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Cell-free expression and functional reconstitution of CALM in clathrin assembly

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Abbreviations: CCV; clathrin-coated vesicle, CALM; clathrin assembly protein lymphoid myeloid, GST; glutathion sulfur transferase, SH3; src homology 3

Abstract

Clathrin-mediated vesicle formation is an essential step in the intracellular trafficking of the protein and lipid. Binding of clathrin assembly protein to clathrin triskelia induces their assembly into clathrin-coated vesicles (CCVs). In order to better understand a possible role of post-translational modification of CALM (clathrin assembly protein lymphoid myeloid), the homologue of AP180, in the assembly of CCVs, CALM was expressed in the cell-free reticulocyte translation system that is capable of carrying out post-translational modification. The apparent molecular weight of the expressed recombinant CALM was estimated as 105 kD. Alkaline phosphatase treatment of CALM resulted in a mobility shift on SDS-PAGE. We found that CALM was associated with the proteins harboring SH3 domain, promote assembly of clathrin triskelia into clathrin cage and bound to the preformed clathrin cage. CALM was also proteolyzed by caspase 3 and calpain but not by caspase 8. These results indicated that the post-translationally modified CALM, expressed in the eukaryotic cell-free reticulocyte translation system was able to mediate the assembly of clathrin and the coated-vesicle formation.

Keywords: expression, clathrin-coated vesicle, CALM, SH3 domain, cleavage

Introduction

Clathrin-mediated vesicle formation is an essential step in the intracellular trafficking of the membrane compartment. Most of the transport vesicles arise from the specialized coated regions of the membrane and bud off as coated vesicles with distinctive vesicle contents (Alberts *et al*., 1994). Clathrin, the most abundant component of coat - forming proteins makes 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. These three heavy chains and three light chains form three lagged trimer complex, called clathrin triskelion. This triskelion assembles into a basketlike framework of hexagons and pentagons to form coated pits on the cytoplasmic surface of membrane (Marsh *et al*., 1999). The second major coat proteins are assembly proteins (assembly or adaptor proteins, AP).

Clathrin assembly proteins belong to two gene family, the tetrameric or monomeric AP family. Four tetrameric APs have been described and designated AP-1 (Ahle *et al*., 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 as the major clathrin coated vesicle adaptor proteins. AP-1 was found to localize at clathrin coated vesicles budding from Golgi membrane and AP-2 at clathrin-coated vesicles budding from plasma membrane, but AP-3 has only recently been identified and appeared to be associated with clathrin coated protein as well (Faundez *et al*., 1998). AP-3 plays a role in trafficking from trans-Golgi network to the lysosome (Simpson *et al*., 1997). AP-4 has also been identified but not been characterized in detail.

Monomeric APs include AP180 and CALM. AP180 was mainly found to be present at synapse (Stephan *et al*., 1990), while CALM is expressed in most of all tissues (Dreyling *et al*., 1996; Kim *et al*., 1999). The native AP180 was shown to interact with clathrin triskelia and thereby to induce clathrin assembly through C-terminal domain (Ye *et al*., 1995). AP180 is a glycosylated and phosphoorylated acidic protein (Keen *et al*., 1986; Bar-Zvi *et al*., 1988; Murphy *et al*., 1991; Morris *et al*., 1993). AP180 contains high affinity binding sites for inositides, the binding of which inhibits their ability to promote clathrin assembly (Norris *et al*., 1995).

Recently, the CALM, which is homologous to AP180, was reported in human and rat tissue (Dreyling *et al*., 1995; Kim *et al*., 1999). Bacterially expressed CALM was able to induce the assembly of clathrin cage and bind to clathrin triskelia, like AP180 through C terminal domain. GST-CALM could interact with SH3 domain, and be proteolysed by calpain and caspase (Kim *et al*., 2000b). The CALM was found to be colocalized with

AP-2 and clathrin heavy chain in plasma membrane and coated pits (Tebar *et al.*, 1999). However, whether contribution of any post-translational modification of CALM, especially the cellular signal mediated phosphorylation would affect the clathrin-mediated vesicle formation is not clearly understood. For further elucidation of the interrelationship between the structural modification of CALM and the protein-protein interaction during vesicle formation, CALM was expressed using the cell-free reticulocyte lysate translation system. The results showed the glycosylated and phosporylated CALM retained its functional properties as AP in clathrin vesicle formation.

Materials and Methods

Materials

All buffers used in the protein work contained 0.1 mM PMSF (phenylmethylsulfonylfluoride). Protein concentrations were determined by BCA kit according to the protocol by manufacturer's instruction (Pierce). The recombinant caspase 3 and 8 were the generous gifts from Dr. Y. G. Chung (Kwang-Ju KAIST, Kwang-Ju, Korea). Plasmid expressing SH3 domain was provided by Dr S. R. Kim (Chungbuk Univ. Cheongju, Korea)

Preperation of clathrin

All procedures were performed at 4°C. Fresh bovine brains were chopped and minced after stripping out the meninges. And then it was homogenized using the equal volume of homogenizing buffer in a Waring blender. The homogenizing buffer consisted of 0.1 M 2-(N-morpholino) ethansulfonic acid (MES), 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% NaN_3, pH 6.5. The homogenate was centrifuged at 19,000 *g* in a Sorval GSA rotor for 40 min. The supernatant was saved and centrifuged at 43,000 *g* in a Beckman 45 Ti rotor for 1 h. The pellet containing the coated vesicles was resuspended in homogenizing buffer and gently homogenized using Dounce glass homogenizer. The resulting pellet is the crude coated-vesicle. To extract clathrin and associated proteins, the crude coated-vesicle was resuspended in the extration buffer, which composed of 0.5 M Tris, pH 7.0, 2 mM dithiothreitol (DTT), 1 mM EDTA, and homogenized using Dounce glass homogenizer. The homogenate was stored at 4°C overnight. The extract was centrifuged at 43,000 *g* for 4 h, and the supernatant was applied to the sepharose CL-4B column (2×100 cm) which was equilibrated with the extraction buffer. The column was eluted with the equilibration buffer, and the first major peak was used as the clathrin sample.

In vitro translation

Proteins were expressed using *in vitro* translation kit containing TNT T7 coupled reticulocyte lysate system (Promega) by manufacturer's instruction. The plasmid encoding the full length cDNA of CALM (Kim *et al.*, 1999) was used for the *in vitro* transcription/translation system containing translation grade [^{35}S] methionine (1,000 Ci/mmol, Amersham Pharmacia).

Clathrin cage binding assay

Clathrin triskelia (1.8 μM) was dialysed overnight in isolation buffer (0.1 M MES, pH 6.7, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM PMSF) with 3 mM $CaCl_2$ at 4°C. Newly assembled cages were precipitated by ultracentrifugation (100,000 *g*) in a Beckman TLA 100.3 rotor. The pellet was suspended in isolation buffer, and incubated with assembly protein ([^{35}S]-methionine labeled CALM protein, reticulocyte lysate) for 1 h on ice. The mixtures were ultracentrifuged for 20 min. The pellet and supernatant fractions were analyzed by SDS-PAGE, followed by Coomassie blue staining and autoradiography.

Clathrin assembly assay

Three molar solution of clathrin triskelia were dialyzed overnight at 4°C against isolation buffer with the addition of assembly protein ([^{35}S]-methionine labeled CALM protein, reticulocyte lysate). Following centrifugation at 13,600 *g* for 3 min to remove nonspecific aggregates, newly assembled clathrin cages were precipitated by ultracentrifugation for 20 minutes at 100,000 *g*. The pellet and the supernatant fractions were analysed by SDS-PAGE, followed by Coomassie blue staining and autoradiography.

SH3 (Src homology 3) domain binding assay

GST-SH3 domain proteins and GST proteins were expressed in E. *coli* and immobilized on glutathion sepharose 4B beads. Then the beads were incubated for 2 h at 4°C with 5 μl of CALM protein in 500 μl of the binding buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP 40). Sample was centrifuged for 10 min at 10,000 *g* at 4°C. The supernatant was carefully aspirated and the beads were washed with 500 μl of binding buffer. And the samples were centrifuged for 10 min, at 10,000 *g* at 4°C. The samples were washed the same way four times. Bound protein was eluted by boiling in standard SDS-gel loading buffer, separated by 10% SDS-PAGE and visualized by autoradiography.

Dephosphorylation of the CALM protein

Five microliters of the labelled CALM were mixed with 40 unit of calf intestine alkaline phosphatase in reaction buffer (50 mM Tris, pH 9.0, 1 mM $MgCl_2$) or mixed with only reaction buffer as negative control. The mixtures were incubated for 1 h at 30°C. The change in mobility was analyzed by SDS-PAGE followed by autoradio-

graphy.

Proteolysis of CALM

The labelled CALM protein (5 μl) was mixed with lysate containing caspase in 20 μl of reaction buffer (16 mM HEPES, 8 mM NaCl, 0.004% IGEPAL-CA630). The mixtures were incubated for 1 h at 30°C. The CALM protein (5 μl) was mixed with calpain (Sigma) in 20 μl of reaction buffer (1X PBS, 1 mM $CaCl_2$). The mixtures were incubated for 1 h at 37°C. The digestions of CALM were analyzed by SDS-PAGE followed by autoradiography.

Results and Discussion

Expresseion of CALM and preperation of clathrin, and AP180

We expressed the CALM using cell-free reticulocyte lysate translation system. SDS-PAGE of the [^{35}S]-methionine labeled proteins revealed a major band with an apparent molecular weight of 105 kD (Figure 1A). This is consistent with the previous report from rat liver (Kim *et al.*, 2000a), that produced native CALM with an apparent MW of 110 kD on SDS-PAGE. The molecular weight is however somewhat larger than that estimated 71 kD mass weight based on amino acid composition. The discrepancy in the MW between native and the estimated size was also observed in AP180. The unusual migration of CALM in PAGE was likely caused by high ratio of proline, glycine and charged amino acid. The CALM contains 23% of charged amino acids, and 14% of proline residues (Kim *et al.*, 1999). The posttranslational modification could also affect the eletrophoretic mobility in the gel. These might result in anomalous migration of the CALM on SDS-PAGE, with differences between true and apparent molecular weights. The purity of the AP180 and clathrin used in the experiment were shown in Figure 1B. AP180 was prepared by using GST (Glutathione-S-transferase) expression system, and the staining patterns of Coomassie blue and immunoblotting by anti-AP180 monoclonal antibody were identical. Clathrin heavy chain from bovine brain was identified as single band on SDS-PAGE, and the light chains were not shown on the gel condition.

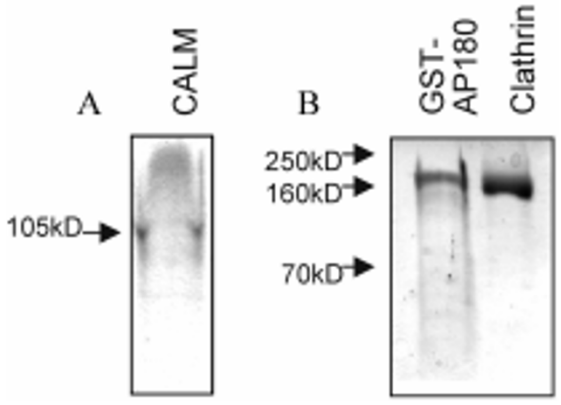

Figure 1. SDS-PAGE analysis of the [^{35}S]-methionine labeled CALM protein and the purified proteins. [^{35}S]-methionine labeled CALM was expressed in cell-free rabbit reticulocyte system by *in vitro* translation. GST-AP180 was purified from extracts of *E. coli* BL21 expressing the recombinant protein. Clathrin was purified from bovine brain - coated vesicle. The *in vitro* translated [^{35}S]-methionine labeled CALM was analyzed by SDS-PAGE followed by autoradiography (A) and GST-AP180 and clathrin are analyzed by SDS-PAGE followed by Coomassie blue staining (B).

Binding of the CALM protein with preassembled clathrin cage and clathrin assembly

The binding ability of CALM to clathrin cages was monitored by the cosedimentation of CALM and clathrin cages. AP180 (a positive control) were cosedimented with preassembled clathrin cages in the pellet fraction after ultracentrifugation at 100,000 *g*, in the presence of clathrin cages (Figure 2A, lane 4). CALM dialyzed with clathrin cages, sedimented with the clathrin cages by the 100,000 *g* centrifugation but in the absence of clathrin cages (Figure 2A and 2B, lane 5, 6, 7, and 8) indicating that CALM was able to bind the preassembled clathrin cages. The ability of clathrin cage assembly of CALM was monitored by dialysis with clathrin triskelia and the CALM protein, and the clathrin cage formed from the CALM and clathrin triskelia could be seperated by 100,000 *g* centrifugation on the ultracentrifuge. We used AP180, as a positive control in assembly assay. The cosedimention of CALM protein with clathrin was detect-

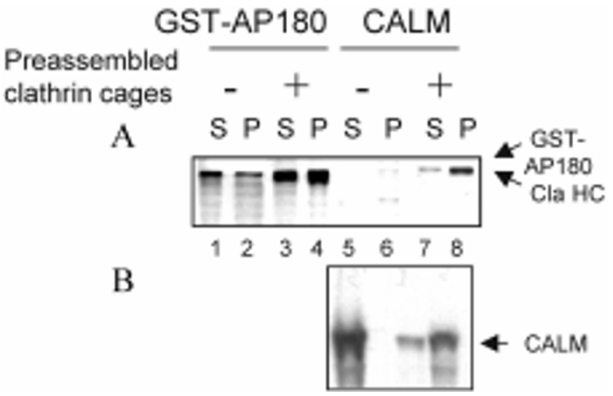

Figure 2. Binding of preassembled cage by the CALM protein. Clathrin cage binding assays were carried on as described in "Materials and Methods". Twenty μM concentration of GST-AP180 (lane 1-4), 5 μl of labeled CALM in reticulocyte lysate (lane 5-8) were incubated in the absence (–) or presence (+) of 1.8 μg (corresponding to molarity of triskelion, 1.2 mg/ml) 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 (upper panel) and autoradiography (lower pannel). Positions of each protein are indicated by clathrin heavy chain (Cla HC), GST-AP180, CALM.

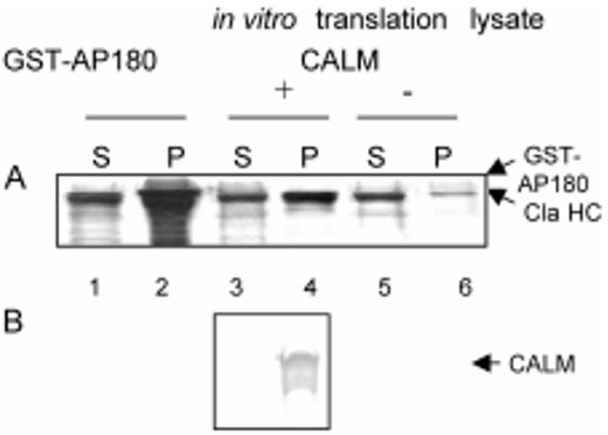

Figure 3. Assembly of clathrin cage by the CALM protein. Clathrin assembly assays were performed as described in "Materials and Methods". 3 μM clathrin triskelia were dialyzed overnight at 4°C against isolation buffer with the addition of 20 μM concentrations of GST-AP180, 10% of the translated [^{35}S]-methionine labeled CALM in reticulocyte lysate. 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 (upper panel) and autoradiography (lower panel). Positions of each protein are indicated in the same way as in Figure 2.

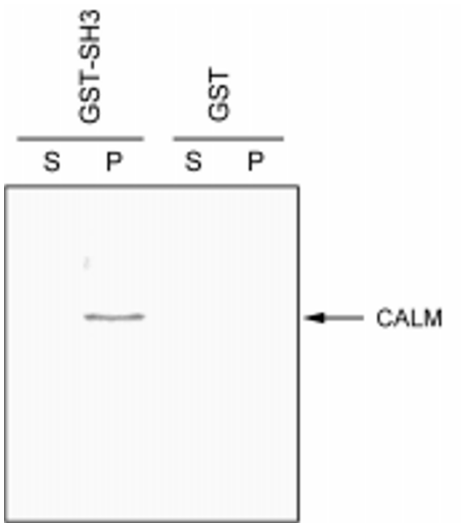

Figure 4. Interaction of the CALM protein with SH3 domain. SH3 binding assays were performed as described in "Materials and Methods". The proteins (GST-SH3, GST) bound to the beads and 5 μl of labeled CALM were used in the binding assay. The supernatant (S) and the pellet (P) fractions were analyzed by 10% SDS-PAGE followed by autoradiography.

ed by it's radioactivity in 100,000 *g* pellet fraction (Figure 3B, lane 4). Protein staining of the gel also showed a significant increase of clathrin proteins in the 100,000 *g* pellet fraction (Figure 3A, lane 4) demonstrating the ability of CALM to promote clathrin triskelia into cages.

CALM isolated from liver was reported to bind with clathrin heavy chain (Tebar *et al*., 1999) and could assemble clathrin triskelia into cages (Kim *et al*., 2000b). Moreover, the observation that bacterially expressed CALM could bind to preassembled clathrin cage and assemble clathrin cage (Kim *et al*., 2000b) suggests that on a gross appearance, the posttranslational modification of the CALM may not significantly alter the clathrin binding activity and cage-formation activity of the CALM although a kinetic profile of binding activities of CALM via cell signal-induced phosphorylation cannot be ruled out with these data. The CALM protein has more than 95% homology with AP180 in N terminus, but the sequence of C terminal region is different from that of AP180 (Dreyling *et al*., 1996; Kim *et al*., 1999). Apparently, both N terminal (33 kD) and C terminal (55 kD) parts of the AP180 have clathrin-binding activity (Ye *et al*., 1995) and C terminal domain has the activity of clathrin cage assembly. Vesicle formation could be regulated through binding of inositol phosphate, other adaptor protein, and phosphorylation of assembly protein. AP180 has high affinity to inositol hexakisphosphate and diphosphoinositol pentakisphosphate. The CALM protein possesses inositol binding motif and able to bind hexakisphosphate (Kim *et al*., 1999). Binding of these phosphoinositides inhibits clathrin assembly (Norris *et al*., 1995). Because of similar molecular structure between CALM and AP180, the formation of CCV by CALM might also be regulated by the phosphoinositides.

The interaction of the CALM protein with SH3 domain

CALM has multiple proline rich regions in the C terminal, which would interact with both SH3 and WW domain. Furthermore, it has some PxxP motif, which is a known ligand for SH3 domain. To confirm the direct interaction between CALM and SH3 domain through the proline rich region, the GST-pull down assay was carried out *in vitro*. In this assay, GST-PKCγ (protein kinase C) SH3 domain - immobilized agarose beads were incubated with CALM and the bound protein was analyzed by SDS-PAGE followed by autoradiography. The result showed that CALM did interact with SH3 domain (Figure 4, lanes 1 and 2). It failed to interact with a fusion partner GST alone (Figure 4, lanes 3 and 4). The clathrin vesicle - associated proteins harboring SH3 domain are dynamin and the amphiphysin. Dynamin is a cytosolic guanosine triphosphatase (GTPase), which 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 AP-2 (Zhang *et al*., 1994; Cestra *et al*., 1999).

Dephosphorylation of the CALM protein by alkaline phosphatase

AP180, a CALM homologous protein, was known to be a phosphoprotein and to be phosphorylated by casein kinase II (Hao *et al*., 1999). CALM has eight possible phosphorylation sites (Kim *et al*., 1999). The major sites

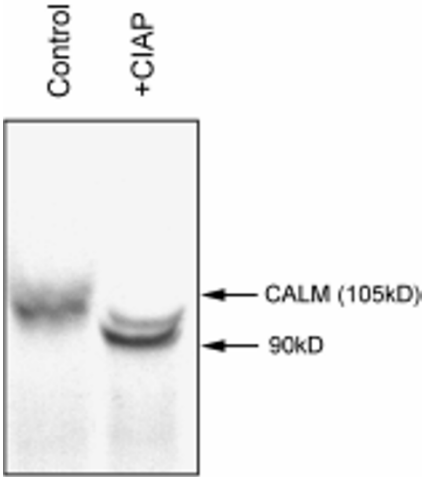

Figure 5. Dephosphorylation of the CALM protein by alkaline phosphatase. Five μl of the labeled CALM in reticulocyte lysate was treated with only calf intestine alkaline phsphatase buffer as a negative control (lane 1), and 4 unit of calf intestine alkaline phosphatase (CIAP) and reaction buffer (lane 2). Reactions were analyzed by 10% SDS-PAGE, followed by autoradiography.

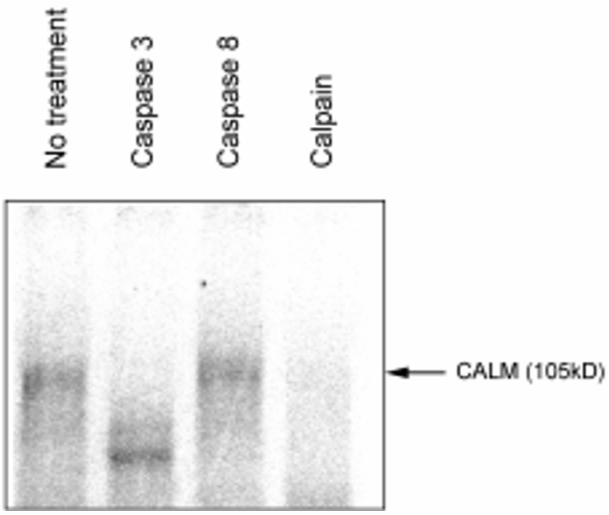

Figure 6. Proteolysis of the CALM protein by caspase 3 and calpain. Five μl of the labeled CALM in reticulocyte lysate was treated with caspase 3 (lane 2), caspase 8 (lane 3), and calpain (lane 4), and no treatment as a negative control (lane 1). Reactions were analyzed by 10% SDS-PAGE followed by autoradiography.

of phophorylation are located on the N terminal. In an effort to test whether the expressed CALM, by reticulocyte lysate translation system, was phosphorylated, it was treated with calf intestine alkaline phosphatase and analyzed by SDS-PAGE for any change in the electrophoretic mobility. Indeed, alkaline phosphatase treatment led to a distinct increase of the elctrophoretic mobility of CALM band as well as an increase in its sharpness (Figure 5), corresponding to a decrease in the apparent molecular size of CALM protein from 105 kD to 90 kD but since the electrophoretic mobility of CALM is anomalous, the decrease in molecular size can not be correlated to the number of phosphorus groups. However, the result definitively supports that CALM expressed in the cell-free reticulocyte lysate system is phosphorylated. Phosphorylation of AP180 was reported to decrease its interaction with AP-2. The assembly activity of the combination of phosphorylated AP180 and AP-2 was affected due to its reduced affinity for AP-2 (Hao *et al.*, 1999). Phosphorylation mediated modulation of CALM interaction with other adaptor protein is a strong possibility. In turn, CALM may participate in and be regulated in the formation of CCVs, by its phosphorylation state or interacting with SH3-harboring protein.

Proteolysis of the CALM protein by caspase 3 and calpain

The CALM has 3 DxxD motif at amino acids 263, 392, and 526 from amino terminus. DxxD motif could be the substrate of caspase 3. To confirm the proteolysis by caspase, CALM was treated with caspase 3 and 8. Protealysis of CALM by caspase 3 was evident, but not by caspase 8 (Figure 6, lanes 2 and 3). Caspase 3, an effector caspase, was activated by caspase 8 or caspase 9 by different pathways (Nunez *et al.*, 1998). Caspases are known to be activated during only apoptosis cascade. Apoptotic proteolysis of vesicle-associated proteins by caspases were not reported to date and it is the first case of CALM being a substrate for caspase. To investigate whether the CALM can be cleaved by calpain, a Ca^{2+}-dependent protease, the radiolabeled CALM was digested with calpain. The intensity of the CALM band was markedly reduced without showing any laddering fragmentation pattern (Figure 6, lane 4), suggesting the presence of possible multiple calpain-cleavage sites in CALM. The calpain sensitivity of CALM may provide mechanism through which its activity is regulated by changing intracellular calcium concentration. The proposed involvement of caspase and calpain in the processes of the neurodegeneration by apoptosis may imply a role for the CALM. The CALM protein, known to possess some protein binding motifs and distributed widely in various tissues, may play a role as a clathrin assembly protein as well as an adaptor protein (Marsh *et al.*, 1999). Our findings with CALM protein expressed in the controlled eukaryotic system showed it's participation in the formation of the clathrin vesicle and raised strong possibility of its involvement in modulation of interacting with other proteins harboring SH3 domain, through its phosphorylation, and by proteolytic cleavage.

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