosphoinositol pentakisphosphate[11)12)]. Binding of either of these ligands inhibits the ability of AP-3 to assemble clathrin [12)]. Thus, inositol hexakisphosphate and diphosphoinositol pentakisphosphate may regulate clathrin-coated vesicle assembly and/or disassembly by means of the clathrin assembly protein AP-3. The possible function of the marked different region of the C-terminal half could be interation with proteins in the plasma membrane, possibly contributing to the regulation of the endocytic activity and receptor turnover of the cell. This also might be regulated by means of phophoinositol pathways.

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Molecular Cloning of Novel Clathrin Assembly Protein Gene from Rat Brain

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김 형 래·홍 영 숙

= 국 문 초 록 =

신경연접소포는 신경전달물질을 신경연접간극으로 이동시키는데 중요한 역할을 한다. 신경연접소포는 clathrin 단백질이 골격을 이루고, 이 clathrin을 중합체 형태로 만들어 유지하는데 여러 연결 단백질이 필요하다. Clathrin 연결단백질 중 AP180 단백질은 신경세포에 특이적이며 신경연접 부위에만 존재한다. 신경전달물질의 유리과정의 조절 기전을 이해하는데 신경연접소포를 구성하는 연결단백질의 분자적 특성을 규명하는 것이 중요하다. 본 연구진은 새로운 형태의 clathrin 연결단백질을 클론닝하였다. 분리한 유전자의 핵산염기열로부터 아미노산 및 단백질 구조를 분석한 결과 이는 새로운 clathrin 단백질을 코드하며 그 분자량이 6만 4천으로 추정된다. 이 단백질은 AP180 단백질과 약 73%의 유사성을 보였으며 특히 아미노기 말단은 아미노산 서열이 84%나 동일하였고 카르복시기 말단은 약 50% 동일하여 이 새로운 단백질은 clathrin의 중합체 형성에 중요한 역할을 하며 소포의 이동 및 분비과정의 조절에 다른 중요한 역할을 하리라 생각된다.