Production of Reagents and Optimization of Methods for Studying Calmodulin-Binding Proteins

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Received June 22, 1998, and in revised form September 16, 1998

Owing to subtle but potentially crucial structural and functional differences between calmodulin (CaM) of different species, the biochemical study of low-affinity CaM-binding proteins from Dictyostelium discoideum likely necessitates the use of CaM from the same organism. In addition, most of the methods used for identification and purification of CaM-binding proteins require native CaM in nonlimiting biochemical quantities. The gene encoding D. discoideum CaM has previously been cloned allowing production of recombinant protein. The present study describes the expression of D. discoideum CaM in Escherichia coli and its straightforward and rapid purification. Furthermore, we describe the optimization of a complete palette of assays to detect as little as nanogram quantities of proteins binding CaM with middle to low affinities. Purified CaM was used to raise high-affinity polyclonal antibodies suitable for immunoblotting, immunofluorescence, and immunoprecipitation experiments. The purified CaM was also used to optimize a specific and sensitive nonradioactive CaM overlay assay as well as to produce a high-capacity CaM affinity chromatography matrix. The effectiveness of this methods is illustrated by the detection of potentially novel D. discoideum CaM-binding proteins and the preparatory purification of one of these proteins, a short tail myosin I.

Key Words: calmodulin; calmodulin-binding proteins; affinity chromatography; myosins; Dictyostelium discoideum.

Ca2+ ions play a critical role in the regulation of many cellular processes. Calmodulin (CaM), as a low molecular weight and high-affinity Ca2+-binding protein, is responsible for mediating Ca2+ signals to a multitude of systems. CaM is present in all eukaryotic cells (reviewed in 1 and 2), including cellular ameba such as Dictyostelium discoideum. Mammalian and D. discoideum CaMs are 87% identical (3,4) and are quite similar in enzyme activation. However, some functional differences have also been observed (3) and in order to study CaM and CaM-interacting proteins in D. discoideum, it is probably necessary to use the endogenous protein.

In D. discoideum, CaM is an essential protein (4) and is expressed at constant level from a single gene throughout the developmental cycle. It is necessary for spore swelling (5) and is required for cell and pronuclear fusion (6). Localization studies revealed that CaM is enriched on the membranes of the osmoregulatory system, i.e., the contractile vacuole (7). It was suggested that this localization results from its association with an unconventional myosin (7,8). The myosins form a superfamily of molecular motors that have a common modular structure. These proteins are composed of three functional domains: the conserved head (or motor domain), the highly divergent tail (or potential cargo-binding domain); and an intervening neck domain. The neck domain contains one or more so-called IQ motifs which have been shown to bind light chains belonging to the EF-hand family, in particular, CaM. As mentioned above, CaM interacts with a multitude of effectors, often with a Kd in the range of nanomolars and in a strictly Ca2+-dependent fashion. Interestingly, it has been reported that the interaction of CaM with many unconventional myosins may be

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2 Abbreviations used: CaM, calmodulin; IPTG, isopropyl-b-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; TBST, Tris-buffered saline plus Tween 20; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; ENPP, 1,2-epoxy-3-(p-nitrophenoxy)propane; BAEE, Nα-benzoyl-L-arginine ethyl ester; TAME, Nα-p-tosyl-L-arginine methyl ester; TPCK, N-p-tosyl-L-phenylalanine chloromethyl ketone; DTT, 1,4-dithiothreitol; HESES, Hepes-sucrose-EGTA-salt buffer; PBSG, PBS-gelatin.
independent of Ca\(^{2+}\) (reviewed in 9) and with a likely 
K_d in the range of micromolars.

The detailed biochemical study of such interactions in D. discoideum therefore necessitates the availability of D. discoideum CaM in nonlimiting quantities. The present study describes the expression of D. discoideum CaM in Escherichia coli and the simple and efficient purification of the recombinant protein. Special care was given to the optimization of a complete series of assays used to detect and purify D. discoideum proteins binding CaM with middle to low affinities. Thus purified CaM was used to raise high-affinity polyclonal antibodies and to perfect a specific and sensitive nonradioactive CaM overlay assay. In addition, we produced a high-capacity CaM affinity chromatography matrix which can be specifically and gently eluted with excess free CaM.

**MATERIAL AND METHODS**

Expression of D. discoideum Calmodulin in E. coli

The cDNA-encoding D. discoideum calmodulin (4) (Dd CalA, a generous gift of Dr. Margaret Clarke, Oklahoma Medical Research Foundation, Oklahoma, OK) was subcloned into the E. coli expression vector pET8c (Novagen Inc., Madison, WI) digested with NcoI and BamHI. The construct was introduced into the E. coli strain BL21 (DE3) (Novagen Inc.). Expression was induced as described by Studier and colleagues (10). Briefly, 4 ml of an overnight culture of the bacteria, grown in LB medium supplemented with ampicillin (100 \(\mu\)g/ml), was used to inoculate 250 ml LB medium plus ampicillin (100 \(\mu\)g/ml). The bacteria were grown to an OD_{600} of 0.4 - 0.6 and the induction started by adding IPTG to a final concentration of 1 mM. Induction was continued for 4 h at 37°C and then the cells were cooled on ice for 10 min. Bacteria were collected by centrifugation at 8000g at 4°C for 10 min, washed in 25 mM Tris–Cl, pH 7.5, and recentrifuged. At this stage, the cell pellet can be either flash frozen in liquid nitrogen and stored at \(-20°C\) or immediately treated for purification of CaM as follows.

Purification of D. discoideum CaM

The bacterial pellet of a 250-ml culture was suspended in 10 ml lysis buffer (25 mM Tris–Cl, pH 7.5, 2 mM CaCl_2, 1 mM MgCl_2, 1 mM benzamidine, 0.5 mM PMSF). A comparison of lysozyme and DNase I, and freeze/thaw cycles (not shown) demonstrated cycles of freeze/thawing to be simple, rapid, and the most efficient method for disruption of the bacteria. It should be noted that this method is likely to work best in lysogen bacteria such as the BL21 (DE3) used here and may require optimization if using other strains. The suspension was lysed by three rapid cycles of freeze/thawing between liquid nitrogen and 37°C. The lysate was cleared by ultracentrifugation at 120,000g (Beckmann Optima TLX-Ultracentrifuge, rotor TLA 100.4) for 30 min at 4°C. NaCl and CaCl_2 were added to the supernatant to a final concentration of 500 and 5 mM, respectively. The 10-ml sample in a 50-ml Falcon tube was placed in boiling water. When the temperature in a second, similarly filled reference tube reached 60°C the timer was started and the sample further incubated for 5 min. The solution was ultracentrifuged as described above and the CaM-containing supernatant was either stored at \(-20°C\) or immediately fractionated by reverse-phase chromatography, as adapted from the method of Gopalakrishna and Anderson (11). For fractionation the supernatant was loaded on phenyl–Superose HR 10/10 (Pharmacia, Freiburg, Germany) in the presence of Ca\(^{2+}\) (Buffer A, 25 mM Tris–Cl, pH 7.5, 2 mM CaCl_2, 1 mM MgCl_2, 500 mM NaCl) and eluted in an EGTA gradient (Buffer B, 25 mM Tris–Cl, pH 7.5, 5 mM EGTA, 1 mM MgCl_2). The purity of the fractions, corresponding to the peak of CaM (as monitored by OD_{280}), was verified by SDS–PAGE analysis. Protein concentrations were determined spectrophotometrically using the Bio-Rad protein assay (Bio-Rad, Munich, Germany). Purified CaM was flash frozen in liquid nitrogen in the presence of 5 mM CaCl_2 and stored at \(-80°C\).

Production of Polyclonal Anti-CaM Antibodies

Polyclonal antibodies were raised in rabbits (Biotrend, Köln, Germany) according to standard procedures which necessitated the purification and lyophilization of approximately 2 mg of D. discoideum CaM. Briefly, the antigen was used uncoupled and the immunization was carried out as follows: 300 \(\mu\)g of the immunogen, dissolved in 300 \(\mu\)l of PBS, was mixed with 1 ml adjuvant and injected subcutaneously into rabbits. Boosting was performed at monthly intervals with 100 \(\mu\)g of CaM. The sensitivity and specificity of the anti-CaM sera were tested by Western blotting against defined amounts of the recombinant CaM and extracts from human, bovine, rat, mouse, and D. discoideum cells, respectively.

Immunofluorescence and Microscopy

D. discoideum cells of wild-type strain AX-2 were grown in HL5c medium in shaking culture at 200 rpm. For immunofluorescence experiments conventional glass coverslips grad 0 which are 80 to 100 \(\mu\)m thick (diameter 12 mm) were used. Subconfluent cells were plated and allowed to adhere to the coverslips for several hours prior to fixation. The coverslips were plunged directly in methanol at \(-85°C\) followed by rewarming to \(-35°C\) in 30 min (12). Following this methanol-fixation/permeabilization treatment, sam-
samples were taken out of the methanol, immediately washed in PBS at room temperature for at least 15 min to remove methanol, and incubated with PBS–gelatin (0.2% PBSG) for an additional 15 min. Methanol fixation/permeabilization allows immunocytochemistry to be performed without the need for detergent permeabilization. Fixed cells were incubated for 30 to 60 min with the anti-CaM serum diluted 1:1000 in PBSG, washed in PBS, and incubated for 30 to 60 min with Cy3-labeled secondary antibody diluted 1:500 in PBSG. The unbound antibodies were then washed out with PBS, and the coverslips were plunged for a few seconds in water and mounted in Mowiol containing 100 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma, Deisenhofen, Germany) and a polyclonal antibody that was generously provided by Dr. M. Baehr, Ludwig Maximilian University, Munich, Germany). As secondary antibodies we used a goat antibody against whole rabbit IgG, coupled to horseradish peroxidase (Bio-Rad) at a 1:1000 dilution in TBS/3% milk. The samples were analyzed with a Leica confocal microscope DM/IRB using a 63X Plan-Apo objective with NA 1.40. Confocal optical sections were recorded at 0.5 μm per vertical step and 8× averaging.

SDS–Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

The fractions of the purification were analyzed by SDS–PAGE (15% SDS–PAGE) (13) and proteins were visualized by staining with Coomassie brilliant blue R-250. Broad range molecular size markers (Bio-Rad) were used. For Western blotting experiments, the separated proteins were transferred to nitrocellulose membranes (BA-S 85, pore size 0.45 μm, Schleicher and Schuell, Dassel, Germany) according to Towbin et al. (14), blocked overnight with 5% nonfat dry milk in TBST. The anti-CaM antibody was used at a 1:1000 dilution in TBS/3% milk. In addition, we used a rabbit polyclonal anti-calceinurin A (15; generously provided by Dr. R. Mutzel, University of Konstanz, Konstanz, Germany) and a polyclonal antibody that recognizes an epitope conserved in most myosin I (G-371, (16); generously provided by Dr. M. Bähler, Ludwig–Maximilian University, Munich, Germany). As secondary antibodies we used a goat antibody against whole rabbit IgG, coupled to horseradish peroxidase (Bio-Rad) at a 1:1000 dilution in TBS/3% milk. The immune complexes were detected by the enhanced chemiluminescence procedure (ECL, Amersham, Braunschweig, Germany).

CaM Overlay Assay

The nonradioactive CaM overlay assay was carried out according to Zhu and Clarke (7), with the following modifications. In order to study the influence of the state of protein denaturation on the sensitivity of the overlay, samples were either prepared as standard by boiling in DTT-containing sample buffer or, alternatively, prepared under nonreducing conditions with sample buffer lacking DTT at 65°C. After transferring the proteins to nitrocellulose membranes, unspecific binding sites were blocked overnight with 5% nonfat dry milk in TBST. The membranes were then incubated with different concentrations of CaM (from 2 to 20 μg/ml, corresponding to approximately 0.1 to 1.0 μM) and BSA (1 mg/ml) in TBS for 2 h at room temperature in the presence of 0.1 mM CaCl2 or 5 mM EGTA. After washing four times for 5 min in TBS and blocking for 1 h in 5% nonfat dry milk in TBST, the presence of bound CaM was revealed with anti-CaM antibodies as described above under Western blotting. In addition, we monitored the influence of chemically cross-linking CaM, on the nitrocellulose membrane, on the sensitivity of the CaM overlay to binding to its binding partner. For this, bound CaM was cross-linked by incubation in 0.2% gluteraldehyde/PBS for 15 min, followed by four washes of 5 min in TBS and again by blocking for 1 h in 5% nonfat dry milk in TBST.

Cultivation of D. discoideum and Preparation of a Cytosolic Extract

D. discoideum AX2 cells were grown in shaking culture in HL5c medium (17). They were harvested at concentrations of 2–3 × 10^6 cells/ml and processed as follows. The cells were pelleted by centrifugation at 1000g for 10 min at 4°C, washed in ice-cold Heses buffer (20 mM Hepes, pH 7.2, 0.25 M sucrose, 1 mM EDTA), and centrifuged again. The pellet was resuspended in Heses buffer (20 mM Hepes, pH 7.2, 0.25 M sucrose, 1 mM EDTA, 10 mM ATP, 1 mM DTT, 1 μg/ml aprotinin, 0.1 mM PMSF, 0.2 mM o-phenanthroline, 150 μM TLCK, 100 μM ENPP, 100 μM BAAE, 100 μg/ml TAME, 80 μg/ml TPCK, 2 μg/ml pepstatin, 5 μg/ml leupeptin) supplemented with 0.7 M KCl, at a ratio of 4 ml buffer per 10^9 cells. The cells were broken with a ball homogenizer (EMBL, Germany); 8 to 10 passages with a clearance of 5 μm) and again spun at 1000g for 10 min at 4°C, and the resulting postnuclear supernatant was centrifuged at 120,000g for 40 min at 2°C to pellet the membranous and cytoskeletal components. The supernatant was dialyzed against 10 mM Hepes, pH 7.5, 10 mM KCl, 1 mM DTT, 10% sucrose, 0.1 mM ATP, 0.1 mM PMSF, 0.2 mM o-phenanthroline, shock frozen in liquid nitrogen, and stored at −80°C.

CaM Affinity Chromatography

Purified CaM was immobilized onto Affi Gel 10 matrix (Bio-Rad) with coupling concentrations varying from 1 to 10 mg/ml of beads. Briefly, the Affi Gel 10 slurry was poured on a 0.22-μm filter (Stericup-GS, Millipore, Eschborn, Germany), washed liberally with ice-cold distilled water, and quickly transferred to the solution containing the desired amounts of CaM in coupling buffer (50 mM Hepes, pH 7.5, 150 mM NaCl,
1 mM CaCl₂). Incubation with constant gentle shaking was continued for 4 h at 4°C. The matrix was centrifuged at 800 g and washed twice with coupling buffer. Blocking of unreacted succinimidyl esters was achieved by incubation with 0.1 M ethanolamine–HCl, pH 8.0, for 1 h at 4°C in batch mode. After washing in 50 mM Tris–Cl, pH 7.5, 150 mM NaCl, and 1 mM CaCl₂ the CaM affinity matrix was stored at 4°C in the presence of 0.01% azide. The coupling efficiency was determined as the ratio between the concentration of CaM in the solution before and after incubation with the activated matrix.

CaM affinity chromatography was performed by incubating 0.5 ml of the CaM–Affi Gel 10 matrix with 3 to 5 mg of D. discoideum cytosol (incubation buffer, 25 mM Tris–Cl, pH 7.5, 10 mM KCl, 2 mM CaCl₂, 1 mM EGTA, 0.1 mM PMSF, and 1 mM DTT) for 2 h at 4°C in batch mode on a shaker. After transfer of the slurry to a small column, unbound proteins were washed by a series of incubation steps in the same buffer. The CaM-binding proteins were then eluted by performing successive elutions with increasing stringency. First, the column flow was stopped and 1.2 ml of incubation buffer containing free CaM (in a concentration ranging from 80 to 240 μM) was added, and then the matrix was resuspended by gentle stirring and incubated for a total of 1 h, stirring gently several times during this incubation. Similar incubations/elutions were subsequently performed, with the same column, for 10 min each by using 5 mM EGTA (in 25 mM Tris–Cl, pH 7.5, 10 mM KCl, 0.1 mM PMSF, and 1 mM DTT), 1 M KCl (in 25 mM Tris–Cl, pH 7.5, 5 mM EGTA, 0.1 mM PMSF, and 1 mM DTT) and finally the matrix was stripped with 6 M urea (in 25 mM Tris–Cl, pH 7.5, 0.1 mM PMSF, and 1 mM DTT).

RESULTS

Expression of D. discoideum CaM in E. coli

The basal expression level of CaM in E. coli BL21 (DE3 strain) transformed with the pET8c-CaM vector was very low. Addition of IPTG, however, induced the synthesis of a protein of the expected size of 18 kDa (as estimated from the SDS–PAGE gel) in amounts that continuously increased over the 4-h incubation (Fig. 1), reaching up a maximum of approximately 50 mg/liter of culture. Three separate preliminary tests were performed to confirm the identity, as well as to ascertain the quality, of the crude CaM preparation. First, we showed that it reacted with an antibody raised against Paramecium CaM (18, 19), second we verified that it produced the characteristic Ca²⁺-dependent electrophoretic mobility shift upon SDS–PAGE analysis (2), and third we tested its heat stability which is a prerequisite for the purification procedure described below. The results of the last two experiments are stringently dependent on the completeness of native folding of CaM.

Purification of CaM

In order to use D. discoideum CaM as an immunogen, to create an affinity matrix and to further in detection assays such as blot overlay procedures, it was purified to homogeneity. The purification was carried out in three steps. After disruption of the bacteria by freeze/thaw cycles and ultracentrifugation, the lysate was heat denatured and recentrifuged. At this step, CaM was found in the soluble fraction and its purity was nearly 50–70% of the total amount of protein. Note that, following the induction protocol presented here, no CaM was detectable in the insoluble inclusion bodies. The soluble fraction was loaded on a phenyl–Superose column in the presence of 2 mM Ca²⁺ (Buffer A) resulting in quantitative binding of CaM. The elution was done with a gradient of EGTA (from 0 to 100 % Buffer B) and three main peaks of absorption at 280 nm were obtained (Fig. 2A). Fractions 4–17 correspond to the flowthrough, fractions 34–37 contained CaM, and the content of the last peak could not be unambiguously identified. Figure 2B shows the corresponding Coomassie blue staining after SDS–PAGE separation of fractions from the first two peaks (fractions 4–37).

Mass Spectrometry Analysis

Posttranslational processing of calmodulin is likely to influence the affinity of binding to its numerous
cellular targets. Therefore, purified *D. discoideum* CaM was subjected to electrospray ionization mass spectrometry. The analysis clearly revealed that the starter methionine had been proteolytically cleaved, as the molecular mass observed (17,019 ± 5 Da) corresponded exactly to the one calculated from the cDNA (17,019.83 Da). No other modification of the sequence was detectable; the lysine 115 was not trimethylated, which is identical to calmodulin directly purified from *D. discoideum* and contrary to calmodulin purified from bovine tissues, for example. The spectra also indicated that purified calmodulin molecules were associated with different numbers of calcium, varying from one to four bound ions.

Raising and Testing Antibodies against *D. discoideum* CaM

Antibodies against the recombinant CaM were raised in rabbits and the sera tested for affinity and specificity in Western blots with various amounts of recombinant CaM and extracts of *D. discoideum* cytosol, respectively (Fig. 3). Note that the sera failed to detect CaM in extracts from human, bovine, rat, and mouse cells by Western blotting (not shown). The crude serum obtained after three regular boosts specifically detected a single band in the *D. discoideum* cytosolic fraction and readily visualized purified CaM in amounts smaller than 1 ng. Finally, CaM appears to represent nearly 0.03% of the total cell protein, in agreement with quantitations described by others (20, 21). The titer of anti-CaM antibodies in the crude serum is very high and allows for immunocytochemistry without further purification. In combination with a novel cryoctxification method developed in our laboratory (12), we assessed the intracellular localization of CaM in *D. discoideum* by using immunofluorescence microscopy. As can be seen in Fig. 4, CaM is mainly concentrated at the periphery of the osmoregulatory contractile vacuole system, possibly by association with an unconventional, CaM-binding myosin (8).

CaM Overlays

Detection of protein–protein interactions by blot overlay is an increasingly popular method and has already been applied to CaM-interacting proteins. In this experiment, we wanted to assess the suitability of a nonradioactive CaM overlay method (7,22) in the study of low-affinity, potentially Ca$^{2+}$-independent CaM-binding proteins. In addition, we rigorously tested the influence of several experimental parameters on the specificity and sensitivity of the CaM overlay, such as
preparation of reduced/nonreduced samples and chemical cross-linking of bound CaM prior to antibody detection (Fig. 5). As a control we used bovine calcineurin (molecular mass 66 kDa) which bound CaM in a strictly Ca\textsuperscript{2+}-dependent fashion as expected (compare lanes 2 and 4, 6 and 8, 2’ and 4’, and 6’ and 8’). Use of nonreducing sample preparation did not significantly affect the overall signal strength but did lead to increased band smearing and detection of potential calcineurin multimers (lanes 2’ and 6’). Fixation of CaM subsequent to the binding incubation resulted in a weakening of most signals, with the exception of a 180-kDa protein (compare lanes 1 and 5 and 1’ and 5’ respectively). In the cytosolic extracts, the major signal corresponds to D. discoideum calcineurin (molecular mass about 80 kDa), which behaves identically to its bovine homolog under the conditions tested. Finally, as we are interested in potential CaM-binding myosins, we were somewhat surprised that, in absence of Ca\textsuperscript{2+}, no strong signal in the high molecular weight range of the blot could be detected, even though the binding of CaM to many myosins has been reported to be Ca\textsuperscript{2+} independent (9). In our hands, strong signals were only obtained with a combination of nonreducing electrophoresis conditions and use of free Ca\textsuperscript{2+} during incubation with CaM.

CaM Affinity Chromatography

Affinity chromatography on immobilized CaM has been shown to be a useful and powerful tool for the identification and isolation of CaM-binding proteins from a cytosolic preparation (23–26). For the identification and purification of low-affinity CaM-binding proteins, it seems crucial to couple CaM to a matrix with a high final concentration. Commercially avail-
able affinity matrices routinely carry approximately 1 mg/ml of immobilized CaM. We coupled CaM to Affi Gel 10 agarose beads at concentrations of 1, 4, and 7 mg/ml and then tested these three affinity matrices. No significant differences between the matrices in terms of binding of the control, high-affinity CaM-binding protein calcineurin (not shown) were observed. In contrast, preliminary experiments demonstrated that, at a concentration of 7 mg/ml (corresponding approximately to 350 μM immobilized CaM), more of the high molecular mass cytosolic proteins were bound and could be eluted under gentle specific conditions. Therefore, the 7 mg/ml matrix was used in the following experiments.

D. discoideum cytosol was incubated with the immobilized CaM in batch mode, in the presence of Ca$^{2+}$. After binding, the slurry was transferred to a column and washed until the concentration of protein in the flow through was down to baseline (Fig. 6A, lanes 3–7). Elution was performed in steps of increasing stringency, first with an excess of free CaM (1.2 ml of 80 μM CaM or 1.7 mg; lanes 8 and 9), followed by a strong Ca$^{2+}$ chelator (5 mM EGTA, lane 11), high salt (1 M KCl, lane 12), and finally a stripping solution (6 M urea, lane 13). No remarkable difference was observed between the band pattern of the cytosol and the flowthrough (compare lanes 1 and 2). The gentle elution by competition with excess CaM resulted in the detachment of a surprisingly high number of different proteins (lanes 8 and 9). The pattern of bands is very different from that observed in the starting material and was essentially unchanged if higher concentrations of free CaM were used for elution (up to 240 μM, not shown). Washes performed with a higher stringency, for example, by including 0.5% Tween 20, 250 mM KCl, or 1 mg/ml BSA, did not significantly alter the pattern of proteins eluted by excess CaM (not shown). The subsequent elution with EGTA detached yet another set of proteins that differ somewhat from the CaM elution (compare lanes 9 and 11). The elution profiles of the high salt and urea stripping were also quite distinct, with a strong signal around 220 kDa which corresponds to D. discoideum myosin II (as detected by Western blotting, not shown). The different fractions are (lane 1) cytosol, (lane 2) flowthrough, (lanes 3–7) repeated washes, (lanes 8 and 9) CaM elution, (lane 10) wash, (lane 11) EGTA elution, (lane 12) KCl elution, and (lane 13) stripping with urea.

**FIG. 6.** Fractionation of D. discoideum cytosol by CaM affinity chromatography. 3 mg of D. discoideum cytosol was incubated for 2 h at 4°C with 500 μl of Affi Gel 10 matrix to which CaM had been coupled (7 mg CaM/ml Affi Gel 10). After washing with incubation buffer, bound proteins were subsequently eluted with 80 μM free CaM, 5 mM EGTA, 1 M KCl, and finally 6 M urea. The fractions were separated by 8% SDS–PAGE and stained with silver (A) or transferred onto nitrocellulose. Immunodetection was performed with antibodies against D. discoideum calcineurin A (B) and with an antibody that recognizes an epitope conserved in most myosin I (C). The different fractions are (lane 1) cytosol, (lane 2) flowthrough, (lanes 3–7) repeated washes, (lanes 8 and 9) CaM elution, (lane 10) wash, (lane 11) EGTA elution, (lane 12) KCl elution, and (lane 13) stripping with urea.
pris two globular domains which are connected by an extended α-helix. Upon Ca\(^{2+}\) binding, the conformation changes and exposes hydrophobic patches which mediate the interaction with the appropriate target peptide. Generally, the interaction of CaM with its target sequences has been roughly classified as Ca\(^{2+}\)-dependent or Ca\(^{2+}\)-independent (28), even though, in the latter case, Ca\(^{2+}\) is often required for maximal binding. The affinity of the interaction varies greatly, with \(K_d\) comprised between low nanomolars and a few micromolars.

Among its multitude of functions, CaM has been described as a light chain of numerous unconventional myosins. Our laboratory is investigating the potentially regulatory and structural functions of CaM as a light chain of D. discoideum myosins. Because it has been suggested that CaM binds to myosins with relatively low affinity and because there may be slight structural and functional differences between CaM of different organisms (for example, D. discoideum CaM has been shown to be manyfold more potent than bovine CaM in activating pea NAD kinase (3)) we decided to produce and use D. discoideum CaM for use in our investigations, even though bovine CaM has already been used successfully to identify and purify high-affinity-binding proteins from D. discoideum (29). The protocols presented in this study describe the production and fast and efficient purification of CaM. The high quality of our CaM preparation was confirmed independently in an investigation of the high-affinity interaction of CaM with the cleaved and processed signal peptides of preprolactin and HIV-1 p-gp160 (30).

In that study, an additional decisive advantage of using D. discoideum CaM was the availability of a matching high-affinity antibody effective both in immunoprecipitation and in immunoblotting, with no observable or significant cross-reactivity to other mammalian proteins. CaM, as a highly conserved protein, generally represents a poor antigen. CaM from D. discoideum appears to be extremely immunogenic so that specific and affine antibodies could easily be raised in rabbits. Use of these antibodies in immunofluorescence studies (12) revealed the expected localization at the periphery of the contractile vacuole of D. discoideum (7,31) which represents a potential correlation with the presence of Myoj on the vacuole surface (8). Myoj is an unconventional myosin with five IQ motifs in its neck domain (32,33).

In addition, in order to study the low-affinity and potentially Ca\(^{2+}\)-independent interaction of CaM with myosins, we thought it necessary to establish and/or optimize a range of specific biochemical assays. A large variety of CaM overlay protocols have been used to detect high-affinity target proteins, many of which make use of \(^{125}\)I-CaM and are adaptations of methods presented in a volume of “Methods in Enzymology” devoted to calcium, calmodulin, and calmodulin-binding proteins (34). More recent additions to this compilation include use of CaM radioactively labeled with either \(^{32}\)P (as a GST fusion protein (22)) or \(^{35}\)S (metabolically labeled in E. coli (35)) as well as biotinylated or fluorescently labeled CaM (36) or even epitope-tagged CaM (37). Only rarely do these studies monitor the degree of dependence of the recognition on the renaturation and accessibility of the target peptide on the membrane (22). Our experiments indicate that the use of nonreducing electrophoresis conditions significantly increase the strength of binding and the number of binding sites, predominantly in the case of high molecular weight proteins (see Fig. 5). These results are somewhat surprising, as it is widely accepted that CaM-binding peptides are likely to be α-helical and should, therefore, not be dependent on secondary structure and should be readily renatured on the blotting membrane. The sensitivity of the method presented is high. In Western blotting, we easily detect less than 1 ng of CaM which should allow the detection of nanogram quantities of a 100-kDa CaM-binding protein, assuming efficient stoichiometric binding of CaM to the denatured peptide. In effect, 25 ng of bovine calcineurin, used as a control protein, led to very strong signals, with exposures in the range of few seconds, superior to other described methods (22). The final identification of the major species detected at 180 kDa and above, including a strong signal well above 200 kDa, none of which corresponding to identified D. discoideum CaM-binding proteins, will require further investigation. Interestingly, two unconventional myosins of D. discoideum are expected to have a size over 250 kDa: Myoj (32,33) and MyoI (M. Titus, personal communication).

Affinity chromatography on immobilized CaM has been used successfully for the purification of high-affinity CaM-binding proteins (see 34 and 38 for a series of basic protocols). In order to optimize the method for the capture of low-affinity binding proteins, we created an affinity matrix with approximately a 10-fold increased concentration of coupled CaM, compared to commercial resins. As it is present in low amounts in our cytosolic extracts, we used D. discoideum calcineurin A as an internal control in the investigation and optimization of different parameters influencing binding and release from the affinity matrix. Under the conditions described, a whole range of proteins was specifically enriched, over and above their concentration in the cytosol by binding to the column. An extremely gentle and exquisitely specific elution was carried out using free CaM to compete with the immobilized binding sites, followed by subsequent elutions of increasing stringency. Even though some myosins I are expected to bind CaM with much lower affinity than calcineurin A, they were highly enriched.
in the fractions of CaM elution. In addition, under these conditions, the method revealed striking differences in the binding capacity of short and long myosin I. Western blotting experiments indicated that a short tail myosin I bound to the matrix and was eluted with free CaM while the long tail myosins I interacted only weakly with the immobilized CaM. It seems likely that the short myosin is MyoE, which is the only myosin I in D. discoideum that possesses more than one clearly identifiable IQ (CaM-binding) motif. Alternatively, the different behavior of short- and long-tailed myosins I could be explained by the fact that IQ motifs differ in their Ca\(^{2+}\) requirements for CaM binding (39). Surprisingly, myosin II was found to bind to the matrix, which likely reflects the ability of its two IQ motifs to bind CaM in vitro. That this binding is nonphysiologically is corroborated by the fact that myosin II was not eluted by free CaM, but only under more stringent and even denaturing conditions. In conclusion, the elution with free CaM revealed an interesting pattern of polypeptides, predominantly in the high molecular weight range. Preliminary experiments, using CaM overlays on these fractions, indicated that many proteins were very likely not binding CaM directly but, due to the very "native" conditions used, some proteins were binding indirectly to the immobilized CaM. Therefore, we speculate that this method has an enormous potential to help in the isolation of functionally important ternary complexes with CaM-binding proteins.

ACKNOWLEDGMENTS

We thank Dr. M. Clarke (Oklahoma Medical Research Foundation, Oklahoma City, OK) for the generous gift of the CaM cDNA construct. Furthermore, we thank Dr. Martin Bähler (Ludwig-Maximilian University, Munich, Germany) for the gift of the pan-myosin I antibody G-371; Dr. J. Cohen (Associé à l’Université Pierre et Marie Curie, Gif-Sur-Yvette Cedex, France) who provided us with the anti-Paramecium CaM antibody; and Dr. R. Mutzel (University of Konstanz, Konstanz, Germany) for the gift of the anti-D. discoideum calcineurin A antibody. We are grateful to Drs. J. Monnat and R. Ortega Perez for valuable suggestions for streamlining the CaM purification by using a heat denaturation step, to E. Neuhauß for help with the immunofluorescence experiments, and to Dr. N. Whittaker (DKFZ, Heidelberg, Germany) for critical reading of the manuscript.

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