Production of Antibody against Calmodulin Which Is a Protein with Poor Antigenicity

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Calmodulin (CaM), a Ca^{2+} -binding protein, is a multifunctional modulator mediating the calcium regulation of a large number of intracellular enzyme systems. Because of its highly conserved structure, it has been difficult to produce anti-CaM antibody. We describe here a simplified method for production of this antibody. BALB/c mice were immunized with a polymerized CaM, and the serum was prepared and tested for the presence of anti-CaM antibody by immunoblot analysis. The serum obtained from the immunized mice was shown to contain anti-CaM antibody. Immunoblot analysis proved that a 500-fold diluted serum was able to recognize both types of CaM with a polymerized form and monomer form. The method developed in this study may be applicable to a wide range of proteins for enhancement of antigenicity.

Key words calmodulin; calmodulin antibody; polymerization

Calmodulin (CaM) is a Ca^{2+} -dependent multifunctional modulator mediating the calcium regulation of a large number of fundamental intracellular enzyme systems. This protein is found in many species of eucaryotes.^{1,2} Proteins with CaM-like activity are also produced in some procaryotes.³ Recently we purified CaM-like protein from *Bordetella pertussis*, which activated 3',5'-cyclic nucleotide phosphodiesterase and adenylate cyclase in a Ca^{2+} -dependent manner.⁴ CaM appears to be a poor antigen in experimental animals such as rabbits and mice, probably because the protein is small (MW 16700) and acidic (pI 4.3), and its amino acid sequence is highly conserved in the tissues of widely divergent animals.⁵⁻⁹ Several attempts to elicit CaM antibody had failed. For example, a poor antigenicity was observed in CaM that is absorbed by poly L-lysine,¹⁰ polymerized with ethyl chloroformic acid to produce insoluble complex,¹¹ or cross-linked to thyroglobulin.¹² The only exception was immunization with a large amount (more than 10 mg) of CaM^{5,7} or with a chemically modified CaM by fluoro-dinitrobenzene or performic acid.^{8,9} Rather complex procedure was required to prepare the chemically modified CaM by the latter method, however. In the present study, we developed a simple method to prepare a polymerized form of CaM, which was confirmed to evoke the production of antibody in mice.

MATERIALS AND METHODS

Chemicals CaM derived from bovine brain (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as an antigen. Polyglycine (MW 2000—5000, Sigma Chemical Co.), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Dojindo Laboratories, Kumamoto, Japan), and *N*-hydroxysulfosuccinimide (Pierce Chemicals Co., Rockford, IL, U.S.A.) were used for polymerization of CaM.

Polymerization of CaM CaM was polymerized by coupling with polyglycine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysulfosuccinimide according to the method of Staros *et al.*¹³ with a slight modification. One and six-tenths ml of CaM

solution (0.25 mg/ml) was mixed with 200 μl of 1 mM polyglycine, 100 μl of 1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 100 μl of 50 mM *N*-hydroxysulfosuccinimide in a glass tube. After incubation for 24 h at room temperature, the solution was dialyzed against phosphate-buffered saline (PBS).

Immunization Procedure Fifty μg of CaM (monomer or polymerized form) absorbed by an equal amount of aluminum hydroxide gel in PBS was subcutaneously injected twice into BALB/c mice at a 3-week interval. On day 7 after the second immunization, the mice were boosted by an intravenous injection with 50 μg of CaM (monomer form) without the adjuvant. The immunized mice were bled on day 7 after the booster immunization, and antisera were prepared.

Immunoblot Analysis Two μg of polymerized or unpolymerized CaM was spotted onto nitrocellulose sheets (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The sheets were incubated with a blocking buffer (PBS containing 0.5% bovine serum albumin and 0.5% NaN_3) for 30 min at room temperature, and then with the anti-CaM serum diluted 1:100 with PBS containing 0.05% Tween 20 and 0.5% NaN_3 (PBS-Tween). After incubation for 2 h at room temperature, the sheets were rinsed three times, at 5 min intervals with PBS-Tween, and then incubated for 2 h at room temperature with an alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, U.S.A.) which was diluted 1:1000 with PBS-Tween. The sheets were then again rinsed three times with PBS-Tween. Finally, the PBS-Tween was replaced by 100 mM Tris-HCl buffer (pH 9.5) containing 100 mM NaCl and 5 mM MgCl_2 (alkaline buffer). Sheets were developed with the alkaline buffer containing 5-bromo-4-chloro-3-indolylphosphate (25 $\mu\text{g}/\text{ml}$) and nitroblue tetrazolium (50 $\mu\text{g}/\text{ml}$).

Others Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4—20% continuous gradient of acrylamide gel (SDS-PAG Plate 4/20, Daiichi Pure Chemicals Co., Tokyo, Japan) was performed by the method of Laemmli.¹⁴ Protein bands were detected

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by staining with Coomassie brilliant blue dye. Protein concentration was determined by the method of Bradford¹⁵⁾ using ovalbumin as the standard.

RESULTS AND DISCUSSION

Fifty μg of CaM (monomer form) absorbed onto aluminum hydroxide gel in PBS was injected into BALB/c mice (5-week-old females, SLC, Shizuoka, Japan). The six-times repeated injection at 1-week intervals did not produce any detectable antibody against CaM. This confirmed that antigenicity of soluble proteins with lower molecular weight is poor in an animal model. We therefore hypothesized that if a polymerized and insoluble form of CaM was used as an antigen, the antibody could be produced in mice.

Carbodiimides catalyze peptide-protein coupling reactions by the formation of amide bonds between carboxylic acids and amines.^{16,17)} Staros *et al.*¹³⁾ reported that addition of *N*-hydroxysulfosuccinimide enhances the carbodiimide-mediated coupling reaction. On the basis of their report, CaM was polymerized by coupling with polyglycine used as a spacer peptide. Bovine brain CaM and polyglycine were incubated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in the presence of *N*-hydroxysulfosuccinimide as described in Materials and Methods. After dialyzing against PBS the incubation mixture was analyzed by SDS-PAGE. The SDS-PAGE profile represented in Fig. 1 showed that only a small amount of CaM monomer remained 1-h after the beginning of incubation for the polymerization reaction (lane 2). The amount of polymerized form of CaM (insoluble complex) was gradually accumulated for 6 h on the top of the gel, in contrast to the decrease of the monomer form (Fig. 1, lanes 3–5). The polymerization of CaM appeared to be completed after 24-h of incubation (Fig. 1, lane 6). The polymerized CaM prepared by the incubation was used to immunize mice.

BALB/c mice were immunized with 50 μg of CaM (polymerized form) absorbed onto aluminum hydroxide gel in PBS, and the serum was prepared and tested for the presence of anti-CaM antibody as described in Materials and Methods. It was shown that the serum obtained from the immunized mice contained anti-CaM antibody. Immunoblot analysis proved that a 100-fold diluted serum was able to recognize CaM in both a polymerized form (Fig. 2, a-1 in panel A) and a monomer form (Fig. 2, b-1 in panel A). Five hundred-fold diluted serum also contained enough anti-CaM antibody to detect CaM in the immunoblot analysis. Ca^{2+} binds to CaM, and the Ca^{2+} -bound CaM is known to undergo a conformational change to a more helical structure.⁸⁾ We therefore tested the reactivity of the anti-CaM serum with CaM in the presence of 10 mM Ca^{2+} . The addition of Ca^{2+} did not impede the reactivity of CaM with serum, and both types of CaM (polymerized and monomer form) reacted with the anti-CaM serum (compare a-1 with a-2, or b-1 with b-2 in Fig. 2, panel A). Serum of mice immunized with the monomer form of CaM and normal serum did not recognize CaM (Fig. 2, panel B and C).

For the production of anti-CaM antibody, either a large

Fig. 1. Time Course of CaM Polymerization

CaM with polyglycine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was added with *N*-hydroxysulfosuccinimide to start the polymerization reaction. At 0 (lane 1), 1 (lane 2), 2 (lane 3), 4 (lane 4), 6 (lane 5) or 24 (lane 6) h after the addition, 10 μg of each CaM sample was subjected to SDS-PAGE on 4–20% gradient gel and subsequently stained with a Coomassie brilliant blue R solution. Lane 7, standard proteins [rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa)]. The arrow with a, b and c indicates the position of polymerized CaM, intact CaM and bottom, respectively.

Fig. 2. Immunoblot Analysis of CaM Antibody

2 μg of CaM (a) or polymerized CaM (b) with (1) or without (2) Ca^{2+} was spotted onto nitrocellulose sheets. The sheets were incubated with anti-polymerized CaM mouse serum (1 : 100 dilution) (A), serum of monomer form CaM immunized mouse (1 : 100 dilution) (B) or with normal mouse serum (1 : 100 dilution) (C).

quantity of a native CaM or chemically modified CaM has been used as the antigen to increase the antigenicity.^{5–12)} The polymerized form of CaM, in contrast, was prepared simply with polyglycine as a spacer peptide by the addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysulfosuccinimide for 24 h at room temperature, and the polymerized CaM proved to be highly antigenic in mice. Therefore, our method understood appears applicable to a wide range of proteins to enhance antigenicity.

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