Detection of Calmodulin-Binding Proteins Using a ^{32}P -Labeled GST-Calmodulin Fusion Protein and a Novel Renaturation Protocol

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ABSTRACT

To identify calmodulin-binding proteins in cellular extracts and tissue homogenates and to analyze purified calmodulin target proteins, overlay procedures using ^{125}I -calmodulin or, more recently, nonradioactive biotinylated calmodulin have been widely used. Here we describe a rapid, alternative method for detecting calmodulin-binding proteins with a ^{32}P -labeled calmodulin probe generated as a glutathione-S-transferase (GST)-fusion protein. We used a modified pGEX-2TK vector, which contains the flag epitope and the consensus sequence R-R-A-S, that can be phosphorylated by the cAMP-dependent protein kinase A. The fusion protein is easily purified from bacterial lysates by affinity chromatography using glutathione-Sepharose® 4B beads. Phosphorylation of GST-calmodulin is performed directly on the beads and, after elution with reduced glutathione, the labeled calmodulin probe can be used for overlay experiments. We also describe a rapid renaturation protocol that enhances the signal for some but not all calmodulin-binding proteins and is used after the proteins have been transferred to

nitrocellulose filters. Furthermore, we have compared the specificity and sensitivity of the ^{32}P -labeled GST-calmodulin overlay with those of ^{125}I -calmodulin and biotinylated calmodulin, clearly indicating that our newly developed protocol is a suitable alternative to conventionally used calmodulin overlay procedures.

INTRODUCTION

Calmodulin (CaM), a ubiquitous 17-kDa cellular calcium-binding protein, plays a central role in transducing the Ca^{2+} signal in eukaryotic cells (6). The specific binding of Ca^{2+} -CaM to target proteins regulates a variety of cellular responses that are initiated by increased levels of free cytosolic calcium. To understand the pleiotropic effects of Ca^{2+} -CaM in living cells, it is of fundamental importance to identify and characterize

the Ca^{2+} -CaM-interacting partners that exert these downstream effects. To identify and analyze CaM-binding proteins, a technique referred to as the ^{125}I -CaM overlay procedure has been widely used (9). More recently, nonradioactive biotinylated CaM has been described as a suitable probe for overlays (1). The fusion protein A-CaM has been used to investigate CaM functions in yeast (10). The basis of these methods is that labeled CaM binds specifically to its target proteins, which have been separated by sodium dodecyl sulfate (SDS) gel electrophoresis according to their sizes. Nonspecifically bound CaM is removed by washing the gel or the membrane onto which the proteins have been transferred, and the binding proteins are visualized by autoradiography or by staining with avidin reagents, respectively.

Here we describe a rapid, alternative

Figure 1. Construction of the expression vectors GST-CaM and GST-PV. Exact amino acid sequence of the transition between GST and CaM, and GST and PV, respectively. The GST moiety has a molecular weight of 26 kDa; the fusion proteins have a molecular weight of 43 kDa (GST-CaM) and 38 kDa (GST-PV). The phosphorylation site is indicated as P-site. Rat CaM cDNA and PV cDNA were cloned into the EcoRI site of the pGEX-2TK vector.

procedure for detecting CaM-binding proteins that uses a ^{32}P -labeled CaM probe generated as a glutathione-S-transferase (GST) fusion protein. The GST gene fusion system is a widely used technique for the expression and purification of fusion proteins produced in *E. coli*. Fusion proteins are easily purified from bacterial lysates by affinity chromatography using glutathione-Sepharose® 4B beads (Pharmacia Biotech, Dübendorf, Switzerland). We used a modified pGEX-2TK vector

(Pharmacia Biotech) that contains the flag epitope and the consensus sequence R-R-A-S, which is phosphorylated by the cAMP-dependent protein kinase A. Since the site is located upstream of the ATG initiation codon of the gene of interest, phosphorylation does not affect the full-length protein itself. Furthermore, the method described here is not restricted solely to the detection Ca^{2+} -CaM-binding targets, but can also be used to study protein-protein interactions in general.

Figure 2. Detection of calmodulin-binding proteins from cellular lysates and tissues using ^{32}P -labeled GST-calmodulin fusion protein. Lysates, prepared from various cell lines and tissues, were subjected to the above-described modified overlay assay. Panel A shows CaM-binding proteins in the presence of 1 mM CaCl_2 either under renaturing or non-renaturing conditions. Panel B shows an overlay performed in the presence of 5 mM EGTA and renaturing conditions. Panel C shows an overlay blot using ^{32}P GST-PV. Lane 1, placenta; lane 2, muscle; lane 3, brain; lane 4, calcineurin; lane 5, rat fibroblast cell line R6; lane 6, *src*-transformed R6 cells; lane 7, *src*/PKC-transformed R6 cells. Each lane contains 50 μg of total protein except for lane 4 (calcineurin), which contains 1 μg . Protein concentration was determined using the Bradford reagent (3). The blots were autoradiographed at -80°C for 24 h.

Research Reports

MATERIALS AND METHODS

Generation of Fusion Protein

Rat CaM (CaM III) cDNA (7) and rat parvalbumin (PV) cDNA (4) were cloned into the *EcoRI* site of the expression vector pGEX-2TK (Figure 1). CaM and PV were generated as GST-fusion proteins according to the manufacturer's recommendation (Pharmacia Biotech). Briefly, 2-mL aliquots of LB ampicillin (50 µg/mL) were inoculated with 100 µL of a freshly made overnight bacterial culture containing GST-CaM or GST-PV, respectively. Expression of the fusion protein was induced after 1.5 h by adding 20 µL of 100 mM isopropylthiogalactoside (IPTG), and the cultures were grown for approximately 3 h at 37°C. After centrifugation, the cells were resuspended in 200 µL of a solution containing 50 mM Tris-HCl pH 7.5, 2 mM EDTA and 0.5% Triton® X-100 and lysed by a freeze-thaw step. After centrifugation, the supernatant was incubated with 20 µL of glutathione-Sepharose 4B beads and mixed gently for 15 min at room temperature (RT). Beads were washed 3 times with 1 mL 1× phosphate-buffered saline (PBS) and once with 1× HMK buffer (heart muscle kinase buffer), which consists of 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl₂.

Labeling of the Fusion Protein

³²P-Labeling of the GST-fusion proteins (20 µL corresponding to 5 µg protein as measured by the Bradford assay; Reference 3) was carried out in a total reaction volume of 30 µL 1× HMK buffer, 1–2 U of bovine heart muscle kinase (protein kinase A; Sigma Chemical, Kaltenbach, Switzerland) and 1 µL (10 µCi) [γ-³²P]ATP (specific activity 3000 Ci/mmol; Amersham, Zürich, Switzerland). The reaction was carried out for 20 min at RT. The glutathione-Sepharose 4B beads were then washed several times with 1× HMK buffer until no radioactivity was present in the supernatant (routinely, 10 mL of HMK buffer are sufficient). The labeled fusion proteins were then eluted by adding 100 µL of elution buffer that contained 10 mM reduced glutathione (Sigma Chemical) in 50 mM Tris-HCl

pH 8.0. The elution step was repeated once to increase the recovery of labeled fusion proteins. Routinely, a specific radioactivity of 10⁵ to 10⁶ dpm/µg protein was achieved. Specific activity may be increased by adding higher amounts of [γ-³²P]ATP.

Modified Overlay Assay

The probes were applied in a modified overlay assay to identify CaM-binding proteins from cell lysates of

various cell lines and tissues. The overlays were performed in the presence of either 2 mM CaCl₂ or 2 mM EGTA in order to demonstrate the Ca²⁺-dependent nature of the interaction between CaM and its targets. GST-PV was chosen as a negative control because so far no target protein has been reported for the small Ca²⁺-binding protein PV. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were done according to standard procedures (8), general-

Figure 3. Overlay with ¹²⁵I-CaM or biotinylated CaM. Lysates, prepared from various cell lines and tissues, were subjected to the ¹²⁵I-CaM (A) and biotinylated CaM (B) overlay assays. The left-hand panels show CaM-binding proteins in the presence of 1 mM CaCl₂ under renaturing conditions. The right-hand panels (controls) show an overlay performed in the presence of 5 mM EGTA. Lane 1, placenta; lane 2, muscle; lane 3, brain; lane 4, calcineurin; lane 5, rat fibroblast cell line R6; lane 6, *src*-transformed R6 cells; lane 7, *src*/PKC-transformed R6 cells. Each lane contains 50 µg of total protein except for lane 4 (calcineurin), which contains 1 µg. Protein concentration was determined using the Bradford reagent (3). The blots were autoradiographed at -80°C for 24 h.

ly using 50 µg of protein per lane (measured by the Bradford method; Reference 3). After blotting, the nitrocellulose (NC) filters were incubated 5 min at RT in 20 mL of 6 M guanidinium hydrochloride, pH 7.0. Renaturation of the immobilized proteins was achieved by incubation of the blot in 2-fold serial dilutions of guanidinium hydrochloride (dilution buffer contained 50 mM imidazole, 200 mM KCl, 1 mM CaCl₂, 0.5% bovine serum albumin [BSA], 0.05% Tween 20). Seven to eight dilution steps are necessary (until the concentration of guanidinium hydrochloride finally reaches about 45 mM). Incubation time of each step was approximately 5–10 min. The NC filters were subsequently blocked for 2 h at RT in 1× Tris-buffered saline (TBS) containing 0.5% (wt/vol) low-fat milk. After blocking, the NC filters were incubated with the ³²P-labeled probe (10⁶ dpm/blot of 10 cm × 7.5 cm) at RT for 3–4 h. The filters were subsequently washed 3 times for 10 min with 1× TBS. Autoradiography was performed at -80°C for 24 h. ¹²⁵I-CaM was kindly provided by Dr. J. Krebs, Department of Biochemistry, Swiss Federal Institute of Technology, Zürich, Switzerland. CaM was iodinated using the Bolton-Hunter reagent (2). The specific radioactivity of the iodinated CaM sample was 5 × 10⁷ dpm/µg, and the overlay was performed according to published procedures using 10⁷ dpm/blot of 10 cm × 7.5 cm (9). Biotinylated CaM was purchased from (Life Technologies, Gaithersburg, MD, USA). The overlay assay was performed with 10 µg biotinylated CaM per gel (according to Reference 1). For all overlays, the same amount of total protein was loaded (50 µg, measured by the Bradford method; Reference 3).

RESULTS AND DISCUSSION

The constructs generated in this study are shown in Figure 1. They consist of a plasmid encoding a fusion protein containing GST and CaM or PV, a Flag epitope and a phosphorylation site for protein kinase A. Figure 2 shows a ³²P-labeled GST-CaM overlay either in the presence or absence of calcium and

under denaturing or non-denaturing conditions. Calcineurin (lane 4), a well-known CaM-binding protein, has been used as a control in our overlay assays. The renaturation protocol clearly leads to the enhancement of the signal for some (but not all) CaM-binding proteins (see Figure 2A: some low-molecular-weight proteins, especially of cell extracts, give enhanced signals). On the other hand, some CaM targets in brain extracts exhibit stronger signals on blots that were not subjected to the renaturation protocol. Therefore, the renaturation protocol might be advantageous in some instances. The overlay assay performed in the presence of 5 mM EGTA (Figure 2B) shows that most CaM-target interactions are strongly dependent on calcium. Some of the bands seen in the presence of EGTA most likely represent Ca²⁺-independent interactions. Figure 2C shows an overlay assay using GST-PV. No significant protein-protein interaction was obtained, except from very weak background signals. This is similar to what was found when GST alone was used as a probe (not shown). We have chosen PV because this protein is a close relative of CaM and belongs to the EF hand superfamily of Ca²⁺-binding proteins (5). In addition, no target proteins for this Ca²⁺-binding protein have been found.

We compared the signal pattern obtained with the ³²P-GST-CaM overlay assay with the one generated with the use of either ¹²⁵I-CaM or biotinylated CaM. As shown in Figure 3A, several bands that can be visualized by using ³²P-GST-CaM are not detectable by ¹²⁵I-CaM. Another technique that has been described to identify CaM-binding targets is the use of biotinylated CaM in overlay assays. We therefore also compared ³²P-GST-CaM and biotinylated CaM in the overlay assay. Figure 3B shows the overlay assay performed with biotinylated CaM. In our hands, the biotinylated CaM overlay assay resulted in a much higher background than seen with the other two probes. In addition, the overall signal intensity is lower as compared with the one generated by ³²P-GST-CaM or ¹²⁵I-CaM.

We also performed semi-quantitative overlay assays to compare the sen-

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sitivity of ^{32}P -GST-CaM, ^{125}I -CaM and biotinylated CaM using calcineurin as a CaM target. Figure 4 demonstrates that ^{32}P -GST-CaM exhibits the highest sensitivity in detecting calcineurin in an overlay assay, whereas ^{125}I -CaM exhibits a slightly lower sensitivity and biotinylated CaM, a markedly lower sensitivity. Upon extended exposure (3 days), 7 ng of calcineurin can be detected by both ^{32}P -GST-CaM and ^{125}I -CaM, but not by biotinylated CaM.

In summary, we have generated a ^{32}P -labeled GST-CaM fusion protein to detect CaM-binding proteins in a modified overlay procedure. This method offers advantages over conventional techniques: Preparation of ^{32}P -labeled probes and separation of unincorporated label is rapid and easy, in contrast to iodine labeling and probe separation, which are somewhat inconvenient. Furthermore, iodine labeling on tyrosine residues or biotin labeling could change some properties of the native protein, thereby affecting target recognition when using this modified overlay procedure. However, a potential drawback to the modified overlay assay described is its use of a fusion protein as a probe. Therefore, one has to consider potential problems arising from the modified N-terminus of the probe, although we have not encountered this problem so far. As compared to GST-CaM (GST-PV as a control), overlays using biotinylated CaM cause considerably higher background (see Figure

3B), and might even produce some unspecific signals due to the ubiquitous biotin-binding proteins present in the sample analyzed. We also demonstrate, by direct comparison of ^{32}P -GST-CaM with ^{125}I -CaM and biotinylated CaM, that ^{32}P -GST-CaM exhibits equal or superior sensitivity and specificity. The generated GST-CaM reagent can be used in methods other than the overlay assay described, for instance, for affinity purification of CaM targets and studies on protein-protein interaction in solution.

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Figure 4. Semiquantitative comparison of ^{32}P -GST-CaM, ^{125}I -CaM and biotinylated CaM. Indicated amounts of calcineurin were run on SDS-PAGE and transferred to nitrocellulose filters. The NC filters were then analyzed in the overlay assay using the indicated CaM probes and the renaturation protocol. ^{32}P -GST-CaM and ^{125}I -CaM blots were autoradiographed at -80°C for 24 h.