Purification of clathrin assembly protein from rat liver

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Abstract

Recently, the gene encoding clathrin assembly protein of lymphoid myeloid leukemia (CALM), which is homologous to the AP180, was cloned from rat brain, and its expression differential to AP180 was reported (Kim and Lee, 1999). This gene product promotes the polymerization of clathrin into clathrin cage and found to be a regulator in membrane trafficking between intracellular compartments in eukaryotic cells (Kim *et al.*, 2000). In this study, we have purified the CALM protein from clathrin-coated vesicles of rat liver using the monoclonal antibody against the recombinant N-terminal region of the CALM. The coated proteins extracted from the coated vesicle fraction was further purified by multi-step procedures involving gel-filtration and ion-exchange chromatography and SDS-PAGE. The purified protein with an apparent molecular weight of 100 kD promoted the assembly of clathrin triskelia into clathrin cage. In this respect the CALM protein bears a functional resemblance to the AP180 that has been previously described.

Keywords: clathrin-coated veside, CALM, AP180, assembly

Introduction

In eukaryotic cells, clathrin-coated vesicles (CCVs) are involved in membrane trafficking of ligands including receptor-mediated intracellular transport, transfer of proteins from trans-Golgi network to pre-lysosomal compartment and recycling of synaptic vesicles (Pearse and Robinson, 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. A major coating protein is 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 assembly proteins (Robinson, 1994). They are adaptors and believed to link receptors to the clathrin network. The assembly protein promotes to assemble clathrin triskelia into artificial clathrin cage that resembles a coated vesicle. To date, six different adaptor proteins are known: AP-1, AP-2, AP-3, AP-4, AP180 and CALM. The AP-1, AP-2, AP-3, and AP-4 are tetramers, whereas AP180 and CALM are monomers.

AP180 is well studied because it is monomeric and neuron specific protein (Kondury and Roland, 1988; Zhou *et al.*, 1992). AP180 was first discovered as a specific coating component of CCVs from neural tissue (Stephan *et al.*, 1990). The native protein was shown to interact with clathrin triskelia and to induce clathrin assembly into a homogeneous 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 vesicular coat was promoted by the phosphorylation of AP's (Wilde and Brodsky, 1996). Two primary functional properties of the AP180 are clathrin assembly and high affinity for specific inositol polyphosphates (Morris *et al.*, 1993; Norris *et al.*, 1995; Ye *et al.*, 1995).

The CALM gene was cloned from human leukemia cell line, and was shown to be homologous to the monomeric clathrin adaptor protein of AP180 (Dreyling *et al.*, 1996; Kim and Lee, 1999). In order to elucidate the role of CALM, we have expressed the full length of the CALM cDNA as GST-CALM fusion protein in bacterial system and reported the properties of the CALM protein, for the first time (Kim *et al.*, 2000). The CALM protein expressed in bacteria has a molecular weight of 80 kD. The protein could promote clathrin triskelia into clathrin cages and could bind the preformed clathrin cage. However, N-terminal domain of CALM with a molecular weight of 33 kD could not bind to preassembled clathrin cage, but assemble clathrin triskelia into clathrin cages. The CALM protein was bound to SH3 domain through N-terminal domain, *in vitro*. The CALM protein is proteolyzed by caspase 3, caspase 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 known.

In this study, to understand the role of CALM protein in mammalian cells, we have purified the CALM protein from rat liver, through various procedures including CCV

preperation, Sepharose CL-4B gel-filtration chromatography, phosphocellulose cation exchange chromatography, Superose 6 FPLC and observed that the CALM protein was able to assemble the clathrin into clathrin cage.

Materials and Methods

Materials

All buffers used in the protein study, even though it is not explicitly indicated in the citation, contained 0.1 mM 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 Amesham 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.

Preperation of clathrin-coated vesicles

Rat liver was minced and homogenized with six passes of Potter-Elvejhem tissue grinder in an equal volume of ice-cold homogenating buffer to the weight of the liver. 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 x *g* in a Sorval GSA rotor for 40 min. The supernatant was saved and centrifuged for 70 min at 43,000 x *g* in a Sorvall SS34 roter. The pellet containing the coated vesicles was resuspended in a small volume of homogenizing buffer using a loose-fitting Dounce homogenizer. The resulting pellet is crude coated-vesicle.

Extraction of coating 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 phenylmethylsulfonyl fluoride (PMSF), pH 7.0, 2 mM dithiothreitol (DTT), 1 mM EDTA, and homogenized using Dounce glass homogenizer. The homogenate was stored at 4 overnight, and clarified by ultracentrifugation at 100,000 x *g* for 3 h.

Gel-filtration chromatography

The tris buffer extract was applied to the Sepharose CL-4B (Pharmacia, Upsala, Sweden) column (2 x 100 cm) equilibrated with the extraction buffer and eluted with the equilibration buffer with the flow rate of 0.5 ml/min. Aliquots of all fractions were analyzed by SDS-PAGE and immuno-blotted with CALM monoclonal antibody.

Fractions that contained partially separated assembly proteins were pooled and concentrated by Centricon 30.

Phosphocellulose chromatography

For a further purification, fractions of the assembly protein were chromatographed on phosphocellulose column (1.0 x 10 cm) using FPLC system. The sample was applied to the column equilibrated with 50 mM potassium phosphate buffer, pH 6.0. and washed with 10 ml of equilibration buffer, and then, eluted with a 30 ml gradient consisting of 1 mM sodium phosphate, 1 mM DTT, pH 8.8 and 350 mM potassium phosphate in 1 mM DTT, pH 8.8. Fractions of 0.5 ml were collected.

Superose 6 Gel filtration fast performance Liquid chromatography (FPLC)

The sample from the gel-filtration chromatography was applied to the Superose 6 column equilibrated with the extraction buffer. Then the column was eluted with equilibration buffer.

Preperation of clathrin

Rat clathrin was used for the binding of clathrin assembly proteins and the clathrin-cage formation. The extraction of coating proteins and the gel-filtration procedures were 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 an addiation of 20 μM assembly protein (purified CALM protein and GST). Following the 3 min centrifugation at 13,600 x *g* to remove nonspecific aggregates, newly assembled clathrin cages were collected by ultracentrifugation for 20 min at 100,000 x *g*. Fractions of the pellet (P) and supernatant (S) were analyzed by SDS-PAGE, followed by Coomassie blue staining.

Results and Discussion

Purification of assembly proteins

Clathrin-coated vesicles were isolated from rat liver by the procedure described by Ahle and Ungewickell (1986). Clathrin and its associated membrane proteins were liberated from the purified coated vesicles with 0.5 M Tris at pH 7.0 and the membrane fractions were removed by ultracentrifugation. The supernatant contained most of the clathrin and other clathrin associated proteins. In order to isolate clathrin, molecular sieve on a Sepharose CL-4B column equilibrated with the extraction buffer to
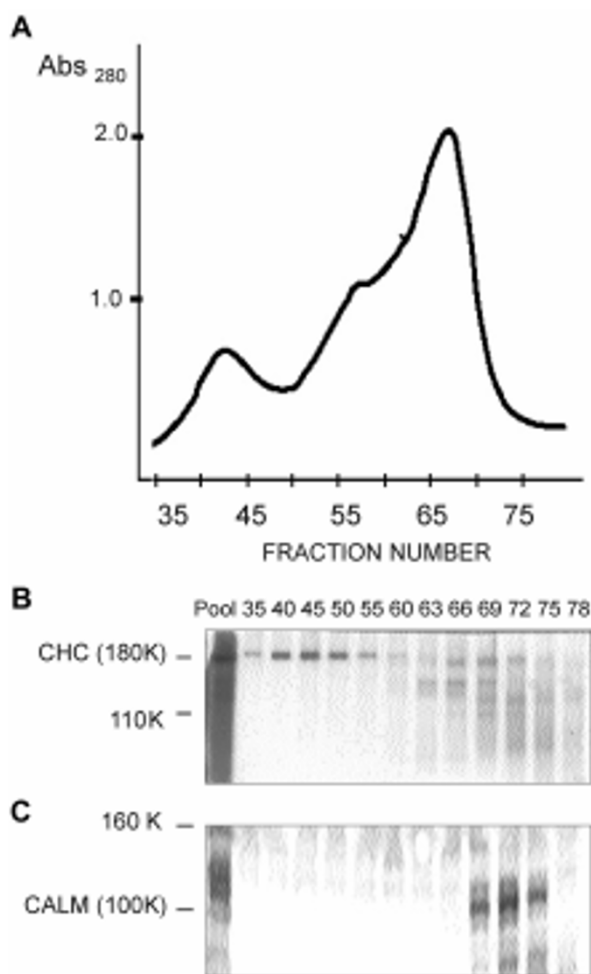
Figure 1. Gel filtration of solubilized coat protein, electrophoresis and immunoblot analysis of individual Sepharose CL-4B column fractions. (A) The coated vesicles were incubated with 0.5 M Tris-HCl, 2 mM EDTA, 1 mM DTT, pH 7.0. The extracted proteins were clarified with ultracentrifugation and then subjected to gel-filtration on Sepharose CL-4B column. (B) Aliquots of fractions of gel filtration elution were analysed 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 of CALM (C)

maintain the assembly proteins (*i.e.* AP-1, -2, -3, *etc.*) in a dissociated state. On the gel filtration profile from the liver tissue, the first peak is the most prominent (Prasad and Lippoldt, 1988). Figure 1 shows a typical elution profile from liver tissue where the first peak is moderate. Analysis of the column fractions by SDS-PAGE shows that the first peak contains clathrin triskelia. The first peak from the liver extract was a bit smaller, which reflect that vesicle trafficking may be lower in liver tissue than in brain. The Western blot analysis of the eluents revealed that the first peak was not immunoreactive against recombinant CALM, but the latter part of the elutes have two immunoreactive bands. The faint band has molecular weight of 100 kD, and the lower intense band was about 90 kD. The two bands might be
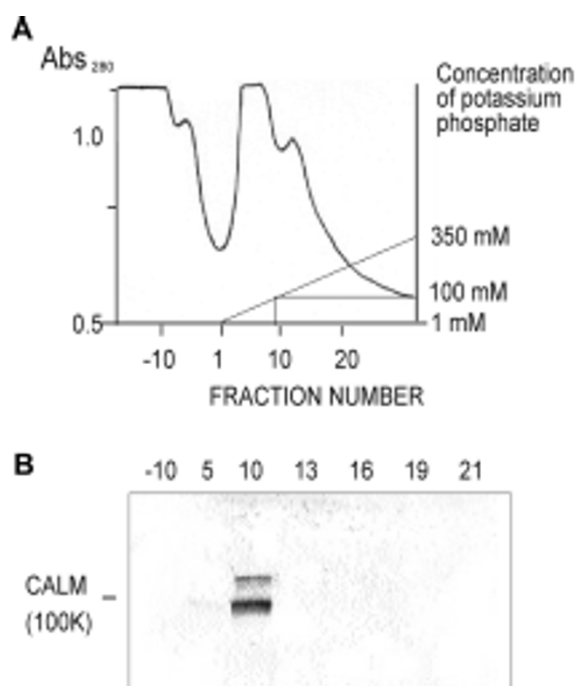

Figure 2. Chromatogram of CALM proteins on phosphocellulose cation-exchange chromatography. The third peak of immunoreaction for CALM antibody from the Sepharose CL-4B gel-filtration chromatography was applied to phosphocellulose cation exchange column. The column was eluted with a linear 1-0.35 M potassium phosphate gradient (see 'Materials and methods' for details). Fractions containing protein were analysed by Western blot.

ascribed to the alternatively splicing variants of the gene (Kim and Lee, 1999).

The size of these bands was somewhat larger than that of bacterial expression product of CALM, 80 kD (Kim *et al*. 2000). On translation of the CALM gene, this protein is composed of 640 amino acids with estimated molecular weight of 71 kD. The increase in apparent molecular weight would be resulted from the atypical amino acid composition and posttranslational modification. The protein contained highly positively charged amino acids and proline residues (45 proline/640 residues), that caused anormalous retarded migration of the CALM on SDS-PAGE, and resulted in the differences between true and apparent molecular weights. In mammals, furthermore, the increase in apparent molecular weight might be caused by posttranslational glycosylation or phosphorylation modifications of the primary translation product. For these reasons, the 100 kD-sized proteins, which is immunoreactive against anti-CALM antibody, were considered to the native form and purified through-out the procedure.

The estimated pI value of the CALM trnascript is 8.7 and that of AP180 is 4.5 (Kim and Lee, 1999). In contrast to using anion exchange chromatography for purification of AP180 due to its acidic nature (Ahle and Ungewickell, 1986), we chose cation exchange chromato-
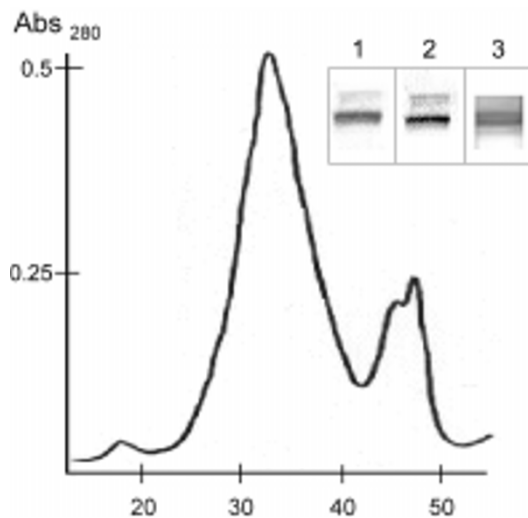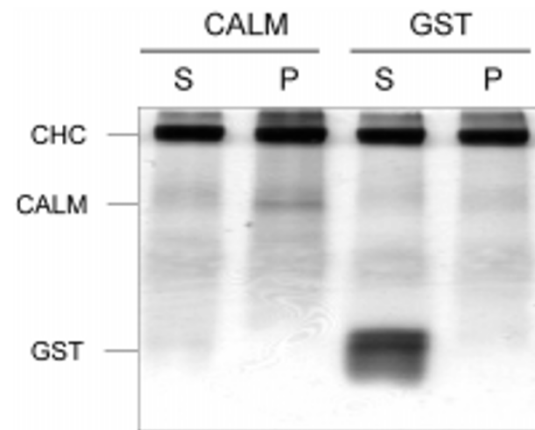
Figure 3. Superose 6 FPLC elution profile of pooled fraction from phosphocellulose cation-exchange chromatography. The pooled fraction was concentrated with Centricon 30 and applied to Superose 6 FPLC column. The column was eluted with 0.5 M Tris, pH 7.5, and the proteins were resolved into two major peaks. The first peak was pooled, separated on SDS-PAGE, and detected with Coomassie blue protein staining (1), anti-CALM antibody (2), and anti AP180 antibody (3).

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

graphy for the next purification step. The fractions containing the 100 kD protein were pooled and subjected to chromatography on a phosphocellulose cation-exchange column in conjunction with FPLC system. The column was equilibrated with 50 mM potassium phosphate buffer, pH 6.0. The bound sample was eluted with linear gradient of 350 mM potassium phosphate buffer, pH 8.8. As shown on Figure 2 more than two third of the protein was in the wash fraction and the 90-100 kD protein was eluted at the front part of phosphate gradient, about 100 mM potassium phosphate concentration.

The protein with molecular weight of 90-100 kD was further purified by Superose 6 FPLC. The pooled fraction was concentrated with Centricon-30 and applied to Superose 6 column. The sample was resolved into two major peaks, and the first peak was immuroreactive to anti-CALM antibody (Figure 3). On SDS-PAGE analysis of the pooled sample, the 90 kD-sized band was prominent whereas the 100 kD sized band was faint. The monoclonal antibody might be more reactive to the short variant of the alternatively spliced CALM.

There was a remarkable homology between the primary structure of the CALM and AP180. The homology of more than 95% is seen between the first 289 amino acids of CALM and those of AP180 (Kim and Lee, 1999). Western blot analysis was carried out for the purified CALM using monoclonal antibody against AP180. The proteins were also reactive to AP180 antibody (Figure 3, lane 3), suggesting that the tertiary structure of N-terminal region of AP180 and CALM would be similar. Furthermore, the N-terminal region of the AP180

where the binding site for the inositol and inositides (Hao *et al.*, 1997) is present, may compete with the CALM for the inositides and inositol phosphates, the regulators of clathrin cage formation, and would play a crucial role in membrane trafficking.

Clathrin assembly assay

CALM is homologous protein of AP180. Apparently, both N terminal (33 kD) and C terminal (55 kD) parts of the AP180 have clathrin binding activity (Ye and Lafer, 1995) and C terminal domain has the activity of clathrin cage assembly. Recently it was reported that the CALM could bind to clathrin heavy chain (Teber *et al.*, 1999). Moreover, CALM of GST fusion protein could bind to preassembled clathrin cage and could assemble clathrin cage (Kim *et al.*, 2000). Clathrin triskelia was dialyzed with putative assembly protein in isolation buffer to induce clathrin assemble into cages. When CALM was mixed with the clathrin triskelia, bound CALM cosedimented with the cages at 100,000 x *g*, while free CALM remained in the supernatant. When clathrin triskelia was sedimented following a dialysis in the presence of purified CALM, it remained in the pellet with the clathrin cages, but a negative control GST was not cosedimented with clathrin cages (Figure 4). These results indicated that the purified CALM has an ability to assemble the clathrin cage.

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