Properties of GST-CALM expressed in *E. coli*

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Abbreviations: CCV, clathrin-coated vesicle; CALM, clathrin assembly protein lymphoid myeloid; GST, glutathion sulfur transferase; AP, adaptor protein

Abstract

Clathrin-coated vesicles (CCVs) are involved in protein and lipid trafficking between intracellular compartments in eukaryotic cells. CCVs are composed of clathrin and assembly proteins. The clathrin assembly protein lymphoid myeloid leukemia (CALM) gene, encodes a homologoue of the neuronal clathrin assembly protein AP180. In this study, we characterized the properties of the CALM expressed in *E. coli.* The molecular weight of bacterially expressed GST-CALM fusion protein was approximately 105 kD on SDS-PAGE. The CALM protein could promote clathrin triskelia into clathrin cages and could bind the preformed clathrin cage. However, 33 kD N-terminal domain of CALM could not bind pre-assembled clathrin cages, but assemble clathrin triskelia into clathrin cages. The CALM protein was bound to SH3 domain through N-terminal domain1, *in vitro*. The CALM protein is proteolyzed by caspase 3, caspase 8 and calpain through C-terminal domain.

Keywords: expression, clathrin-coated vesicle, CALM, AP180, regulation, SH3 domain

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 components of the coat forming protein, a highly ordered structure on the cytoplasmic surface of the vesicle. Soluble clathrin (~650 kD) is composed of identical three 160 kD heavy chains and three 22-28 kD light chains (Pearse, 1975). These three heavy chains and three light chains complexes form three lagged trimers, called clathrin triskelion. These triskelion assemble into a basketlike framework of hexagons and pentagons to form coated pits on the cytoplasmic surface of membrane (McMahon, 1999). 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. Four tetrameric APs have been described and designated as AP-1 (Ahle and Ungewickell, 1986), AP-2 (Beck *et al.,* 1992), AP-3 (Faundez *et al.*, 1998), and AP-4 (Dellangelica *et al.*, 1999). AP-1 and AP-2 were first charaterized (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 had been identified but has not been 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 of all tissue (Dreyling *et al.,* 1996). The native AP180 was shown to interact with clathrin triskelia by one to one stoichiometry and thereby induced clathrin assembly into a uniformly sized of 60-70 nm coat structures (Kondury and Roland, 1988). These appear to be somewhat smaller and to be sediment considerably more slowly that than those containing 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). AP180 is phosphorylated at serine residues *in vivo* (Bar-Zvi *et al.*, 1988) and *in vitro* (Keen and Black, 1986). AP180 is also glycosylated by a single O-linked N-acetylglucosamine (GlcNAc) residue (Murphy *et al.,* 1994). AP180 contains high affinity binding sites for inositides, where such binding induces loss of their ability to promote clathrin assembly (Norris *et al.*, 1995). Despite of such extensive biochemical characterization

of AP180, the function of AP180 *in vivo* still is not clear.

Recently, the AP180 homologous gene was reported in human and rat tissue (Dreyling *et al.*, 1995, Kim and Lee, 1999). The N-terminal domain of CALM is very similar with that of AP180 in relation to their binding sites for clathrin and a possible phosphorylation site whereas the C-terminal domain is different from that of AP180. The CALM is expressed at the most of tissues and CALM is suggested to be a nonneuronal homologue of AP180 (Dreyling *et al.*, 1995, Kim and Lee, 1999), but the properties of the CALM protein has not been clearly defined.

In this study, we expressed the GST-fusion protein encoding cDNA of the CALM in bacterial system and characterized it's properties in order to understand a possibile role of CALM protein in clathrin coated vesicle modulating function.

Materials and Methods

Materials

All chemicals were purchased from USB and Sigma. Protease inhibitors and NBT/BCIP reagents were from Boehringer Mannheim. Centricon-30 and -10 were from Amicon. All chromatography resins were from Amersham Pharmacia. Plasmid expressing SH3 domain was kindly provided by Dr. S. R. Kim and expressed caspase 3, 8 and calpain were kindly provided by Dr. Y. G. Jung.

Preparation of the plasmids

G18, the whole cDNA of CALM, was cloned into pBluscript II SK (Kim and Lee, 1999), but the expression using pBluscript II SK as the vector was unsuccessful, so the CALM open reading frame was further subcloned into pGEX expression vector. The construct of expressing whole size (62 kD) was constructed using polymerase chain reaction using two primer (5-'GCT GCA AAT TCT GGC CGA AGC AGC CTG-3´, 5´-CTT CCC TCG AGT TAC ATA AAC TGT ATC TG'-3) and plasmid G18 (previously described) as a template. The PCR product was purified by Qiagene elution kit protocols by manufacturer's instructions. The purified PCR product and pGEX4T-3 vector were digested by *EcoR*I and *Xho*I, and the vector was dephosphorylated by calf intestine alkaline phosphatase, and resulting 1.7 kb sized PCR product was ligated with double digested pGEX 4T-3 by T4 ligase for 5 h at 15-20°C. The ligation mixture was introduced into *E. coli* DH5 by electroporation, and the clone expressing full length CALM was obtained. This new plasmid called GST-CALM. The expression construct of amino terminus of CALM was constructed same way and named GST-N33 kD (5-'GCT GCA GAG AAT TCT GGC CAG AGC AGC CTG'-3 and 5-'TCC TTG GAA GGG AAG AAA CTC GCG GAT TCC'-3).

Purification of coated vesicle

All operations were performed at 4°C. Fresh bovine brains were stripped of meninges and chopped. These pieces were minced and homogenized in a Warning blender using an equal volume of homogenizing buffer, 0.1 M 2-(N-morpholino) ethansulfonic acid (MES), pH 6.5 containing 1 mM EGTA, 0.5 mM magnessium chloride, and 0.02% sodium azide. The homogenate was centrifuged at 10,000 rpm in a Sorvall GSA rotor for 40 min at 4°C. The supernatant was re-centrifuged at 38,000 rpm in a Beckman 45 Ti rotor for 1 h. The pellets containing the coated vesicles were resuspended in homogenizing buffer, gently homogenized using Dounce glass homogenizer, and centrifuged at 10,000 rpm in a Sorval GSA rotor. The supernatant was re-centrifuged at 38,000 rpm in a Beckman 45 Ti rotor for 1 h. In order to extract clathrin and associated proteins, the crude coated vesicle pellets were resuspended in Buffer B (0.5 M Tris buffer, pH 7.0 containing 2 mM DTT, and 1 mM EDTA), and re-homogenized using Dounce glass homogenizer. The homogenate was stored at 4°C overnight. The extracts were centrifuged at 46,000 rpm for 4 h. The Tris extract was applied to the exclusion chromatography column (Sepharose CL-4B), 2×100 cm, equilibratd with buffer B and was eluted with the same buffer B.

Raising of the antibodies

Young female rabbit was immunized initially 500 µg of purified GST-N33 kD fusion protein emulsified with complete Freund's adjuvant, and successively three times with the same antigen in an incomplete adjuvant. Whole blood was collected under anesthesia and prepared for purification. The serum was salting out with a 40% saturated ammonium sulfate solution and affinity purified using this fragment covalently immobilized on CNBr-Sepharose. Monoclonal antibodies against GST-N33 kD protein were prepared by immunizing female BALB/c mice with100 antigen in a complete Freund's adjuvant *via* i.p. The mice were boosted two weeks later with an intraperitoneal injection of activated CNBr sepharose bead, linked with purified GST-N33 kD (50 µg) in an incomplete Freund's adjuvant. The second booster was performed two weeks later with the purified GST-N33 kD in an incomplete Freund's adjuvant (50 µg). The female mouse was finally boosted with an i.v. injection of the purified GST-N33 kD without Freund's adjuvant (50 µg). Three days later excised spleen cells were fused with myeloma cell line (P3x63Ag8.V653) as described previously Kohler and Milstein's procedure (1975). The fused cells were distributed in 96-well tissue culture plate (1×10^8 spleen cells/20 ml of Dulbecco's modified Eagles Medium (DMEM) supplemented with 20% inactivated fetal bovine serum) and incubated in 5% CO_2 at 37°C. HAT (hypoxantine (100 mM), amino-

pterine (0.4 mM), thymidine (16 mM)) medium was added to fusion cells from the next fusion day. The hybridoma culture supernatant were screened two weeks later by indirect enzyme linked immunisorbant assay (ELISA). Cells from positive cultures were cloned by limiting dilution in HT medium (hypoxantine (100 mM), thymidine (16 mM)). Clones were screened by immunoblotting and confirmed as monoclone. The isotypes of the monoclonal antibodies were determined by mouse antibody isotyping kit according to the protocol by manufacturer's instructions.

Clathrin cage binding assay: Clathrin triskelias (1.8 μM) were dialyzed overnight in the isolation buffer (0.1 M MES buffer, pH 6.7 containing 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.1 mM PMSF) at 4°C. Newly assembled cages were collected by ultracentrifugation (100,000 *g*) in a Beckman TLA 100.3 rotor. The pellets suspended in the isolation buffer was incubated with the assembly protein (purified GST fusion proteins and GST, each of 20 μM) for 1 h on ice. The suspension mixtures were again collected by ultracentrifugation (100,000 *g*) for 20 min. The pellet (P) and supernatant (S) fractions were analysed by SDS-PAGE, followed by Coomassie Blue staining.

Clathrin assembly assay: 3 μM clathrin triskelia were dialyzed overnight at 4°C against isolation buffer with the addition of 20 μM of either assembly protein (GST-fusion proteins 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.

SH3(Src homology 3) binding assay: GST-SH3 (PLC-γ1) was immobilized on activated CNBr-agarose beads. Then the beads were incubated for 2 h at 4°C with 20 μg of GST fusion proteins (GST-CALM, GST-N33 kD, GST) in 500 μl of GPD buffer (20 mM Tris buffer, pH 7.5 containing 150 mM NaCl and 0.5% NP40). Samples were centrifuged for 10 min at 10,000 *g* at 4°C. The supernatants were carefully aspirated and the beads were washed with 500 μl of GPD buffer. And the samples were centrifuged for 10 min, at 10,000 *g* at 4°C. The samples were washed four times in a same manner. Bound proteins were eluted by boiling in standard SDS-gel loading buffer, analyzed by 10% SDS-PAGE, followed by Coomassie Blue staining.

Proteolysis by caspase and calpain: 10 of bacterially expressed GST fusion proteins (GST-CALM and GST-N33 kD) were incubated with caspase 3 and 8 bacterial expressed lysate in 20 μl of reaction buffer (16 mM HEPES, 8 mM NaCl, and 0.004% IGEPAL). The mixtures were incubated 1 h at 30°C. For calpain, GST fusion proteins (GST-CALM and GST-N33 kD) were incubated with purified calpain (Sigma) in 20 μl of reaction buffer (1 X PBS, 1 mM $CaCl_2$). The mixtures were incubated 1 h at 37°C. The digestions of CALM were analyzed by SDS-PAGE followed by immunoblot.

Result

Protein purification and production of antibodies against CALM

N-terminal fragment of CALM (amino acid; 1-300) was used to raise a polyclonal antibody and monoclonal antibody. Immunoblot analysis with affinity purified polyclonal antibody and monoclonal antibody detected a specific band of 110 kD in coated vesicle of bovine brain (Figure 1).

To identify the product of the CALM gene, recombinant rat GST-CALM, and its N-terminal domain were prepared from a bacterial expresseion vector and purified on glutathione-sepharose. SDS-PAGE of the purified protein (GST-CALM and GST-N33 kD) revealed 105 kD and 65 kD bands (Figure 2a). GST-CALM revealed a major band with an apparent molecular weight of 105 kD and some lower molecular weight bands (Figure 2a). The protein pattern of the Coomassie Blue stained gel was shown to be identical to an immunoblot with an anti-CALM monoclonal antibody (Figure 2b).

Clathrin cage binding assay

The ability of these two proteins to bind to pre-assembled clathrin cages was examined by utilizing the cage binding assay as described by Linder and Ungelwickell

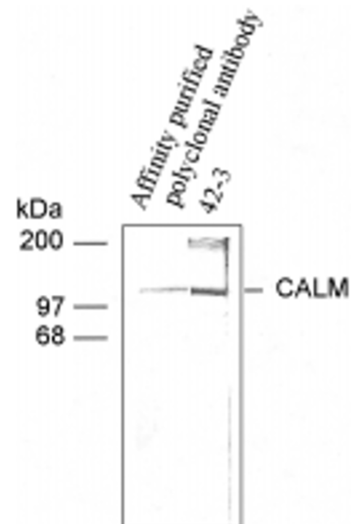

Figure 1. Characterization of antibodies raised against GST-N33 kD. Antibodies were raised against the 33 kD N-terminal domain of GST-CALM fusion protein, as described on “Materials and Methods”. lane 1; affinity purified polyclonal antibody against CALM, lane 2; mAb clone 42-3

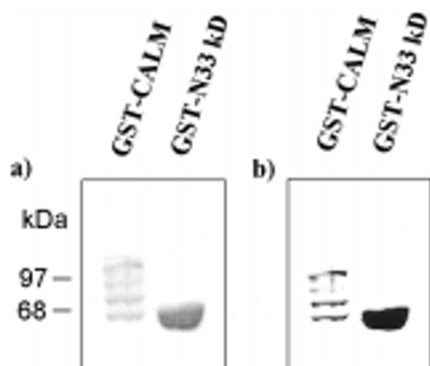

Figure 2. SDS-PAGE analysis of the GST-CALM protein. GST-CALM, GST-N33 kD, were purified from extracts of *E. coli* expressing the recombinant protein, as described on "Materials and Methods". a) Coomassie Blue staining of expressed GST- CALM. b) Immunoblot analysis of GST fusion protein of full length and 33 kD N-terminal CALM using monoclonal Antibody.

(1995). The clathrin cages pellet was obtained by centrifugation at 100,000 *g*. When CALM was mixed with the pre-assembled cages, bound CALM cosedimented with the cages during 100,000 *g* centrifugation, while free CALM remained in the supernatant. Essentially most of the GST-CALM remained in the supernatant in the reaction that contained 20 μM of CALM alone (Figure 3, lanes 1 and 2). When 3 μM of preassembled clathrin cages are incubated, essentially most of GST-CALM was found in the pellet fraction (Figure 3, lanes 3 and 4) indicating partial binding to the cages. But sedimentation patterns of GST-N33 kD were not altered in the presence and absence of cages. GST-N33 kD was found to be aggregated either by itself or with nonspecific aggregates, but not by specific association with clathrin cages (Figure 3, lanes 5-8). The GST negative control showed 0% binding to the clathrin cages (Figure 3, lanes 9-12). GST-CALM was able to bind to the clathrin cages through C-terminal region.

Clathrin assembly assay

Factor-dependent clathrin assembly were carried out as described (Linder and Ungelwickell 1995). Following dialysis of clathrin triskelia mixed with a putative assembly protein in the isolation buffer, clathrin did assemble into cages. When clathrin triskelia are sedimented following dialysis in the presence of GST-CALM, the GST-CALM was found to be cosedimented with clathrin cage. Such association of GST-CALM does indicate CALMability to assemble into clathrin (Figure 4, lanes 1-2). When clathrin triskelia were sedimented following dialysis in the presence of GST-N33 kD, GST-N33 kD remained in the pellet with the clathrin cages, but GST was not cosedimented with clathrin cages (Figure 4, lanes 3-6). Bacterially expressed recombinant CALM was able to assemble clathrin cages, and also the recombinant 33

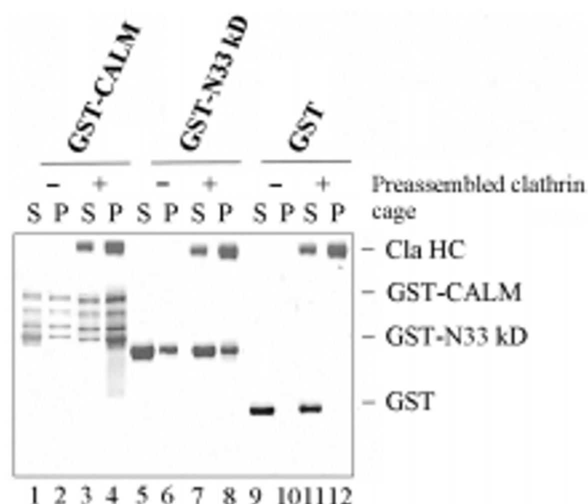

Figure 3. Binding of preassembled cage by the CALM protein. Clathrin cage binding assays were carried as described on "Materials and Methods". 20 μM concentration of GST-CALM (lanes 1-4), GST-N33 kD (lanes 5-8), and GST (lanes 9-12) were incubated in the absence (–) or presence (+) of 1.8 μg (corresponding to molarity of triskelion, 1.2 mg/ml) of preassembled clathrin cage. Following a low spin to remove nonspecific aggregates, all samples were pelleted by ultracentrifugation at 100,000 *g*. The supernatant (S) and the pellet (P) fractions were analyzed by SDS-PAGE, followed by Coomassie Blue staining. Positions of clathrin heavy chain (Cla HC), GST-CALM and GST-N33 kD, GST were indicated.

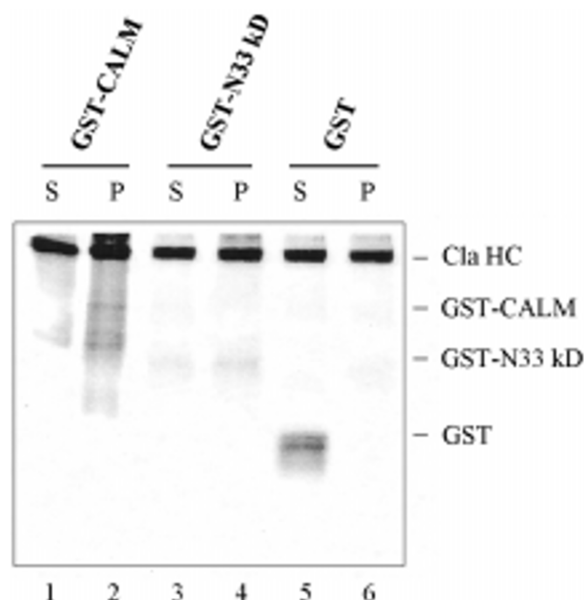

Figure 4. Assembly of Clathrin cage by the CALM protein. Clathrin assembly assays were performed as described on "Materials and Methods". 3 μM clathrin triskelia mixed with either 20 μM concentrations of GST-CALM (lanes 1 and 2), GST-N33 kD (lanes 3 and 4), or GST (lanes 5 and 6) were dialyzed overnight at 4°C against isolation buffer. Following a low spin 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. Position of each protein is indicated in the same way as in Figure 3.

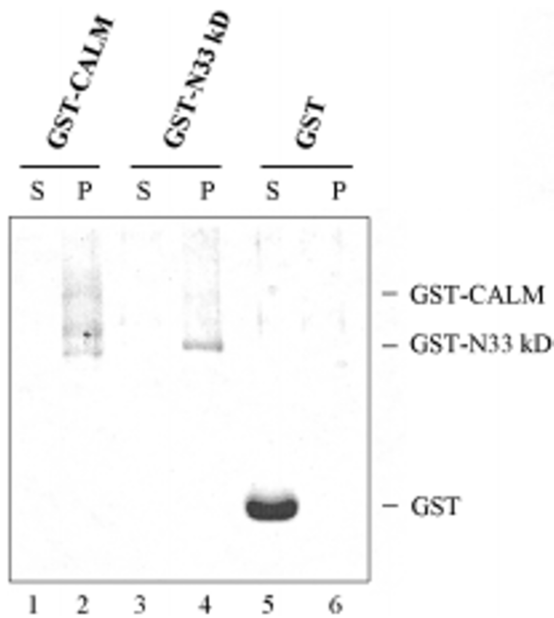

Figure 5. Interaction of the CALM protein with SH3 domain. SH3 binding assays were performed as described on "Materials and Methods". The proteins (GST-SH3) bound to the agarose beads were reacted with either 20 µg of expressed proteins, GST-CALM (lanes 1 and 2), GST-N33 kD (lanes 3 and 4), or GST (lanes 5 and 6). The supernatant (S) and the pellet (P) were analyzed by 10% SDS-PAGE followed by Coomassie Blue staining.

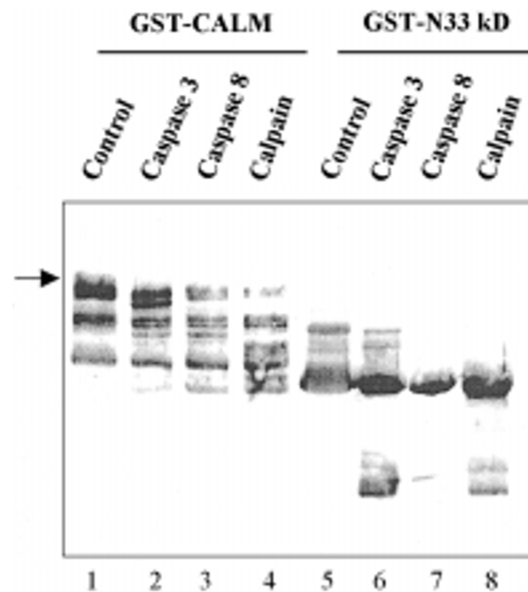

Figure 6. Proteolysis by caspase and calpain. Proteolysis assays were performed as described on "Materials and Methods". The 10 µg of expressed proteins, GST-CALM (lanes 1-4) or GST-N33 kD (lanes 5-8) was used in the assay. Bacterial lysate (lanes 1and 5) was used a control and treated either with caspase 3 (lanes 2 and 6), caspase 8 (lanes 3 and 7), or calpain (lanes 4 and 8). The reactions were analyzed by 10% SDS-PAGE followed by immunoblot. The arrow indicates the full length of the GST-CALM.

kD N-terminus was also able to assembly clathrin cages.

SH3 binding assay

CALM was shown to contain multiple proline rich domains. We found that CALM bounds to SH3 domain of PLCγ1 *in vitro* using a recombinant fusion protein of glutathione-S-transferase with the PLCγ1 SH3 domain (GST-SH3). As shown on figure 5, GST-CALM (Figure 5, lanes 1 and 2) and GST-N33 kD (Figure 5, lanes 3 and 4), but not GST (Figure 5, lanes 5 and 6) bound to PLCγ1 immobilized agarose beads. This result indicated that the CALM bound directly to the SH3 domain of PLCγ1, through proline rich domain in 33 kD N-terminal domain.

Proteolysis of CALM

According to the analysis of the deduced amino acid sequence of the CALM protein, the CALM protein has 3 DxxD motif in C-terminal. DxxD motif could be the target substrate of caspase 3. The CALM homologous protein, AP180 is target protein of Ca^{2+} dependent protease, calpain. To analyze the proteolysis by caspase and calpain, the GST-CALM and GST-N33 kD were treated with caspase 3, 8 and calpain. The results are shown in Figure 6. The GST-CALM was proteolysed by caspase 3, 8 and calapin (Figure 6, lanes 2, 3, and 4, indicated by asterisk), but GST-N33 kD was not proteolysed by the same enzymes (Figure 6, lanes 6, 7, and 8). These results showed that proteolytic target sites were located in C-terminal of the CALM. In negative control experiment with bacterial lysate, no changes in the protein migration pattern was observed (Figure 6, lanes 1 and 5).

Discussion

CALM was found to be involved in the t(10;11)(p13;q14) chromosomal translocation in lymphoid cell line (Dryeling *et al.*, 1996), and its function was suggested by the results obtained from studies on the homologous protein, AP180. The rationale of experiments in this study was based on what is known about AP180.

The primary structure of the CALM, was shown a remarkable homology with the murine and rat clathrin assembly protein AP180. The homology ranges from 97% to 28% in different regions of the protein. The homology of more than 95% is found between the first 289 amino acids of CALM and those of AP180. Whereas, the amino acid sequence of the C-terminal half of the CALM is different from those of AP180 (Dreylling *et al.,* 1996; Kim and Lee, 1999).

We have expressed the full length cDNA of the CALM for the first time. The size of band of bacterially expressed recombinant GST-fusion protein, CALM (rGST-CALM)

was 105 kD. The molecular weight of rCALM protein would be 80 kD, a size slightly larger than that of predicted, 71 kD, perhaps for the reason that this protein contained highly charged amino acids and proline residues (45 proline/640 residue) and caused anomalous migration on SDS-PAGE. The N-terminal region of 340 amino acids is highly charged and predominantly basic. By immunoblot assay, 110 kD band of native CALM was detected from coated vesicles isolated from bovine brain. The increase in apparent molecular weight might be due to posttranslational modifications through phosphorylation.

Studies of AP180 have described the clathrin-binding and coat-assembly activities of this protein *in vitro* in detail, although the mechanism of AP180 binding to clathrin remains unclear. Apparently, both N-terminal (33 kD) and C-terminal (55 kD) parts of the AP180 have clathrin triskelia binding activity (Ye and Lafer, 1995). N-terminal of the CALM has over 90% homology with AP180 (Dreylaing *et al.,* 1996, Kim and Lee, 1999), that suggested that CALM would bind to clathrin. Although this N-terminal domain of AP180 is able to bind to clathrin triskelia, it was unable to assemble them into clathrin cages and bound to preassembled cages. Since the *in vitro* clathrin cage binding and assembly assay could demonstrate that association of CALM protein with the preassembled clathrin cages and assemble clathrin triskelia to clathrin cages. Therefore, we utilized a system in which we could evaluate the binding and assembly activities of the 33 kD N-terminus. Restult that rGST-CALM expressed from recombinant plamids was able to bind clathrin and assembly properties suggests that the CALM might be another form of clathrin assembly protein. Whereas the recombinant 33 kD N-terminus of CALM was not able to bind to preassembled clathrin cages, but able to assemble clathrin triskelia into cages. A probable effect of of post-translational modification on the 33 kD N-terminal which could generate the binding activities to clathrin cages was ruled out since the bacterially expressed full-lengh proteins were able to bind clathrin cages and assemble clathrin. Our results strongly support that the recombinant 33 kD N-terminus of CALM was not sufficient for clathrin cage binding or clathrin assembly.

The deduced amino acid sequences of the CALM protein allowed to predict certain biochemical properties of the CALM. The CALM contain some proline rich domains, which could interact with both SH3 and WW domains. SH3 domains and WW domains are known to be associated with the proline-rich sequences PPLP and PPXX (Ryohei, 1999). The proline-rich domains of the CALM did indeed interact with the SH3 domain of PLCγ1 (Figure 5). Another SH3 domains containing proteins, that are good candidates for binding CALM include clathrin vesicle associated proteins the dynamin and the amphiphysin. Dynamin is a cytosolic guanosine triphosphatase (GTPase), has a role of pinching off the clathrin coated pit. Amphiphysin has a SH3 domain which binds the GTPase dynamin, the inositol-5-phophatase, synaptojanin-1 and tetrameric clathrin assembly protein AP2 (Zhang *et al*., 1998; Cestra *et al*., 1999).

CALM has 3 DxxD motifs, identified as substrate sites of caspase 3. Caspase 3 is an effector caspase and is activated by caspase 8 or caspase 9 by different pathway (Nunez *et al*., 1998). Caspases are activated only in apoptosis by cascade steps. CALM was proteolysed by caspase 3 and caspase 8. Substrates domains of caspase 3 are present in many proteins in broad category but that clathrin associated proteins are possible substrates of caspase, has not been reported. We also found that the CALM was a substrate for Ca^{2+}-dependent proteolysis *in vitro*. It is reported that AP180 was a substrate for Ca^{2+}-dependent protease, calpain. The known target sites of calpain are acidic cluster in basic region (Sorimachi *et al*., 1997). CALM also contains related target sites of calpain. Our results did confirm proteolysis of CALM by calpain (Figure 6). The calpain sensitivity of CALM may therefore provide mechanism through which its activity is regulated by changing intracellular calcium concentration.

The CALM protein contains some protein binding motif, which may provide a unique properties to function as clathrin assembly protein and as well as an adaptor protein (Sudhof, 1995; Marsh and McMahon, 1999). CALM might be an important regulator of the endocytic processes. By interacting with several SH3 harboring proteins, i.e. synaptojanin, amphiphysin, and dynamin, CALM can regulate the curvature of the coat and, therefore, the size of the budding vesicle.

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