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Cleavage of purified neuronal clathrin assembly protein (CALM) by caspase 3 and calpain

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Abbreviations: CCV; clathrin-coated vesicle, CALM; clathrin assembly protein lymphoid myeloid, FPLC; fast performance liquid chromatography

Abstract

The most efficient means of protein internalization from the membrane are through clathrin-coated pits, which concentrate protein interactions with the clathrin-associated assembly protein complex AP-2 and internalization signals in the cytoplasmic domain of transmembrane proteins. Binding of clathrin assembly protein to clathrin triskelia induces their assembly into clathrin-coated vesicles (CCVs). Due to a difficulty of isolating clathrin molecules from their complex or assembly state in the cells, most of the studies were carried out with recombinant clathrin proteins, which may present different conformation and structural variation. In this study, we have developed an efficient method of isolating the native clathrin assembly protein lymphoid myeloid (CALM) from the bovine brain that is enriched with clathrin and clathrin associated proteins and characterized by their sensitivity to proteases and it's ability to form CCV. The purified CALM has molecular weight of approximately 100,000 dalton on SDS-PAGE, which is consistent with the result of *in vitro* translation. The purified CALM protein could promote the assembly of clathrin triskelia into clathrin cage, and cleaved CALM proteolysed by caspase 3 and calpain could not promote them. In this respect, our data support a model in which CALM functions like AP180 as a monomeric clathrin assembly protein and might take part in apoptotic process in neuronal cells.

Keywords: clathrin-coated vesicle, CALM, caspase, expression

Introduction

Clathrin-mediated vesicle formation is an essential step in the intracellular trafficking of the protein and lipid. Most transport vesicles form specialized coated regions of membranes and bud off as coated vesicles with a distinctive vesicle proteins (Albert *et al.*, 1994). Clathrin is the major component of the coat forming protein, a highly ordered structure on the cytoplasmic surface of the vesicle. Soluble clathrin (~650 kDa) is composed of three identical 160 kDa heavy chains and three 22-28 kDa light chains. These three heavy and light chain complexes form three lagged trimers, called clathrin triskelion. This triskelion assembles into a basketlike framework of hexagons and pentagons to form coated pits on the cytoplasmic surface of membrane.

The second major coat proteins are adaptor proteins (AP, assembly or adaptor proteins). Clathrin assembly proteins belong to two gene family, the tetrameric AP family or the monomeric AP family. Tetrameric APs have been classified as AP-1 (Ahle and Ungewickell, 1986), AP-2 (Beck *et al.,* 1992), AP-3 (Faundez *et al*., 1998), and AP-4 (Dell'Angelica *et al*., 1999). AP-1 and AP-2 were first characterized (Ahle and Ungewickell, 1986) as the major clathrin coated vesicle adaptor proteins. AP-1 localizes to clathrin-coated vesicles budding from Golgi membrane. While AP-2 localizes to clathrin-coated vesicles budding from plasma membrane, AP-3 has only recently been identified and also appeared to be associated with clathrin coated protein (Foudez *et al.*, 1998). AP-3 plays a role in trafficking from trans-Golgi network to the lysosome (Simpson *et al.,* 1997). AP-4 has been identified but not characterized in detail.

Monomeric APs are AP180 and CALM (Clathrin Assembly Lymphoid Myeloid leukemia gene). AP180 localizes to synapse (Stephan *et al*., 1990), while CALM is expressed in most tissues (Dreyling *et al*., 1996). The native AP180 interacted with clathrin triskelia and thereby induced clathrin assembly into a uniformly sized 60-70 nm coat structures. These appear to be somewhat smaller and sediment considerably more slowly than those containg AP-2 (80 nm) (Ye and Lafer, 1995). AP180 is an unusually acidic protein with an isoelectric point (pI) of 5.1 (Morris *et al*., 1993). AP 180 was considered to be phosphoprotein (Keen and Black, 1986; Bar-Zvi *et al*., 1988) and glycoprotein (Murphy *et al.,* 1994). AP180 contains high affinity binding sites for inositides, which inhibit their ability to promote clathrin assembly (Norris *et al*., 1995).

Recently, the AP180 homologous gene was reported

in human and rat tissue (Dreyling *et al*., 1995, Kim and Lee, 1999) and the properties of GST-CALM in bacterial system was reported (Kim *et al.,* 2000). The CALM protein expressed in bacteria has a molecular weight of 80 kDa. The protein could promote clathrin triskelia into clathrin cages and could bind the preformed clathrin cage. However, N-terminal domain of CALM with 33 kDa could not bind preassembled clathrin cages, but assemble clathrin triskelia into clathrin cages. The CALM protein could bind to SH3 domain through N-terminal domain *in vitro*.

Caspase exists as proenzyme in most cells including neurons, and is processed and activated to the heterodimeric forms by two distinct pathways of caspase 9 or caspase 8 (Nunez *et al.,* 1998). Caspase 3 has a finite number of cellular protein substrates, including cytoskeletal proteins, enzymes involved in signal transduction, cell-cycle proteins and nuclear DNA-repairing proteins (Deveraux and Reed, 1999).

Calpains could become over-activated with the elevation of cytosolic Ca^{2+} levels, which is generally associated with necrosis and some forms of apoptosis. Calpain preferentially cleaved the site of (V, I, L)x. As expected, most proteins contain sites that are susceptible to the proteolysis of calpain, but only a small part of cellular proteins has been reported to be vulnerable, while the majority of cellular proteins are resistant. Calpain substrates include cytoskeletone-associated proteins, signal transduction and calmodulin-dependent proteins and transcription factors. Recently, it was generally accepted that chronic and acute degenerative neuronal cell deaths were necrotic and apoptotic deaths simultaneously.

The CALM protein is proteolyzed by caspase 3, 8 and calpain through C-terminal domain. Although molecular properties of the CALM were elucidated using the bacterial expression system, native forms of CALM in eukaryotes and its characteristics were not investigated yet. To understand the characteristics of CALM protein in mammalian cells, we have purified the CALM protein from bovine brain, through CCV preparation, Sepharose CL-4B gel-filtration chromatography, Mono S and Superose 6 FPLC. The binding and molecular mechanism of the purified CALM were analyzed.

Materials and Methods

Materials

All buffers used in the protein work, even though it is not explicitly indicated in the citation, contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF). All chemicals were purchased from USB and Sigma. Centricon-30 was from Amicon. NBT/BCIP reagents were from Boehringer Mannheim. All chromatography resins and PVDF membrane were from Amersham Pharmacia. Monoclonal antibody against CALM was prepared by immunizing the bacterially expressed GST-CALM protein (Kim *et al*., 2000). Monoclonal antibody against AP180 was kindly provided by Dr. S. R. Kim. Caspase 3 was kindly provided by Dr. Y. G. Jung.

Preparation of clathrin-coated vesicles

All operations were performed at 4°C. Fresh bovine brain was stripped of meninges, chopped. The pieces were minced and homogenized with six passes of Potter-Elvejhem tissue grinder in an equal volume of ice-cold buffer to the weight of the brain. The homogenizing buffer consisted of 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES), 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% NaN_3 adjusted to pH 6.5 with sodium hydroxide. The homogenate was centrifuged at 19,000 *g* in a Sorval GSA rotor for 40 min. The supernatant was saved and centrifuged for 70 min at 43,000 *g* in a Bechman 45 Ti rotor. The pellet containing coated vesicles was resuspended in a small volume of homogenizing buffer by using a loose-fitting Dounce homogenizer. The resulting pellet is the crude coated-vesicle.

Extraction of coat proteins

The coated vesicles were extracted according to the procedure of Keen *et al*. The crude coated-vesicle was resuspended in the extraction buffer, which composed of 0.5 M Tris, 0.1 mM PMSF, pH 7.0, 2 mM dithiothreitol (DTT), 1 mM EDTA, and homogenized using Dounce glass homogenizer. The homogenate was stored at 4°C overnight, and clarified by ultracentrifugation at 100,000 *g* for 3 h.

Gel-filtration chromatography

The column (2x100 cm) was packed with Sepharose CL-4B (Pharmacia, Upsala, Sweden) and was equilibrated with the extraction buffer. The Tris extract was applied to the column. The column was eluted with the equilibration buffer with the flow rate of 0.5 ml/min. Aliquots of all fractions were analyzed by SDS-PAGE and immunoblot using anti-CALM monoclonal antibody. Fractions that contained the partially separated assembly proteins were pooled and concentrated by Centricon 30.

Mono S ion-exchange fast performance liquid chromatography (FPLC)

For further purification of assembly proteins, the fractions were chromatographed on Mono S FPLC column. The column was equilibrated with 50 mM Tris buffer pH 8.0, 1 mM EDTA, 1 mM DTT. The sample was applied at a flow rate of 0.5 ml/min. The column was subsequently washed with 50 ml of equilibration buffer, and then, eluted with linear gradient of 0-1000 mM NaCl in

equilibration buffer.

Superose 12 gel filtration FPLC

The sample from the cat ion-exchange chromatography was applied to the Superose 12 column equilibrated with the extraction buffer. Then the column was eluted with the same buffer.

Preparation of clathrin

Bovine clathrin was used for the binding of clathrin assembly proteins and the clathrin-cage formation. The procedures of extraction of coat proteins and gel-filtration were the same as above. The first protein peak was applied to the Superose 6 FPLC, and the major protein peak was used for the clathrin-assembly study.

Clathrin assembly assay

Three micro molar clathrin triskelia were dialyzed overnight at 4°C against isolation buffer (0.1 M MES, pH 6.7, containing 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.1 mM PMSF) with the addition of 20 μM assembly protein (purified CALM protein and GST). Following the 3 min centrifugation at 13,600 *g* to remove nonspecific aggregates, newly assembled clathrin cages were collected by ultracentrifugation for 20 min at 100,000 *g*. The pellet (P) and the supernatant (S) fractions were analyzed by SDS-PAGE, followed by Coomassie blue staining.

Proteolysis by caspase

One microgram of purified CALM protein was incubated with caspase 3 and control bacterial lysate in 20 μl of reaction buffer (16 mM HEPES, 8 mM NaCl, and 0.004% IGEPAL). The mixtures were incubated for 1 h at 30°C. The digestions of CALM were analyzed by immunoblotting using anti-CALM polyclonal antibody.

Results and Discussion

Purification of assembly proteins

CCVs were prepared as previously described (Kim and Kim, 2000). Clathrin and other membrane proteins were extracted with 0.5 M Tris and fractionated by gel filtration. Gel filtration on a Sepharose CL-4B column was chosen as a first step of purification procedure to separate clathrin and other adaptor proteins. The column profile and SDS-PAGE pattern of the fractions are shown in Figure 1A and 1B, respectively. The first large peak represents the clathrin fraction (fraction 39-55). The elution profile was distinct from that of rat liver because of a large number of synaptic vesicles.

Western blot analysis of the eluents revealed that the second broad peak represents the associated proteins, such as AP-2 (data was not shown), CALM (Figure 1C) and AP180 (Figure 1D). Especially AP180 and CALM were remained in almost the same fractions (fractions 63-72). Two immunoreactive bands shown in Figure 2C against CALM, 90 and 100 kDa might be ascribed to the alternatively splicing variants of the gene (Kim and Lee, 1999), which is consistent with our previous report (Kim and Kim, 2000). The parts containing CALM, representing fractions 63-72 in the above column, were pooled and dialyzed against 50 mM Tris pH 8.0 for the next step.

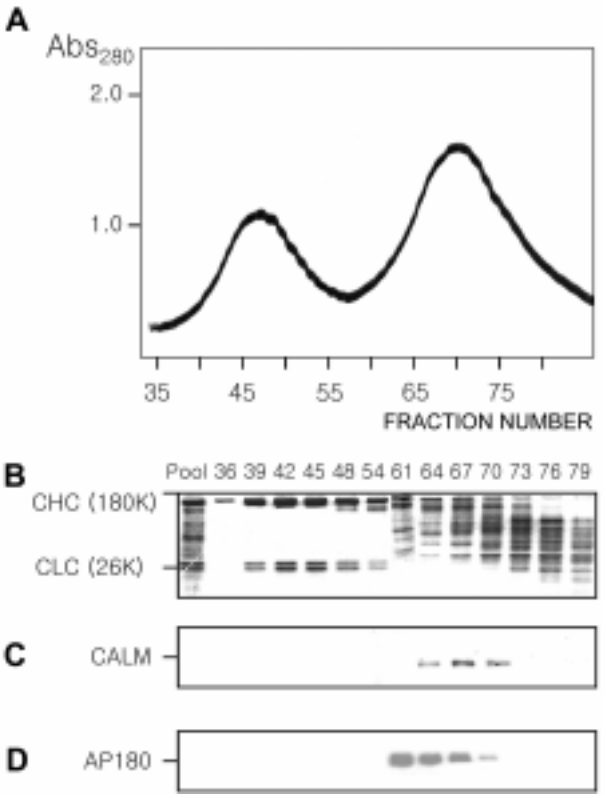

Figure 1. Gel filtration of solubilized coat protein. (A) The coated vesicles were incubated with 0.5 M Tris-HCl, 2 mM EDTA, 1 mM DTT, pH 7.0. The extracted proteins were clarified with ultracentrifugation and then subjected to gel-filtration on Sepharose CL-4B column. (B) Aliquots of fractions of gel filtration elution were analyzed by SDS-PAGE and the separated proteins were either stained for protein by Coomassie blue (B) or electro-blotted on to PVDF membrane and reacted with monoclonal antibody against CALM (C), and AP180 (D).

The estimated pI value of the CALM transcript would be 8.7, whereas that of AP180 was 4.5. (Kim and Lee, 1999). Therefore we chose cat-ion exchange chromatography for the next purification step. The fractions containing CALM were pooled and subjected to Mono S FPLC column. The column was equilibrated with 50 mM Tris buffer, pH 8.0. Bound sample was eluted with linear gradient of 0-1000 mM NaCl in equilibration buffer. As shown in Figure 2, more than two third of the proteins were washed out, and the CALM preferentially eluted at the concentration of about 350 mM NaCl.

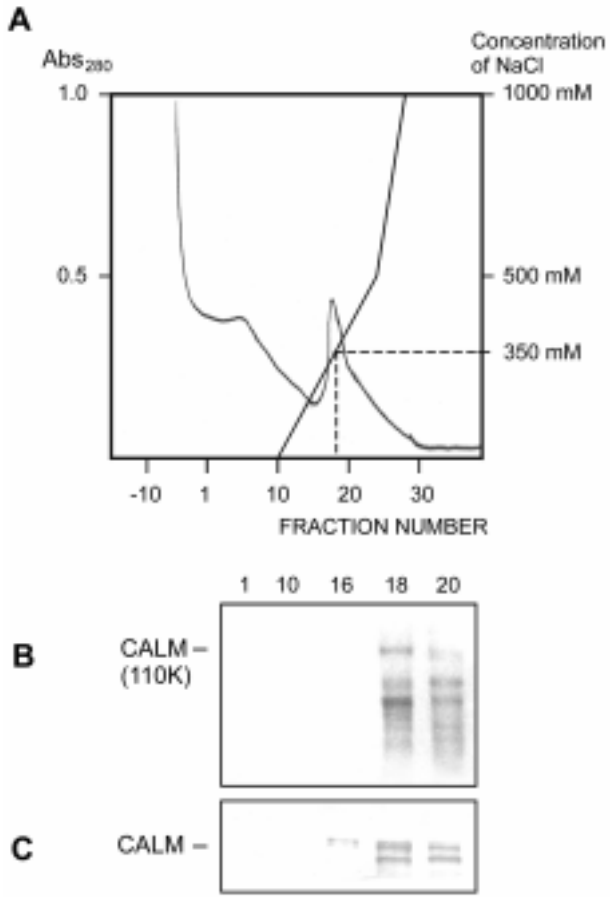

Figure 2. Chromatogram of CALM proteins on Mono S FPLC. The fractions immunoreactive for CALM antibody from the Sepharose CL-4B gel filtration chromatography were adsorbed to Mono S FPLC column. The column was eluted with a linear 0-0.5 M, and 0.5-1 M NaCl gradient (see Materials and methods for details). Fractions containing protein were analyzed by Western blotting.

For further purification of CALM, the fraction of 90-100 kDa protein was pooled and concentrated with Centricon-30 and dialyzed against 50 mM Tris pH 8.0 and applied on a Superose 12 column equilibrated in the sample buffer. The sample was resolved into two major peaks, and the first peak was immuroreactive to anti-CALM antibody (Figure 3) and the molecular weight was 90- and 100-kDa. But the second peak had 30-kDa molecular weight and was not immunoreactive against anti-CALM antibody. The size of these proteins was somewhat larger than that of bacterial expression product of CALM, 80 kDa (Kim *et al.,* 2000). On *in vitro* translation of the CALM gene in eukaryotic system, the expressed protein has molecular weight of 105 kDa (data not shown). The discrepancy of molecular weight might be due to dephosphorylation during the purification process. The CALM has some phosphorylation sites, and eukaryotic expressed CALM was phosphorylated. Thus smaller molecular weight might be caused by dephosphorylation.

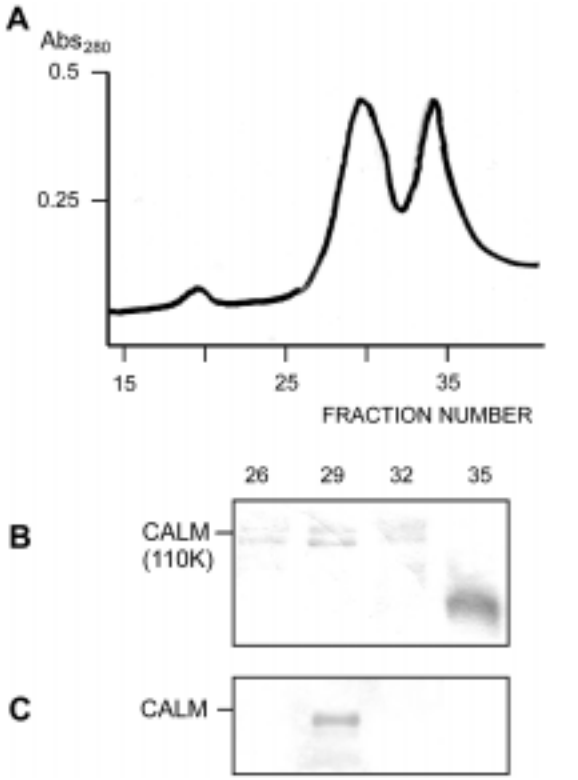

Figure 3. Superose 12 FPLC elution profile. The pooled fraction was concentrated with Centricon 30 and applied to Superose 12 FPLC column. The column was eluted with 50 mM Tris, pH 8.0, and the proteins were resolved into two major peaks (A). The fractions separated on SDS-PAGE, and detected with Coomassie blue protein staining (B), Anti-CALM antibody (C).

Clathrin assembly assay

The ability of clathrin cage assembly of CALM was assayed by incubating clathrin triskelia with the CALM or GST as a control followed by ultracentrifugation at 100,000 *g*. The resulting supernatant and pellet were analyzed by SDS-PAGE followed by Coomassie blue staining. Because the CALM was participated in the formation of clathrin cage, it would be co-sedimented with clathrin (lower band on Figure 4, first lane) leaving the uncaged clathrin triskelia in the supernatant. As the GST used as a control for assembly assay was not contributed to the cage formation, the GST was remained in supernatant fraction (lower band in Figure 4, fourth lane). The purified neuronal CALM could promote clathrin triskelia into cages. This assembly activity of CALM was similar to that of rat liver CALM (Kim and Kim, 2000). Although the efficiency of the assembly activity of the neuronal CALM could not be compared quantitatively with that of non-neural, liver CALM, the CALM might promote the cage formation evenly in most tissues. Considering the similar function of neural specific protein AP180 in nervous system, the role of the CALM might be the basal promoting activity in the clathrin cage formation in most tissues. The even size of

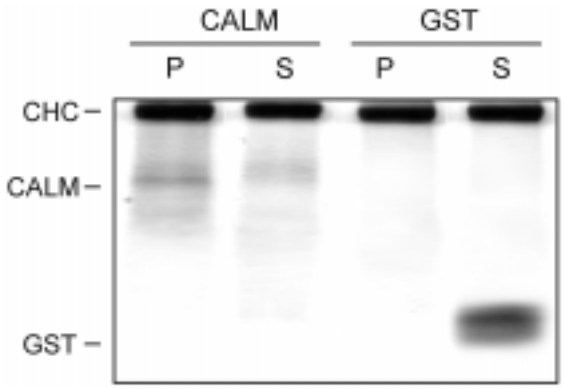

Figure 4. Assembly of clathrin cage by the CALM protein. Clathrin assembly assays were performed as described in Materials and Methods. Three μM clathrin triskelia were dialyzed overnight at 4°C against isolation buffer with the addition of purified CALM (lane 1 and 2) or GST (lane 3 and 4) at the final concentration of 20 μM. Following a low speed centrifugation to remove nonspecific aggregates, newly assembled clathrin cages were pelleted by ultracentrifugation at 100,000 *g*. The pellet (P) and the supernatant (S) fractions were analyzed by 10% SDS-PAGE, followed by Coomassie blue staining. Positions of clathrin heavy chain (CHC), CALM and GST were indicated.

the synaptic vesicles might be ascribed to the basal CALM activity followed by the AP180, or simultaneous action of them.

Proteolysis of the CALM protein by caspase 3 and calpain

Purified CALM was treated with caspase 3 and calpain, and the reactions were examined by Westen blot analysis using anti-CALM monoclonal antibody. The CALM treated by caspase 3 and calpain was fragmented into multiple pieces (Figure 5). The CALM has 5 predicted caspase 3 cleavage sites, DxxD motif, at 260, 263, 392, 427 and 526 positions from amino terminus. It was suggested that CALM has more than three target sites for caspase 3 (Kim and Lee 1999). These proteolysis patterns of purified CALM are consistent with our previous result from in vitro translated system (Kim and Kim 2001). The fragmented CALMs could not promote the assembly of the clathrin triskelia into clathrin cage (data not shown). It has been suggested that apoptosis also proceeded through the regulation of vesicle formation. Apoptotic neuronal cell death has been reported in various chronic neurodegenerative conditions, such as Huntington's disease (HD), Alzheimer's disease (AD), amyotropic lateral sclerosis (ALS) and AIDS-associated dementia. In the brain of Alzheimers disease patient, AP180 and AP2 were dramatically reduced (Yao *et al.*, 1999). It is required to determine whether the reduction of AP180 and CALM were attributed to the cleavage by caspase and calpain in nerodegeneration by apoptotic process.

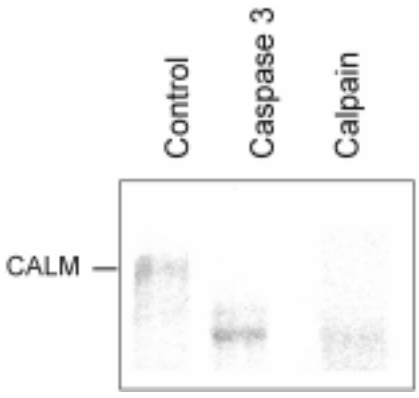

Figure 5. Proteolysis by caspase. Proteolysis assays were performed as described in Materials and Methods. One of purified CALM was used in the binding assay. Purified CALM protein (lane 1) was treated with caspase 3 (lane 2) and calpain (lane 3). The reactions were analyzed by 10% SDS-PAGE followed by immunoblot using anti-CALM polyclonal antibody. The arrow indicates CALM.

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