Identification of Calmodulin-binding Proteins in Chicken Embryo Fibroblasts

WILSON H. BURGESS, D. MARTIN WATTERSON, and LINDA J. VAN ELDIK
Laboratory of Cellular and Molecular Physiology, Howard Hughes Medical Institute and Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37232

ABSTRACT   We recently reported the detection of multiple classes of calmodulin-binding proteins in subcellular fractions of chicken embryo fibroblasts by using a gel binding procedure (Van Eldik, L. J., and W. H. Burgess, 1983, *J. Biol. Chem.*, 258:4539–4547). In this report we identify many of these calmodulin-binding proteins and provide further evidence for the existence of multiple classes of calmodulin-binding proteins based on the interaction of these proteins with calmodulin and other calcium-modulated proteins. The fact that, in some cases, the same calmodulin-binding protein can bind troponin C and S100α suggests that similar functional domains may be present in these distinct calcium-modulated proteins. We also have used protocols based on purification steps for calmodulin-binding proteins and calmodulin-regulated activities from other systems, in conjunction with enzymatic assays and various immunological methods, to identify many of the calmodulin-binding proteins in chicken embryo fibroblasts. The identities of these proteins suggest in vivo roles for calmodulin in the regulation of cell shape and motility, cyclic nucleotide metabolism, and possibly nucleic acid and protein turnover in fibroblasts.

A major advance in our knowledge of the role of calcium in mediating cellular events was the demonstration that chemically homogeneous proteins have physiologically relevant calcium-binding activities (for a review see reference 1). Several classes of calcium-binding proteins have been proposed on the basis of the type of calcium-binding activity and structure they exhibit. The members of one class, the calcium-modulated proteins, are mostly intracellular proteins that reversibly bind calcium with dissociation constants in the micromolar range under physiological conditions and include parvalbumin, calmodulin, troponin C, and S100. No enzymatic activities have been reported for any of these proteins; rather, they are thought to mediate the effects of calcium through their interactions with other proteins.

We recently reported (2) the subcellular distribution of calmodulin and calmodulin-binding proteins in normal and virus-transformed chicken embryo fibroblasts (CEF).[1] In those studies, we identified multiple classes of calmodulin-binding proteins in the crude subcellular fractions. We identified proteins that bind calmodulin in the presence of calcium but not in the presence of calcium chelator (EDTA) and proteins that bind calmodulin in the presence of calcium or EDTA. In this report, we have extended these observations to include an analysis of the binding of troponin C to proteins in the subcellular fractions and the identification of several of these calmodulin-binding proteins. Our results suggest that some functional domains are shared by members of the calcium-modulated proteins whereas others may be unique to calmodulin. The identification of the calmodulin-binding proteins suggests in vivo roles for calmodulin in the regulation of cell shape and motility, cyclic nucleotide metabolism, and perhaps nucleic acid and protein turnover in CEF.

MATERIALS AND METHODS

---

[1] *Abbreviations used in this paper:*   CEF, chicken embryo fibroblasts; MLCK, myosin light chain kinase; ML, N, P, and S, mitochondrial/lysosomal, nuclear, microsomal, and soluble subcellular fractions.

free water was from a Darco Water System (Durham, NC) or a Milli Q system (Millipore Corp., Bedford, MA). All other chemicals were reagent grade. Pure cellulose nitrate paper (0.45-μm pore size) was from Sartorius Filters, Inc. (Hayward, CA). The microslab gel apparatus was from Idea Scientific (Corvallis, OR).

Source of Proteins: Bovine brain calmodulin was prepared as described (3). Troponin C was prepared from rabbit skeletal muscle as described by Perry and Cole (4). Myosin was prepared from rabbit skeletal muscle according to the method of Fredricksen (5). Myosin light chain kinase (MLCK) was purified from chicken gizzard by using the procedure of Adelstein and Klee (6), and MLCK from turkey gizzard was a gift of Drs. E. Payne and R. Adelstein (National Institutes of Health). Mixed light chains were prepared from bovine cardiac muscle myosin by the method described by Blumenthal and Stull (7). We purified the regulatory light chain by applying mixed light chains (in 10 mM 2-(N-morpholino)propane-sulfonic acid [MOPS], 15 mM 2-mercaptoethanol, pH 7.0) to an affigel blue (Bio-Rad Laboratories) column that was equilibrated in the same buffer. The column was washed, and regulatory light chains were eluted with a linear gradient of 0–0.4 M KCl in 10 mM MOPS, 15 mM 2-mercaptoethanol, pH 7.0. Phosphorylase *b* and phosphorylase kinase from rabbit skeletal muscle were provided, respectively, by Drs. R. Uhing and T. Chrisman (this institution). Bovine brain S100α was provided by Dr. D. Marshak (this institution). Carp muscle parvalbumin was provided by Dr. R. H. Kretsinger (University of Virginia).

Preparation of Protein Fractions: Subcellular fractions of CEF were prepared by differential centrifugation exactly as described (2). In some experiments nuclei from CEF were prepared by the procedure of Lahav et al. (8). Ribosomal protein preparations were prepared from CEF as described by Palmiter (9). A bovine brain fraction of cyclic nucleotide phosphodiesterase that is depleted of calmodulin was prepared as previously described (10). A similar procedure was used to obtain the DEAE fraction from CEF except that DE-52 cellulose was used in place of Sephadex A-50 and the gradient contained 0.1 M NaCl in the start buffer and 0.5 M NaCl in the limit buffer. Fractions were collected, dialyzed against 10 mM ammonium bicarbonate, and lyophilized.

Gel Binding Procedure Using ^{125}I-Labeled Calcium-modulated Proteins: The gel binding procedure used in these studies is a modification of a previously described procedure (11). It is based on earlier reports by Burridge (12) for the detection of lectin-binding proteins and antigens after separation in SDS polyacrylamide gels and the studies of Lacks and Springhorn (13) on the renaturation of enzymes after electrophoresis in SDS polyacrylamide gels. Protein samples were subjected to electrophoresis in the presence of 0.1% (wt/vol) SDS on 7.5 or 12.5% (wt/vol) polyacrylamide gels (0.4-mm thick) using the buffer system of Laemmli (14) and the microslab apparatus described by Matsudaira and Burgess (15). We prepared samples for electrophoresis by adding an equal volume of sample buffer immediately before loading them onto the gel. After electrophoresis (~60 min at 90 V constant voltage) the gels were placed into 135 × 130 × 30 mm plastic boxes (Althor Products, Div. of American Hinge Corp., Wilton, CT) on a rotary shaker and fixed for 3 h (three changes of 100 ml each) in 25% (vol/vol) isopropanol, 10% (vol/vol) acetic acid; washed for 3 h (three changes of 100 ml each) in buffer A (50 mM Tris-HCl, 200 mM NaCl, pH 7.6); and incubated for 2 h in 100 ml of buffer A containing 1 mg/ml BSA.

The gels were then incubated for 12 h in buffer A containing 1 mM CaCl$_2$ and ^{125}I-labeled protein (~4–5 × 10^6 cpm calmodulin, troponin C, S100α, or parvalbumin) in the presence or absence of the appropriate unlabeled protein (see Results). The gels were then washed for 3 h (three changes of 100 ml each) in buffer A containing 1 mM CaCl$_2$ to remove excess iodinated protein; stained for 15 min in 0.1% (wt/vol) Coomassie Brilliant Blue R-250, 50% (vol/vol) methanol, 10% (vol/vol) acetic acid; destained for 1 h in 5% (vol/vol) methanol, 9% (vol/vol) acetic acid; and dried and then exposed to x-ray film to detect those proteins in the gel that bind the iodinated protein. In some experiments, 5 mM EDTA instead of 1 mM CaCl$_2$ was added to buffer A in the incubation and washes described above. In this protocol, all steps are the same before the incubation with ^{125}I-labeled calcium-modulated proteins and either 1 mM CaCl$_2$ or 5 mM EDTA. Thus we avoid any potential artifacts of differential renaturation in the presence or absence of excess divalent cations or chelators.

The specific activities of the ^{125}I-labeled calcium-modulated proteins used in these studies were in the range of 100–500 Ci/mmol. The length of exposure of the gels to x-ray film varied depending on the specific activity of the protein used and the amount of binding protein applied to the gel. In general a 24-h exposure was sufficient to detect the majority of binding proteins although a 48- or 72-h exposure was often necessary to detect minor components.

In some experiments proteins were transferred from the gel to nitrocellulose paper and then examined for ^{125}I-calmodulin-binding activity. Electrophoresis was performed as above and the gels were washed in 50 mM Tris-HCl, pH 7.6, for 30 min (three changes of 100 ml each) and transferred to nitrocellulose

paper using a Bio-Rad transblot apparatus. The chamber was filled with buffer containing 25 mM Tris-HCl, 192 mM glycine, 20% (vol/vol) methanol. Transfer was conducted at 180 mA constant current for 16 h at 4°C, after which the nitrocellulose was incubated for 2 h in 100 ml buffer A containing 1% (wt/vol) BSA and 0.2% (wt/vol) gelatin, rinsed with buffer A containing 1 mM CaCl$_2$, and then incubated with ^{125}I-calmodulin as described above.

Immunological Methods: Rabbit antiserum against bovine brain calcineurin was provided by Dr. R. Wallace (University of Alabama at Birmingham) and rabbit antiserum against bovine brain spectrin was provided by Dr. K. Burridge (University of North Carolina). Antibodies against chicken gizzard MLCK were produced in Balb/c mice by injection of a partially purified MLCK fraction prepared as described above through the DEAE-cellulose chromatography step. Antigen (~80 μg per mouse) was emulsified in complete Freund's adjuvant and injections were intraperitoneal and subcutaneous on days 1 and 15. Mice showed a positive serum response at the first bleed (day 21). Iodinations and competition radioimmunoassays were done using procedures similar to those described for calmodulin (16).

For immunoblot experiments, proteins were transferred after electrophoresis to nitrocellulose papers, as described above, which were then incubated in blocking buffer (10 mM Tris-HCl, 0.9% [wt/vol] NaCl, 0.5% [wt/vol] BSA, 0.05% [vol/vol] Nonidet P-40, 5% [vol/vol] horse serum, pH 7.4) for 1.5 h at 37°C. Next, the nitrocellulose paper was washed in PBS for 30 min (three changes of 100 ml each) at room temperature followed by incubation at 37°C for 2 h with antiserum diluted in PBS. The paper was then washed at room temperature for 30 min in PBS containing 0.05% (vol/vol) Tween 20 (three changes of 100 ml each) and incubated for 1 h at 37°C with the appropriate biotinylated antibody (50 μl in 10 ml PBS containing 1% [vol/vol] horse serum). The paper was washed again at room temperature with buffer containing Tween 20 followed by incubations with Vectastain ABC reagent in PBS containing 0.1% (vol/vol) Tween 20 for 1 h at 37°C. Finally, the papers were washed as above and incubated at room temperature with 15 ml PBS, 3 ml of 3 mg/ml 4-chloro-1-napthol in methanol, 6 μl 30% hydrogen peroxide for 5–20 min. We stopped the color reaction by washing the paper with distilled water.

Enzyme Assays: MLCK activity was assayed as described by Blumenthal and Stull (7), using bovine cardiac myosin light chains. There was no calcium-stimulated MLCK activity in the absence of added calmodulin. Calmodulin-activatable cyclic nucleotide phosphodiesterase activity was assayed as previously described (17). Calmodulin-stimulated dephosphorylation of *p*-nitrophenyl phosphate was assayed as described by Pallen and Wang (18).

Detection of DNA-binding Proteins: Proteins were subjected to electrophoresis and transferred to nitrocellulose paper as described above. Detection of DNA-binding proteins was performed as described by Bowen et al. (19). Total DNA isolated from the sperm of the sea urchin *Arbacia punctulata* and labeled with ^{32}P by nick translation was provided by Dr. R. Zielinski (this institution).

General Procedures: Protein concentrations were determined by the method of Lowry et al. (20), using BSA as the standard, or by amino acid analysis (21). Iodination of calmodulin, troponin C, S100α, and parvalbumin was done with lactoperoxidase (22). Calmodulin, tropinin C, and S100α were analyzed for homogeneity by electrophoresis in the presence of SDS, by amino acid analysis (21), and by competition radioimmunoassay (16) using antisera specific for calmodulin or troponin C. Troponin C preparations were free of detectable calmodulin contamination and did not react in competition radioimmunoassay with anticalmodulin sera when tested up to a 2,000-fold molar excess over the amount of calmodulin required for 50% competition.

RESULTS

Binding of ^{125}I-Calmodulin and ^{125}I-Troponin C to Proteins in the Subcellular Fractions of CEF

As previously described (2), we prepared subcellular fractions (N [nuclear], ML [mitochondrial/lysosomal], P [microsomal], and S [soluble]) from CEF. We examined the binding of ^{125}I-calmodulin to proteins in the subcellular fractions in the presence of calcium (Fig. 1*B*). Clearly, many of the calmodulin-binding proteins in CEF localize specifically to the particulate fractions, primarily ML and P. As noted previously (2), many of the calmodulin-binding proteins in the particulate fractions bind ^{125}I-calmodulin in the presence of calcium or EDTA (Fig. 1*C*).
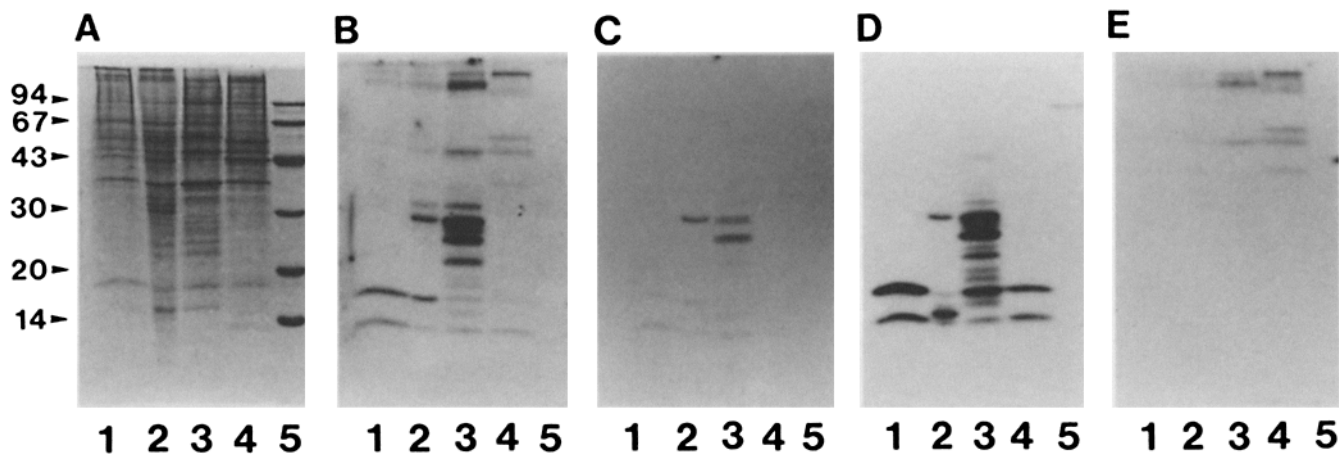
To further examine the specificity and the molecular basis

FIGURE 1 The binding of ^{125}I-calmodulin and ^{125}I-troponin C to proteins from the subcellular fractions of chicken embryo fibroblasts. A shows a Coomassie Blue-stained 12.5% (wt/vol) acrylamide SDS gel. Panels B–E show corresponding autoradiograms of gels that were incubated after electrophoresis with (B) ^{125}I-calmodulin in the presence of calcium; (C) ^{125}I-calmodulin in the presence of EDTA; (D) ^{125}I-troponin C in the presence of calcium; (E) ^{125}I-calmodulin in the presence of calcium and a 500-fold molar excess of unlabeled troponin C. In each panel, lane 1 contains the nuclear (N) fraction of CEF (~9.5 µg), lane 2, the mitochondrial-lysosomal (ML) fraction (~9.5 µg), lane 3, the microsomal (P) fraction (~9.5 µg), lane 4, the soluble (S) fraction (~9.5 µg), and lane 5, the molecular weight standards (labeled by arrows at left of A, $M_r \times 10^{-3}$). The autoradiograms shown are 72-h exposures.

of the interactions between calmodulin and these proteins, we examined the binding of another calcium-modulated protein, troponin C, to the proteins in the subcellular fractions. The results of one such experiment are shown in Fig. 1D. Troponin C is both a structural and in some cases a functional analogue of calmodulin. Most of the proteins that bound ^{125}I-troponin C constituted a subset of those that bound calmodulin. Many of these proteins were found in the P fraction and were ones that bound calmodulin even in the presence of EDTA. When gels were incubated with ^{125}I-calmodulin in the presence of a 500-fold molar excess of unlabeled troponin C, ^{125}I-calmodulin bound only to those proteins that did not bind iodinated troponin C; i.e., unlabeled troponin C was found to compete with ^{125}I-calmodulin for those proteins that also bind ^{125}I-troponin C (Fig. 1E). These results indicate that some proteins may bind to a similar domain in both calmodulin and troponin C. The generality of this type of interaction is illustrated more clearly in the studies described below.

Binding of ^{125}I-Calmodulin and ^{125}I-Troponin C to Purified and Partially Purified Protein Fractions

Using the gel binding procedure described in Materials and Methods, we also examined the binding of ^{125}I-calmodulin to purified proteins. As shown in Fig. 2B, we detected binding of ^{125}I-calmodulin to purified MLCK (lane 2) and to the β and γ subunits ($M_r = 128,000$ and 45,000) of phosphorylase kinase (lane 1). A physiological role for calmodulin in the regulation of these two enzymes appears to be well established. Both enzymes have been purified to chemical and enzymatic homogeneity and the calcium-binding subunit has been shown directly to be calmodulin (23, 24). The binding of ^{125}I-calmodulin to the β subunit of phosphorylase kinase was more apparent when NaCl was not included in the incubation or wash buffer (Fig. 2C, lane 1). The reversible interaction of calmodulin with the holoenzyme is reported to occur through binding to the α and β subunits whereas the calmodulin that binds as a "tight" or integral subunit of the homoenzyme does so by interaction with the γ subunit (24).

We also detected ^{125}I-calmodulin binding to purified phosphorylase b (Fig. 2B, lane 3) and to the heavy chain of myosin (Fig. 2B, lane 4). The physiological significance of the interaction between calmodulin and these two proteins has not been determined. As was the case with the β subunit of phosphorylase kinase, the binding of ^{125}I-calmodulin to phosphorylase b and myosin heavy chain was more readily detected when NaCl was not included in the incubation and wash buffers (Fig. 2C, lanes 3 and 4). The binding of ^{125}I-calmodulin to all of the proteins shown in Fig. 2 was inhibited when CaCl$_2$ was replaced by 5 mM EDTA in the incubation and wash buffers, or when the gels were washed with EDTA-containing buffers after incubation with ^{125}I-calmodulin in the presence of CaCl$_2$. Similar results were obtained when a 500-fold molar excess of unlabeled calmodulin was included in the incubation with the iodinated protein or when incubations were done in the presence of 1 mM MgCl$_2$ or 1 mM MnCl$_2$.

Calmodulin has four structural domains that are similar to one another in amino acid sequence and to the four structural domains of skeletal muscle troponin C (25). In addition to these structural domains, calmodulin and troponin C, as well as S100, are proposed to have functionally similar drug-binding domains (22). Calmodulin has been shown to substitute functionally for troponin C under certain in vitro conditions (26). The results of the experiments on the binding of ^{125}I-calmodulin and ^{125}I-troponin C to common proteins in the subcellular fractions of CEF also suggest the presence of functionally similar protein-binding domains in these calcium-modulated proteins. To further investigate these potential similarities, we examined the binding of ^{125}I-troponin C and ^{125}I-S100α to purified proteins using the same conditions as were used for calmodulin.

We detected binding of ^{125}I-troponin C to several of the proteins that also bound ^{125}I-calmodulin. For example, as shown in Fig. 3B, phosphorylase kinase (lane 1), phosphorylase b (lane 3), myosin heavy chain, and an $M_r = 35,000$ polypeptide in the myosin sample (lane 4) bound both ^{125}I-troponin C and ^{125}I-calmodulin. In contast, MLCK bound
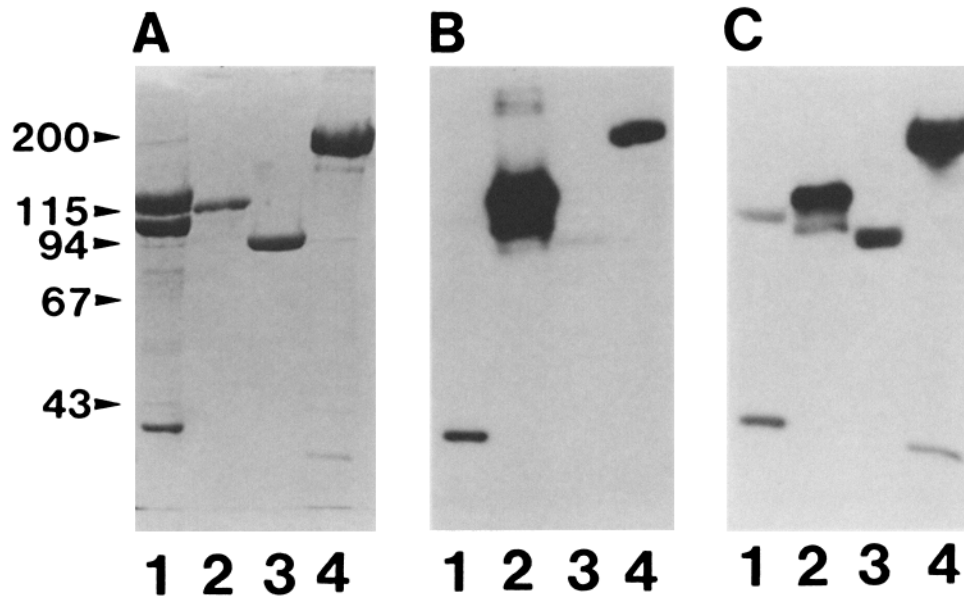
FIGURE 2 The binding of ^{125}I-calmodulin to purified proteins. A shows a Coomassie Blue-stained 7.5% (wt/vol) acrylamide SDS gel. B and C show corresponding autoradiograms of gels that were incubated after electrophoresis with ^{125}I-calmodulin in the presence of (B) 1 mM CaCl$_2$ and 0.2 M NaCl or (C) 1 mM CaCl$_2$ and no added NaCl. The autoradiogram in B is a 72-h exposure; C is a 24-h exposure. In each panel, lane 1 contains phosphorylase b kinase from rabbit skeletal muscle (5 μg), lane 2, MLCK from turkey gizzard (1 μg), lane 3, phosphorylase b from rabbit skeletal muscle (2 μg), and lane 4, myosin from rabbit skeletal muscle (3 μg). ($M_r \times 10^{-3}$ in this and the following gels.)
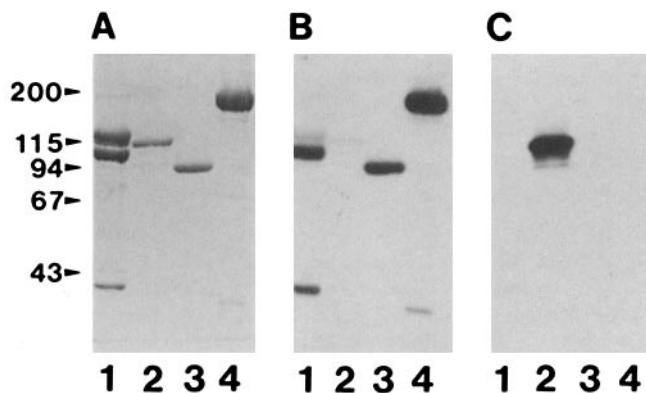

FIGURE 3 The binding of ^{125}I-troponin C to purified proteins. A shows a Coomassie Blue-stained 7.5% (wt/vol) acrylamide SDS gel. B and C show corresponding autoradiograms of gels that were incubated after electrophoresis with (B) ^{125}I-troponin C in the presence of 1 mM CaCl$_2$ and 0.2 M NaCl or (C) ^{125}I-calmodulin in the presence of 1 mM CaCl$_2$, 0.2 M NaCl, and a 500-fold molar excess of unlabeled troponin C. Both autoradiograms are 24-h exposures. In each panel, lanes 1–4 contain the same samples as described in Fig. 2.

^{125}I-calmodulin but showed little ^{125}I-troponin C binding (cf. lane 2, Figs. 2B and 3B). Similarly, proteins in crude bovine brain extracts that bound ^{125}I-troponin C were generally distinct from those that bound ^{125}I-calmodulin. Unlike calmodulin, the binding of ^{125}I-troponin C to these proteins was not diminished by substitution of 5 mM EDTA for 1 mM CaCl$_2$ in the incubation and wash buffers. In addition, binding of ^{125}I-troponin C to the β subunit of phosphorylase kinase (cf. lane 1, Figs. 2B and 3B) was easily detected even in the presence of 0.2 M NaCl.

When a 500-fold molar excess of unlabeled troponin C was

included in the incubation with ^{125}I-calmodulin (Fig. 3C), unlabeled troponin C did not compete with ^{125}I-calmodulin for binding to MLCK but did compete for binding to phosphorylase b, phosphorylase kinase, and myosin. These observations are consistent with the results obtained using ^{125}I-troponin C alone. The binding patterns observed for ^{125}I-S100α are similar to those observed using ^{125}I-troponin C in that ^{125}I-S100α bound phosphorylase kinase, phosphorylase b, and myosin but not MLCK. The binding patterns of ^{125}I-S100α to proteins in crude bovine brain extracts also are qualitatively similar to those obtained using ^{125}I-troponin C (data not shown).

Carp muscle parvalbumin is a calcium-modulated protein that contains calcium-binding structures, but not drug-binding domains, that are similar to those in calmodulin, troponin C, and S100α (22). In contrast to the results obtained using ^{125}I-troponin C and ^{125}I-S100α, we could detect only weak binding in the presence of 1 mM CaCl$_2$ of ^{125}I-parvalbumin to phosphorylase b, and little or no binding to MLCK (Fig. 4B). In the presence of 5 mM EDTA, the binding of ^{125}I-parvalbumin to phosphorylase b was enhanced (Fig. 4C, lane 1).

In conclusion, the binding patterns observed for ^{125}I-calmodulin, ^{125}I-troponin C, ^{125}I-S100α, and ^{125}I-parvalbumin cannot be explained by the fact that they are all relatively small, acidic, calcium-binding proteins. Consistent with this observation is the fact that α-lactalbumin, a small, acidic and calcium-binding protein that is not clearly related structurally or functionally to calmodulin, troponin C, S100α, or parvalbumin did not inhibit the binding of ^{125}I-calmodulin to the proteins examined, even when present at a 1000-fold molar excess. Finally, there is a specific set of calmodulin-binding proteins that bind troponin C and S100α. However, there are also other proteins that bind troponin C or S100α but do not bind calmodulin.

A   B   C

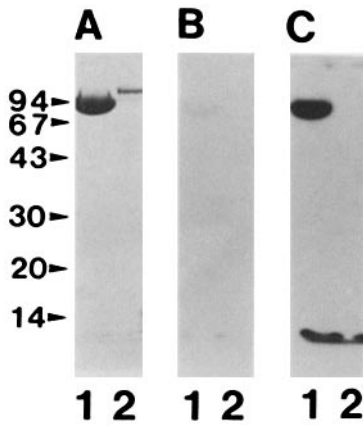94▸
67▸
43▸
30▸
20▸
14▸

1 2   1 2   1 2

FIGURE 4 The binding of ¹²⁵I-parvalbumin to purified proteins. *A* shows a Coomassie Blue-stained 12.5% (wt/vol) acrylamide SDS gel. *B* and *C* show corresponding autoradiograms of gels that were incubated after electrophoresis with ¹²⁵I-parvalbumin in the presence of (*B*) 1 mM $CaCl_2$ and 0.2 M NaCl or (*C*) 5 mM EDTA and 0.2 M NaCl. Both autoradiograms are 72-h exposures. In each panel, lane *1* contains rabbit skeletal muscle phosphorylase *b* (3 μg) and lane *2* contains MLCK from turkey gizzard (1 μg).

were present in a ribosomal protein preparation from CEF and suggests that these binding proteins may be ribosomal proteins.

Burridge et al. (30) detected the presence of nonerythroid spectrin in CEF by using antibodies against brain spectrin (fodrin) and immuno-autoradiography on gels. Several laboratories have demonstrated binding of ¹²⁵I-calmodulin to nonerythroid spectrin (fodrin) from various cell types including bovine brain (31, 32). The S and P fractions of CEF were analyzed for the presence of calmodulin-binding proteins that cross-react with bovine brain spectrin (fodrin) antibodies. The results of one such experiment are shown in Fig. 7. Proteins with apparent molecular weights of ~240,000 and 150,000 that cross-react with the antibodies and also bind ¹²⁵I-calmodulin were detected in the CEF fractions. A protein of apparent molecular weight 150,000 has been postulated to be a proteolytic fragment of bovine brain spectrin (fodrin) (30).

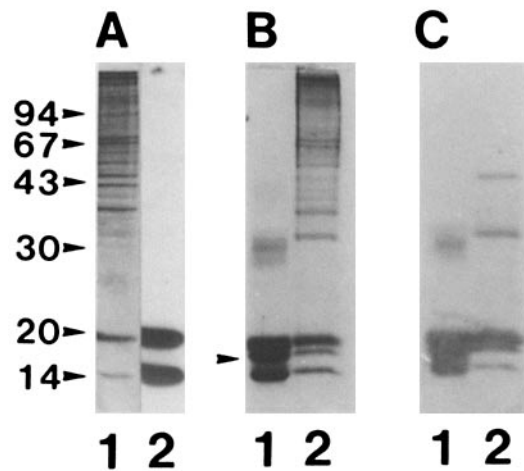Several proteins with apparent molecular weights ~60,000

Identification of the Calmodulin-binding Proteins in CEF

The results described above indicate that different classes of calmodulin-binding proteins can be detected by gel binding experiments using calmodulin and related proteins. The physiological significance of these findings as they relate to CEF cannot be determined without first identifying the binding proteins in the various subcellular fractions.

The apparent molecular weights and predominantly N-fraction localization of the two binding proteins in lane *1* of Fig. 1, *B* and *C* suggest that these proteins are histones. As previously described (2), some nuclear lysis occurs when this particular subcellular fractionation protocol is used. Fig. 5*A* shows the binding of ¹²⁵I-calmodulin to a nuclear preparation of CEF where the integrity of the nuclei was maintained as judged by total DNA determinations. This preparation (cf. Fig. 1*B*, lane *1* and Fig. 5*A*, lane *2*) is enriched for these two calmodulin-binding proteins. These proteins co-migrated with histone standards (Fig. 5*B*) and exhibited similar DNA-binding activities (Fig. 5*C*). It should be noted that one of the histones (H2a) washed out of the gel during the ¹²⁵I-calmodulin-binding protocol (cf. Fig. 5,*A* and *B*). This result is similar to those of Irie and Sezaki (27) who observed that histone H2a was preferentially extracted from acrylamide gels when the gels were fixed with 25% isopropanol, 10% acetic acid before staining.

As can be seen in Fig. 1, there were many proteins in the P fraction with apparent molecular weights ranging from 15,000 to 40,000 that bound ¹²⁵I-calmodulin and ¹²⁵I-troponin C (lanes *3* in Fig. 1,*B–D*). The general pattern is similar to gel profiles of various heterogeneous nuclear ribonucleoprotein particle preparations and also to one-dimensionl gel profiles of ribosomal protein preparations (28, 29). The data shown in Fig. 5 demonstrate that these proteins are not of nuclear origin since they are not present in the nuclear preparation. However, the apparent molecular weights and subcellular distribution were found to be similar to those of ribosomal proteins. We used several procedures for the preparation of eucaryotic ribosomal proteins to determine whether these binding proteins may indeed be ribosomal proteins. The results of one such experiment are shown in Fig. 6. A comparison of lanes *1* and *2* illustrates that many but not all of the ¹²⁵I-calmodulin-binding proteins in the P fraction of CEF

A   B   C

94▸
67▸
43▸
30▸
20▸
14▸

1 2   1 2   1 2

FIGURE 5 The identification of histones from CEF as ¹²⁵I-calmodulin-binding proteins. *A* shows a 12.5% (wt/vol) acrylamide SDS gel containing a nuclei fraction from CEF. Lane *1* shows the proteins stained with Coomassie Blue after the gel overlay incubation and lane *2* shows the corresponding autoradiogram depicting the binding of ¹²⁵I-calmodulin. *B* shows a Coomassie Blue-stained gel containing histone standards (lane *1*) and the nuclei fraction from CEF (lane *2*). *C* shows an autoradiogram depicting the binding of ³²P-DNA to the proteins shown in *B*. Note that one of the histones (arrow) washes out of the gel in the ¹²⁵I-calmodulin binding experiment (compare *A*, lane *1* with panel *B*, lane *2*).
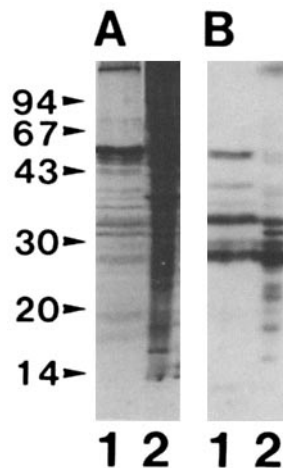

A   B

94▸
67▸
43▸
30▸
20▸
14▸

1 2   1 2

FIGURE 6 The detection of ¹²⁵I-calmodulin binding to proteins from a ribosomal protein preparation of CEF. *A* shows a Coomassie Blue-stained 12.5% (wt/vol) acrylamide SDS gel. *B* shows the corresponding autoradiogram depicting the binding of ¹²⁵I-calmodulin. Lane *1* contains a ribosomal protein preparation from CEF and lane *2* contains the P fraction from CEF.

that bind ^{125}I-calmodulin in the presence of calcium but not in the presence of chelator were detected in the S fraction of CEF. It is possible that these proteins are calmodulin-activatable cyclic nucleotide phosphodiesterase and calcineurin, a calmodulin-activatable phosphatase (33), since both of these calmodulin-binding proteins have calmodulin-binding subunits of M_r ~60,000 (34). To test this possibility, we prepared from bovine brain a partially purified fraction with calmodulin-activatable cyclic nucleotide phosphodiesterase activity and calmodulin-activatable nitrophenylphosphate phosphatase activity. The binding of ^{125}I-calmodulin and calcineurin antibodies to proteins in this fraction and to proteins in the S fraction of CEF are shown in Fig. 8, A and B. In both fractions, proteins that cross-react with the calcineurin antibodies and

also bind ^{125}I-calmodulin were detected. When CEF were subjected to a purification protocol similar to the one used to prepare calmodulin-activatable phosphodiesterase from bovine brain, fractions from the DEAE column were obtained that have calmodulin-activatable cyclic nucleotide phosphodiesterase activity and contain ^{125}I-calmodulin-binding proteins that co-migrate with ^{125}I-calmodulin-binding proteins in the bovine brain fraction (Fig. 8, C and D).

Finally, ^{125}I-calmodulin-binding activity similar to that exhibited by purified MLCK was detected in one of the fractions (Fig. 8D, lane 4). This fraction contains MLCK activity using cardiac regulatory light chain as a substrate. When the subcellular fractions of CEF were examined for MLCK immunoreactivity by immunoblot analysis, a band that co-migrates with MLCK purified from chicken gizzard was detected in the S fraction (data not shown). MLCK immunoreactivity was also present in CEF extracts as judged by competition radioimmunoassay using ^{125}I-labeled chicken gizzard MLCK as tracer (Fig. 9).

DISCUSSION

The results presented here and elsewhere (2) demonstrate that multiple classes of calmodulin-binding proteins can be identified. There are proteins that bind calmodulin in the presence of added calcium, ones that bind calmodulin in the presence of calcium or chelator, ones that appear to bind only calmodulin, and others that bind calmodulin and other calcium-modulated proteins. These results suggest that calmodulin may contain multiple functional domains for interaction with binding proteins, that some of these domains may be shared by troponin C, S100α, or parvalbumin, and that others may be unique to calmodulin.

The studies on the binding of ^{125}I-troponin C to proteins in the subcellular fractions of CEF indicate that many of the "calmodulin-binding proteins" in the particulate fractions are not exclusive for calmodulin. However, the proteins in the subcellular fractions of CEF that bind both calmodulin and troponin C cannot be discounted merely as "nonspecific" binding proteins. For example, we have demonstrated here that calmodulin, troponin C, and S100α bind to phosphorylase, myosin, and subunits of phosphorylase kinase. The inter-
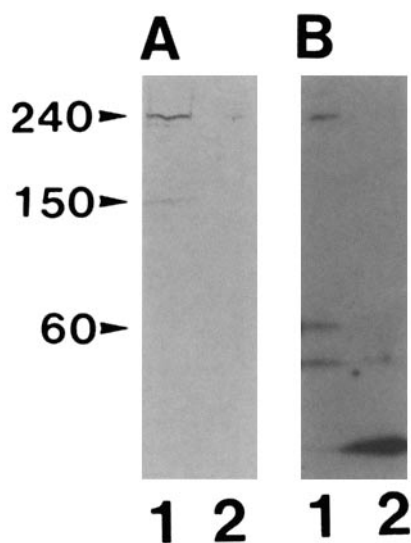

FIGURE 7 Identification of spectrin from CEF as an ^{125}I-calmodulin-binding protein. Proteins were separated on a 7.5% (wt/vol) acrylamide SDS gel, transferred to nitrocellulose paper, and incubated with spectrin antibodies (A) or ^{125}I-calmodulin (B). In each panel, lane 1 contains the S fraction from CEF and lane 2 contains the P fraction from CEF. Proteins with apparent molecular weights of 240,000, 150,000, and 60,000 are labeled with arrows.
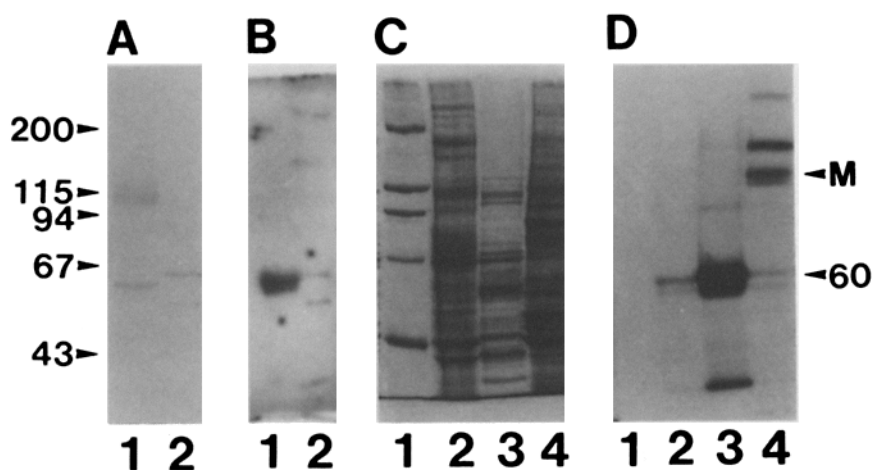

FIGURE 8 Detection of calcineurin immunoreactivity and calmodulin binding to proteins from bovine brain and CEF. In A and B, proteins were separated on a 7.5% (wt/vol) acrylamide SDS gel, transferred to nitrocellulose paper, and incubated with either calcineurin antibodies (A) or ^{125}I-calmodulin (B). Lane 1 contains a fraction from bovine brain with calmodulin-activatable phosphodiesterase and phosphatase (calcineurin) activities and lane 2 contains the S fraction from CEF. The arrow labeled 60 points to the region of the gel containing proteins of apparent molecular weight 60,000. Panel C shows a Coomassie-Blue-stained 7.5% (wt/vol) acrylamide SDS gel and D shows the corresponding autoradiogram depicting the binding of ^{125}I-calmodulin. In C and D, lane 1 contains molecular weight standards (indicated at left), lane 2 contains the DEAE fraction from CEF with maximal calmodulin-activatable phosphodiesterase activity, lane 3 contains the DEAE fraction from bovine brain with maximal calmodulin-activatable phosphodiesterase activity, and lane 4 contains the DEAE fraction from CEF with maximal calmodulin-activatable MLCK activity. The arrows point to the regions of the gel containing MLCK (M) or proteins of apparent molecular weight 60,000 (60).
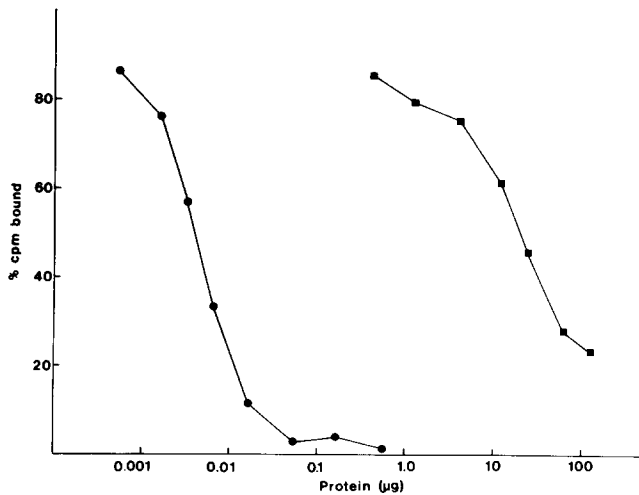
FIGURE 9 Demonstration of MLCK immunoreactivity in CEF by competition radioimmunoassay. Various concentrations of chicken gizzard MLCK (●) and a 90,000 g supernatant of CEF (■) were incubated with mouse anti-MLCK serum (1:750 final dilution) for 18 h at 4°C. A fixed amount of ^{125}I-labeled gizzard MLCK was added and the mixture was incubated for 8 h at 4°C. The mixture was processed as described (16) except that goat anti-mouse serum was used as the second antibody. The degree of competition is expressed as a percentage of the radioactivity bound in the absence of competing protein.

action of calmodulin with phosphorylase has recently been demonstrated by other procedures (35), yet the significance of this interaction is not known. The γ subunit of phosphorylase kinase, whose interaction with calmodulin has been shown by other procedures and is considered to be physiologically relevant, also binds troponin C and S100α in the gel binding procedure. Although we believe this procedure is an excellent method for screening protein fractions for potential calmodulin-binding activity, caution must be used in assigning or discounting physiologically relevant calmodulin-binding activity on the basis of gel binding data alone. Similarly, calmodulin-Sepharose interactions (36) or cross-linking of added calmodulin (37) do not, by themselves, provide any indication as to whether or not proteins bind calmodulin in vivo. Gel overlay analysis combined with enzymology, protein chemistry, immunochemistry, and cell biology studies can provide, as shown here, insight into potential biological roles of calmodulin.

We have extended our initial studies on the detection of calmodulin-binding proteins with the gel binding procedure and have now identified many of the calmodulin-binding proteins and calmodulin-regulated activities in CEF. Some of these have been identified in many cell types other than CEF, some have been identified in CEF but their calmodulin-binding properties have not been investigated, and others have not been previously reported to be calmodulin-binding proteins in any cell type. For example, MLCK, cyclic nucleotide phosphodiesterase, and calmodulin-dependent phosphatase (calcineurin) from many cell types have been shown to be calmodulin-binding proteins with calmodulin-regulated activities. We have demonstrated the presence of MLCK in CEF by kinase activity, by immunoreactivity in immunoblots and competition radioimmunoassay, and by ^{125}I-calmodulin binding. We have identified ^{125}I-calmodulin-binding proteins with apparent molecular weights in the range of 55,000–

60,000 and we can detect calmodulin-activatable cyclic nucleotide phosphodiesterase activity and calcineurin immunoreactivity in CEF. However, we are not yet able to assign cyclic nucleotide phosphodiesterase activity or calcineurin activity to a particular ^{125}I-calmodulin-binding protein in CEF.

Nonerythroid spectrin (fodrin) in CEF has been identified by immunoreactivity with cell extracts after electrophoresis (30). We have correlated immunoreactivity with ^{125}I-calmodulin-binding activity in the S, and to a lesser extent the P, fraction of CEF. Similarly, we have correlated DNA binding activity with ^{125}I-calmodulin-binding activity in nuclei preparations of CEF, and have shown that these proteins comigrate with histone standards. Finally, we have shown that many of the ^{125}I-calmodulin binding proteins in the P fraction of CEF can be identified in ribosomal protein preparations of CEF.

Overall, the results presented here on the identification of calmodulin-binding proteins suggest specific roles for calcium and calmodulin regulation of cyclic nucleotide metabolism and cell shape and motility in CEF. The physiological significance of the interaction of calmodulin with some of these proteins, such as the ribosomal proteins and the histones, has not been established. Clearly, further studies in this area are needed. However, there are reports of effects of calcium on protein synthesis, and calcium and calmodulin on nucleic acid synthesis (38–41). Some of the calmodulin-binding proteins identified in these fractions may represent sites of regulation.

The results obtained here suggest that many particulate calmodulin-binding proteins interact with a calmodulin domain that may be found in the structurally related protein troponin C, and that several binding proteins interact with calmodulin in a less calcium-dependent, possibly calcium-independent, manner. It should be noted that although some of the protein-protein interactions shown here appear to be relatively calcium independent, any potential regulation of these proteins may indeed be calcium dependent. Related to our results, Andreasen et al. (42) recently purified a membrane protein from bovine cerebral cortex whose apparent affinity for calmodulin is higher in the presence of calcium chelators than in the presence of calcium. In contrast, most of the soluble calmodulin-binding proteins from CEF and several particulate calmodulin-binding proteins do not bind troponin C and bind calmodulin in the presence of excess calcium but not in the presence of EDTA. These results are consistent with the possibility that calmodulin could be "compartmentalized" in the cell by virtue of its interaction with particulate binding proteins (2, 11, 42, 43) or through the use of different binding domains on the calmodulin molecule. These interactions could occur even at very low calcium concentrations and this compartmentalized calmodulin could still be potentially accessible to other calmodulin-binding proteins via a different binding domain. In the case of particulate-bound calmodulin, this could include accessibility to "soluble" calmodulin-binding proteins.

In summary, these studies provide insight into the potential role of calmodulin in CEF cell function and a framework for future studies addressing the specificity of its interactions with binding proteins. The correlation of structural and functional domains in the calmodulin molecule and further characterization of the interactions with the various classes of binding proteins are necessary for a complete understanding of the

molecular mechanisms of action of calcium-modulated proteins. In addition, the results presented here identify specific targets or activities for potential calcium and calmodulin regulation in CEF. That some of the activities may change as a function of the cell cycle and upon viral transformation of CEF, coupled with the demonstrated changes in calmodulin levels during the cell cycle and upon viral transformation (2, 44–46), indicate future directions for a more complete understanding of the role of calmodulin in the regulation of cell function.

REFERENCES

1. Van Eldik, L. J., J. G. Zendegui, D. R. Marshak, and D. M. Watterson. 1982. Calcium binding proteins and the molecular basis of calcium action. *Int. Rev. Cytol.* 77:1–61.
2. Van Eldik, L. J., and W. H. Burgess. 1983. Analytical subcellular distribution of calmodulin and calmodulin-binding proteins in normal and virus-transformed fibroblasts. *J. Biol. Chem.* 258:4539–4547.
3. Burgess, W. H., D. K. Jemiolo, and R. H. Kretsinger. 1980. Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate. *Biochim. Biophys. Acta.* 623:257–270.
4. Perry, S. V., and H. A. Cole. 1974. Phosphorylation of troponin and the effects of interactions between the components of the complex. *Biochem. J.* 141:733–743.
5. Fredricksen, D. W. 1979. Physical properties of myosin from aortic smooth muscle. *Biochemistry.* 18:1651–1656.
6. Adelstein, R. S., and C. B. Klee. 1982. Purification of smooth muscle myosin light chain kinase. *Methods Enzymol.* 85:298–308.
7. Blumenthal, D. K., and J. T. Stull. 1980. Activation of skeletal muscle myosin light chain kinase by calcium and calmodulin. *Biochemistry.* 19:5608–5614.
8. Lahav, M., N. Schoenfield, O. Epstein, and A. Atsmon. 1982. A method for obtaining high recovery of purified subcellular fractions of rat liver homogenate. *Anal. Biochem.* 121:114–122.
9. Palmiter, R. D. 1974. Magnesium precipitation of ribonucleoprotein complexes. Expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. *Biochemistry.* 13:3606–3615.
10. Watterson, D. M., W. G. Harrelson, Jr., P. M. Keller, F. Sharief, and T. C. Vanaman. 1976. Structural similarities between the Ca²⁺-dependent regulatory proteins of 3':5'-cyclic nucleotide phosphodiesterase and actomyosin ATPase. *J. Biol. Chem.* 251:4501–4513.
11. Carlin, R. K., D. J. Grab, and P. Siekevitz. 1981. Function of calmodulin in postsynaptic densities. III. Calmodulin-binding proteins of the postsynaptic density. *J. Cell Biol.* 89:449–455.
12. Burridge, K. 1976. Changes in cellular glycoproteins after transformation: identification of specific glycoproteins and antigens in sodium dodecyl sulfate gels. *Proc. Natl. Acad. Sci. USA.* 73:4457–4461.
13. Lacks, S. A., and S. S. Springhorn. 1980. Renaturation of enzmes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. *J. Biol. Chem.* 255:7467–7473.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
15. Matsudaira, P., and D. Burgess. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* 87:386–396.
16. Van Eldik, L. J., and D. M. Watterson. 1981. Reproducible production of antiserum against vertebrate calmodulin and determination of the immunoreactive site. *J. Biol. Chem.* 256:4205–4210.
17. Watterson, D. M., D. B. Iverson, and L. J. Van Eldik. 1980. Rapid separation and quantitation. 3',5'-cyclic nucleotides and 5'-nucleotides in phosphodiesterase reaction mixtures using high-performance liquid chromatography. *J. Biochem. Biophys. Methods.* 2:139–146.
18. Pallen, C. J., and J. Wang. 1983. Calmodulin-stimulated dephosphorylation of p-nitrophenyl phosphate and free phosphotyrosine by calcineurin. *J. Biol. Chem.* 258:8550–8553.
19. Bowen, B., J. Steinberg, U. K. Laemmli, and H. Weintraub. 1980. The detection of DNA-binding proteins by protein blotting. *Nucleic Acids Res.* 8:1–20.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
21. Van Eldik, L. J., and D. M. Watterson. 1979. Characterization of a calcium-modulated protein from transformed chicken fibroblasts. *J. Biol. Chem.* 254:10250–10255.
22. Marshak, D. R., D. M. Watterson, and L. J. Van Eldik. 1981. Calcium-dependent interaction of S100b, troponin C, and calmodulin with an immobilized phenothiazine. *Proc. Natl. Acad. Sci. USA.* 78:6793–6797.
23. Dabrowska, R., J. M. F. Sherry, D. Aromatorio, and D. J. Hartshorne. 1978. Modulator protein as a component of the myosin light chain kinase from chicken gizzard. *Biochemistry.* 17:253–258.
24. Picton, C., C. B. Klee, and P. Cohen. 1980. Phosphorylase kinase from rabbit skeletal muscle: identification of the calmodulin-binding subunits. *Eur. J. Biochem.* 111:553–561.
25. Watterson, D. M., F. Sharief, and T. C. Vanaman. 1980. The complete amino acid sequence of the Ca²⁺-dependent modulator protein (calmodulin) of bovine brain. *J. Biol. Chem.* 255:962–975.
26. Amphlett, G. W., T. C. Vanaman, and S. V. Perry. 1976. Effect of the troponin C-like protein from bovine brain (brain modulator protein) on the Mg²⁺-stimulated ATPase of skeletal muscle actomyosin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 72:163–168.
27. Irie, S., and M. Sezaki. 1983. A quantitative determination of the relative amount of histones in polyacrylamide gel by silver stain. *Anal. Biochem.* 134:471–478.
28. Colaltz, E., V. Norbert, K. Tsurugi, H. N. Lightfoot, W. MacKinlay, A. Lin, and I. G. Wool. 1977. Isolation of eukaryotic ribosomal proteins. *J. Biol. Chem.* 252:9071–9080.
29. Lin, A., B. Wittman-Liebold, J. McNally, and I. G. Wool. 1982. The primary structure of the acidic phosphoprotein P2 from rat liver 60S ribosomal subunits. *J. Biol. Chem.* 257:9189–9197.
30. Burridge, K., T. Kelly, and P. Mangeat. 1982. Nonerythrocyte spectrins: actin-membrate attachment proteins occurring in many cell types. *J. Cell Biol.* 95:478–486.
31. Carlin, R. K., D. C. Bartelt, and P. Siekevitz. 1983. Identification of fodrin as a major calmodulin-binding protein in postsynaptic density preparations. *J. Cell Biol.* 96:443–448.
32. Glenney, J. R., Jr., P. Glenney, and K. Weber. 1982. Erythroid spectrin, brain fodrin, and intestinal brush border proteins (TW-260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type-specific subunit. *Proc. Natl. Acad. Sci. USA.* 79:4002–4005.
33. Klee, C. B., and M. H. Krinks. 1978. Purification of cyclic 3',5'-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to sepharose. *Biochemistry.* 17:120–126.
34. Manalan, A. S., and C. B. Klee. 1983. Interaction of calmodulin with its target proteins. *Chem. Scr.* 21:139–144.
35. Villar-Palasi, D., D. Oshiro, and R. H. Kretsinger. 1983. Interaction of calmodulin and glycogen phosphorylase. *Biochim. Biophys. Acta.* 757:46–50.
36. Watterson, D. M., and T. C. Vanaman. 1976. Affinity chromatography purification of a cyclic nucleotide phosphodiesterase using immobilized modulator protein, a troponin C-like protein from brain. *Biochem. Biophys. Res. Commun.* 73:40–46.
37. LaPorte, D. C., W. A. Toscano, and D. R. Storm. 1979. Cross-linking of iodine-125-labeled, calcium-dependent regulatory protein to the Ca²⁺-sensitive phosphodiesterase purified from bovine heart. *Biochemistry.* 18:2820–2825.
38. Brostrom, D. O., S. B. Bucckino, and M. A. Brostrom. 1983. Identification of a Ca²⁺ requirement for protein synthesis in eukaryotic cells. *J. Biol. Chem.* 258:14390–14399.
39. Steinhardt, R. A. 1982. Ionic logic in activation of the cell cycle. *In* Ions, Cell Proliferation, and Cancer. A. L. Boynton, W. McKeehan, and J. F. Whitfield, editors. Academic Press, Inc., New York. 311–325.
40. Whitfield, J. F. 1982. The roles of calcium and magnesium in cell proliferation: an overview. *In* Ions, Cell Proliferation, and Cancer. A. L. Boynton, W. McKeehan, and J. F. Whitfields, editors. Academic Press, Inc., New York. 283–294.
41. Martelly, I., A. Molla, M. Thomasset, and A. Le Moigne. 1983. Planarian regeneration: in vivo and in vitro effects of calcium on DNA synthesis. *Cell Differ.* 13:25–34.
42. Andreasen, T. J., C. W. Luetje, W. Heideman, and D. R. Storm. 1983. Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. *Biochemistry.* 22:4615–4618.
43. Palfrey, H. C., W. Schiebler, and P. Greengard. 1982. A major calmodulin binding protein common to various vertebrate tissues. *Proc. Natl. Acad. Sci. USA.* 79:3780–3784.
44. La Porte, D. C., S. Gidwitz, M. J. Weber, and D. R. Storm. 1979. Relationship between changes in the Ca²⁺ dependent regulatory protein and adenylate cyclase during viral transformation. *Biochem. Biophys. Res. Commun.* 86:1169–1177.
45. Chafouleas, J. G., and A. R. Means. 1982. Calmodulin is an important regulatory molecule in cell proliferation. *In* Ions, Cell Proliferation, and Cancer. A. L. Boynton, W. McKeehan, and J. F. Whitfield, editors. Academic Press, Inc., New York. 449–464.
46. Veigl, M. L., W. D. Sedwick, and T. C. Vanaman. 1982. Calmodulin and Ca²⁺ in normal and transformed cells. *Fed. Proc.* 41:2283–2288.