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A rapid and sensitive method for detection and quantification of calcineurin and calmodulin-binding proteins using biotinylated calmodulin

(biotinylation/protein blots)

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ABSTRACT **Purified bovine brain calmodulin was biotinylated with biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide. Biotinylated calmodulin was used to detect and quantify calmodulin-binding proteins following both protein blotting and slot-blot procedures by using alkaline phosphatase or peroxidase coupled to avidin. When purified bovine brain calcineurin, a calmodulin-dependent protein phosphatase, was immobilized on nitrocellulose slot blots, biotinylated calmodulin bound in a calcium-dependent saturable manner; these blots were then quantified by densitometry. Biotinylated calmodulin was able to detect as little as 10 ng of calcineurin, and the binding was competitively inhibited by addition of either native calmodulin or trifluoperazine. When biotinylated calmodulin was used to probe protein blots of crude brain cytosol and membrane preparations after gel electrophoresis, only protein bands characteristic of known calmodulin-binding proteins (i.e., calmodulin-dependent protein kinase, calcineurin, spectrin) were detected with avidin-peroxidase or avidin-alkaline phosphatase procedures. Purified calcineurin was subjected to one- and two-dimensional gel electrophoresis and protein blotting; as expected, only the 61-kDa calmodulin-binding subunit was detected. When the two-dimensional protein blot was incubated with biotinylated calmodulin and detected with avidin-alkaline phosphatase, several apparent forms of the 61-kDa catalytic subunit were detected, consistent with isozymic species of the enzyme. The results of these studies suggest that biotinylated calmodulin can be used as a simple, sensitive, and quantifiable probe for the study of calmodulin-binding proteins.**

Much of our current understanding of calmodulin-dependent control of cellular functions has stemmed from identification of the calmodulin-binding proteins, which are activated by Ca^{2+}-calmodulin (1). Calmodulin has been shown to mediate activation of a number of calcium-dependent enzymes such as phosphodiesterase (2–4), adenylate cyclase (5), calmodulin-dependent protein kinases (6–8), and calmodulin-dependent protein phosphatase, calcineurin (9). For purposes of analysis, a calmodulin-binding protein must associate with calmodulin in a high affinity Ca^{2+}-dependent fashion. Several techniques have been useful in the identifications of calmodulin-binding proteins; notable among these are the use of calmodulin affinity columns, photoaffinity crosslinking protocols, and the ^{125}I-labeled calmodulin gel overlay techniques (10–12). Analysis of the $NaDodSO_4$/PAGE protein patterns observed after EGTA elutions from calmodulin affinity resins has provided a means for both identifying and purifying calmodulin-binding proteins; however, this technique is not particularly suitable for material in limited quantity or for screening of many samples.

The gel overlay technique has facilitated analysis of calmodulin-binding proteins in crude tissue fractions resolved by $NaDodSO_4$/PAGE. The early calmodulin overlay techniques required that the gels be incubated for long periods with buffers and washed extensively (≈72 hr) after incubation with ^{125}I-labeled calmodulin (12). Backgrounds were often high and the time needed to visualize the autoradiogram was 5–10 days after sample preparation (13). Recent advances have utilized the immunoblot procedures of Towbin *et al.* (14) to immobilize proteins on nitrocellulose paper followed by incubation of the Tween-20 blocked paper with ^{125}I-labeled calmodulin (15).

Avidin-biotin probes have been used to visualize biotin-labeled DNA in nitrocellulose blot hybridization studies (16). We have exploited the high-affinity avidin-biotin interaction (17) to afford detection of biotinylated calmodulin and calmodulin-binding proteins. We now report that biotinylated calmodulin can effectively bind both purified and crude preparations of calmodulin-binding proteins with sensitivity of detection in the nanogram range. Biotinylated calmodulin has also been used to develop a quantitative slot-blot procedure for the quantification of individual calmodulin-binding proteins. We have also used this approach to probe two-dimensional gels of purified calcineurin and have found several apparent (isozymic) forms of the 61-kDa catalytic subunit (18). The use of biotinylated binding proteins as probes and detection with avidin–enzyme systems should be applicable to a wide class of binding protein–substrate protein interactions.

MATERIALS AND METHODS

Materials. Electrophoretic supplies were purchased from Bio-Rad, except for molecular weight standards, which were obtained from Pharmacia. Biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester was purchased from Calbiochem. Trifluoperazine HCl was purchased from Boehringer Mannheim. Vectastain alkaline phosphatase and horseradish peroxidase kits were obtained from Vector Laboratories (Burlingame, CA). The following substrates were used for visualization of the avidin–enzyme complexes; *p*-chloronaphthol, 4,4-diaminobenzidine hydrochloride, nitroblue tetrazolium chloride, grade III (Sigma), and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Amresco,

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Abbreviation: Biocam-Ca^{2+}, calmodulin biotinylated in the presence of calcium.

Euclid, OH). Nitrocellulose sheets (BA-85) and a slot-blot manifold were obtained from Schleicher & Schuell.

Purification of Proteins. Calmodulin was prepared from bovine brain as described (19). Homogenous calmodulin-dependent protein phosphatase (calcineurin) and calmodulin-dependent phosphodiesterase were prepared according to the method of Kincaid *et al.* (20). Rat brain and heart cytosol and membrane fractions were prepared as described (21). Protein was measured according to the method of Bradford (22); calmodulin values were underestimated by 40% using this method, and values were either adjusted accordingly or determined by using an extinction coefficient of 0.18 for a solution of calmodulin at 1 mg/ml. Calcineurin phosphatase activity was measured as described (23).

Biotinylation of Calmodulin. Homogenous bovine brain calmodulin (4.0 ml; 1.2 mg/ml) was dialyzed overnight at 4°C against 0.1 M phosphate buffer (pH 7.4). Biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester was dissolved in *N,N*-dimethylformamide (3.2 mg per 100 μl) and added to the calmodulin solution at a final concentration of 1 mM. Incubations were for 2 hr at 4°C with constant stirring, followed by exhaustive dialysis. The molar ratio between the biotinylating agent and calmodulin was kept at 14:1, which is twice the number of lysine sites on calmodulin. Reactions were carried out either in the presence of 1 mM Ca^{2+} (liganded calmodulin) or 5 mM EGTA (unliganded calmodulin). Absorbance of native and modified calmodulin was determined by using a Gilford scanning spectrophotometer.

NaDodSO$_4$/PAGE and Protein Blotting Procedures. Both one- and two-dimensional NaDodSO$_4$/PAGE was carried out as described by O'Farrell (24), using 0.001% pyronin-Y as a tracking dye. Gels were electroblotted for 2 hr at 50 V (14) followed by incubation for 1 hr in blocking solution [5% nonfat dry milk/50 mM Tris·HCl, pH 7.4/150 mM NaCl/1 mM $CaCl_2$/0.02% antifoam A (25)]. Blots were washed with buffer A (50 mM Tris·HCl, pH 7.4/150 mM NaCl/1 mM $CaCl_2$). Specific calmodulin-binding proteins were visualized by using biotinylated calmodulin as follows: after blocking and washing, the blots were incubated with various amounts of biotinylated calmodulin (0.2–25 μg) for at least 30 min in 2.5 ml of blocking solution containing either 1 mM $CaCl_2$ or, for control studies, 5 mM EGTA. After this incubation, the blot was washed with buffer A; for "specific" incubations, 1 mM $CaCl_2$ was included in all buffers and washes. For control incubations, 5 mM EGTA was included in all solutions. After incubation and washing, the blots reacted with avidin-peroxidase or with avidin-alkaline phosphatase dissolved in blocking solution for 30–60 min, followed by color development with one of the following chromogens: horseradish peroxidase [either 4,4-diaminobenzidine (0.5 mg/ml in buffer A containing 0.03% H_2O_2) or *p*-chloronaphthol]; alkaline phosphatase- nitro blue tetrazolium chloride (200 μl at 50 mg/ml in 50% dimethylformamide) and 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (100 μl at 50 mg/ml in 50% dimethylformamide); both dissolved in 30 ml of 0.1 M Tris·HCl, pH 9.5/100 mM NaCl/50 mM $MgCl_2$ (26). All blots were air-dried and photographed using Kodak Technical Pan film at ASA 25.

Slot-Blot Procedures. To alleviate problems with the nonisotopic quantification of dot blots (27), a slot-blot apparatus was used to produce protein bands of uniform density, which subsequently were detected by colorimetric means and quantified by scanning densitometry. Nitrocellulose filters were rinsed in distilled H_2O for 5 min and inserted into the apparatus; serial dilutions of purified calcineurin were applied to individual wells of the slot-blot apparatus (2–2000 ng in 20 μl) under light vacuum. The filter was then washed in 10% acetic acid/25% isopropanol for 15 min, followed by three rinses in distilled H_2O. The filter was equilibrated in buffer A, and the excess protein binding sites were blocked with blocking solution (5% nonfat dry milk in buffer A) for 1 hr. In some experiments, the slot blot was sectioned into quadrants for separate incubations after the blocking step. Filters were then incubated with the desired concentration of biotinylated calmodulin dissolved in blocking solution, usually for 60 min, and washed three times in buffer A containing either 1 mM $CaCl_2$ or, for control strips, 5 mM EGTA. Avidin-peroxidase (1:500) or avidin-alkaline phosphatase (1:500) was dissolved in blocking solution and incubated with the blot for 30 min. After three washes in buffer A, color was developed as described for the protein electroblots. The filters were dried and scanned using a Beckman DU8-B scanning spectrophotometer; absorbance was measured at 570 nm for blue substrates (*p*-chloronaphthol, alkaline phosphatase products) and at 420 nm for the diaminobenzidine product. The bands produced by this procedure were uniform, and analysis of the scans indicated that peak heights (as opposed to area) were sufficient to permit accurate quantitation of the strips.

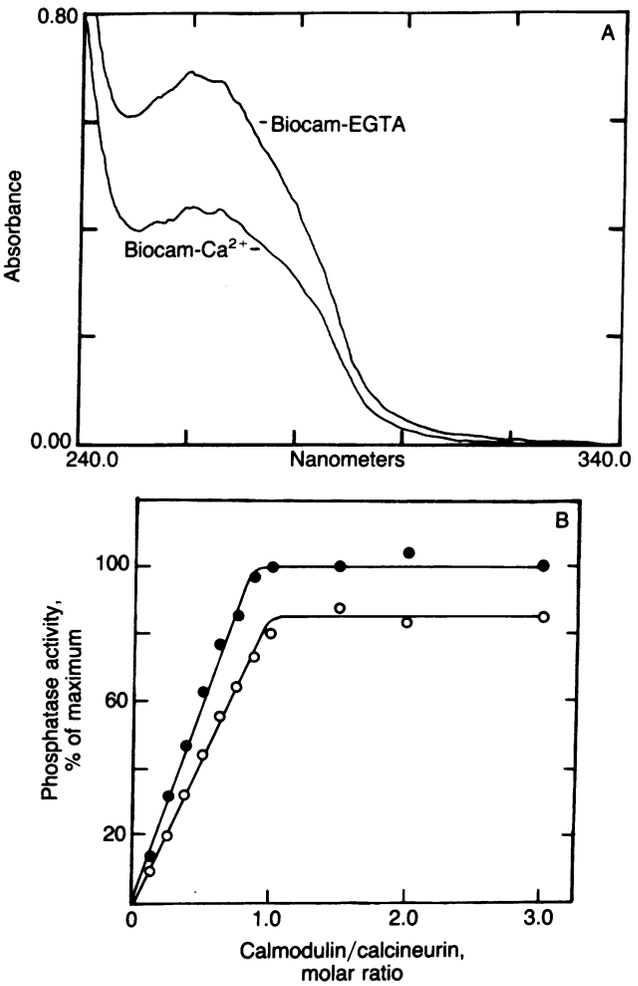

FIG. 1. (*A*) Ultraviolet spectra of biotinylated calmodulin. Samples of calmodulin were biotinylated in the presence (Biocam-Ca^{2+}) and absence of Ca^{2+} (Biocam-EGTA). (*B*) Activation of protein phosphatase (calcineurin) by native and biotinylated calmodulin (Biocam-Ca^{2+}). The phosphatase activity of calcineurin (10 μg, 0.6 μM) was measured with the indicated molar ratios of native (closed circle) and biotinylated calmodulin (Biocam-Ca^{2+}; open circles). Phosphatase activity is expressed as percentage maximal activity with 2 mol of native calmodulin per mol of calcineurin; specific activity, 0.7 μmol of phosphate released per min per mg of protein (23).

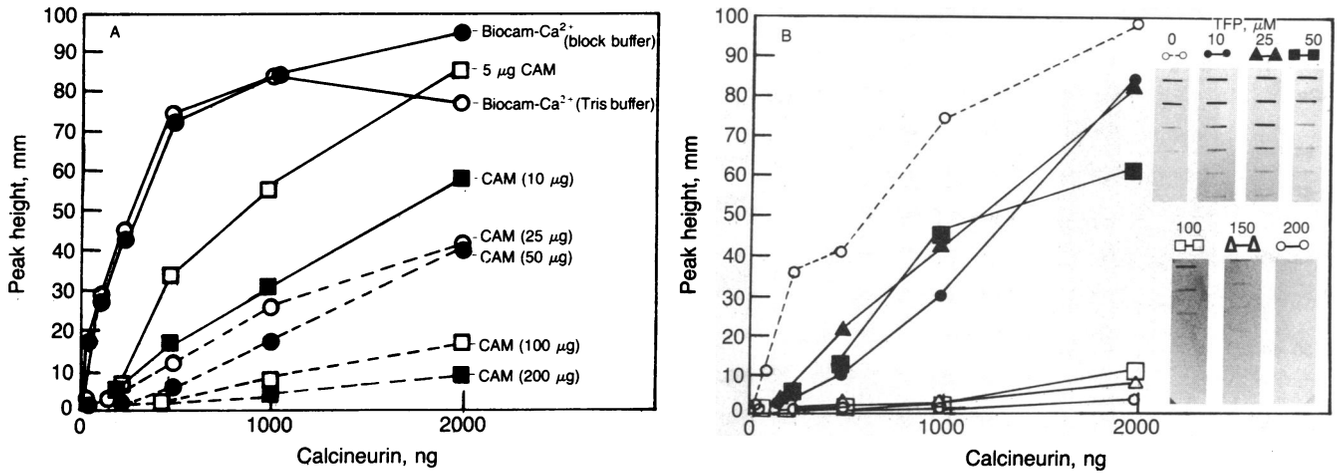

FIG. 2. (*A*) Binding of biotinylated calmodulin to purified calcineurin immobilized on slot blots. Various concentrations of calcineurin (2–2000 ng in 20 μl) were immobilized on nitrocellulose, slot blots were sectioned, and the separate sections were incubated as described. Increasing amounts of native calmodulin (CAM) (5–200 μg) were added to separate sections and changes in binding were quantified by densitometry. (*B*) Inhibition of biotinylated calmodulin binding by trifluoperazine (TFP). Various concentrations of calcineurin (2–2000 ng) were immobilized on nitrocellulose. Separate sections were incubated with biotinylated calmodulin (25 μg of Biocam-Ca^{2+}) and incubated for 60 min with increasing concentrations of TFP (10–200 μM). Slot blots are shown in the right quadrant.

RESULTS

Biotinylation of Calmodulin. Calmodulin was biotinylated using the biotin derivative biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester, and the ultraviolet spectra of calmodulin biotinylated in the presence and absence of Ca^{2+} is shown in Fig. 1*A*. There was an increase in absorbance below 280 nm (absorbance maxima at 260 nm) of both biotinylated derivatives, consistent with incorporation of biotin into the molecule. The incorporation of biotin, estimated by using the extinction coefficient for the caproic acid derivative (2.3 at 260 nm for a 1 mM solution) was ≈2 mol/mol for modification in the presence of calcium, and 3–4 mol/mol in the presence of EGTA. The biologic activity of the biotinylated derivative was well preserved, with a retention of 85–90% of the activity of native calmodulin (Fig. 1*B*).

Detection and Quantification of Calcineurin on Slot Blots by Using Biotinylated Calmodulin. Purified bovine brain calcineurin was immobilized on nitrocellulose filters using a slot blot apparatus. After incubation for 60 min with 10 μg of calmodulin biotinylated in the presence of Ca^{2+} (Biocam-Ca^{2+}) followed by incubation with avidin-peroxidase, a colored peroxidase product was visualized on the paper and quantified by densitometric scanning at 570 nm. The amount of colored product estimated by densitometry was proportional to the amount of calcineurin originally applied to the filter (Fig. 2*A*) whether biotinylated calmodulin was added in 1.0 ml of blocking buffer (closed circles) or buffer A (50 mM Tris·HCl/150 mM NaCl/1 mM Ca; open circles); thus, the presence of protein in the incubation medium did not interfere with interaction of calcineurin and modified calmodulin. The relationship between peak height and calcineurin remained linear between 25 and 500 ng, and it exhibited saturation at higher concentrations. When increasing concentrations of native calmodulin were added to the incubation mixture containing biotinylated calmodulin, a concentration-dependent competition in biotinylated calmodulin binding was observed. When 5 mM EGTA was included in the biotinylated calmodulin incubation, no signal was detected on the nitrocellulose paper.

Similar results were obtained when trifluoperazine, a drug that inhibits calmodulin binding, was added to the incubation mixture. As shown in Fig. 2*B*, increasing concentrations of trifluoperazine completely inhibited the binding of biotinylated calmodulin to immobilized calcineurin, with an approximate IC_{50} of 50 μM. These results indicate that biotinylated calmodulin can be used to quantitatively detect calmodulin-binding proteins immobilized on nitrocellulose; this binding resembles that of native calmodulin in that it is Ca^{2+}-dependent and is competitively displaced by unmodified calmodulin or by known inhibitors of calmodulin.

Detection of Calmodulin-Binding Proteins after $NaDodSO_4$/PAGE and Protein Electroblotting Using Biotinylated Calmodulin. Crude cytosolic and membrane fractions from brain and heart (100 μg of protein) were electrophoresed on 10% $NaDodSO_4$/polyacrylamide gels and electroblotted onto nitrocellulose. Blots were incubated for 1–2 hr with 25 μg of biotinylated calmodulin (Biocam-Ca^{2+}) dissolved in blocking solution, followed by detection with avidin-peroxidase or avidin-alkaline phosphatase chromogens. Fig. 3 (lanes 2–6) shows the protein staining and biotinylated calmodulin-binding patterns of brain cortical cytosol, cerebellar cytosol, heart cytosol, cortical membranes, and cerebellar membranes, respectively. A prominent band of 60 kDa is present in all brain fractions, which comigrated with purified calcineurin (lane 8). However, other calmodulin-binding proteins such as phosphodiesterase and a subunit of calmodulin-dependent protein kinase migrate at or near this molecular mass and may also be components of the band. In cortical cytosol and membranes, a band of 52 kDa was also observed, but it was not present in cerebellar fractions, suggesting that this band is a subunit of calmodulin-dependent protein kinase (21, 28). Additional bands were observed in brain membranes in the high molecular weight range, which probably correspond to brain spectrin and its breakdown products (29). Crude heart cytosol, which reportedly lacks calcineurin and has lower amounts of phosphodiesterase, showed no reaction product in the 60 kDa region. One major band of 75 kDa was observed in heart cytosol (30) and brain membranes; this may correspond to a form of caldesmon (31).

Purified calmodulin-binding proteins could be easily detected on electroblots (Fig. 3, lanes 8 and 9). Calcineurin (5 μg) appeared as a single band of 60 kDa after protein staining; detection of the same sample with biotinylated calmodulin produced a very intense signal after 3 min of color development with avidin peroxidase and *p*-chloronaphthol. The 18-kDa subunit did not bind calmodulin. A 36-kDa fragment, barely observable in the protein staining pattern, also bound calmodulin; this corresponds to a known proteolytic frag-

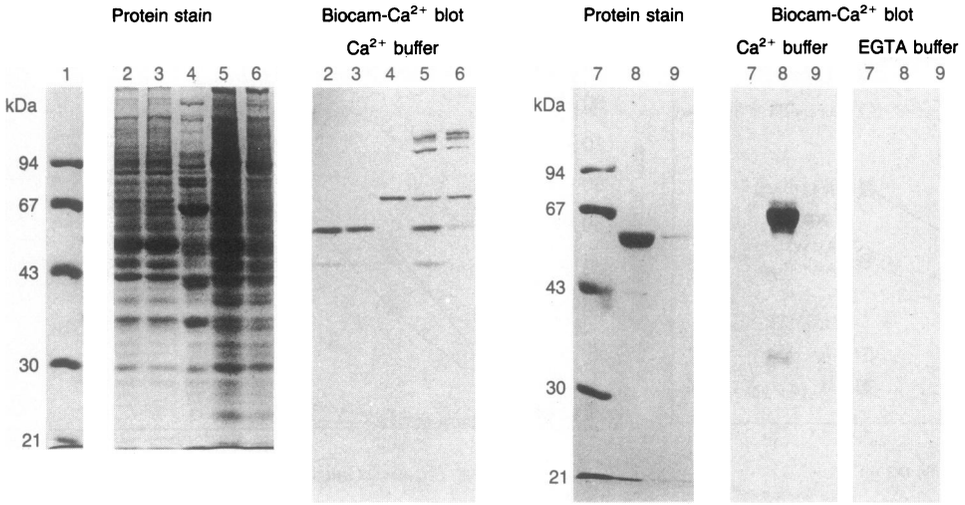

FIG. 3. Binding of biotinylated calmodulin to calmodulin-binding proteins present in crude tissue fractions after 10% $NaDodSO_4$/PAGE and electroblotting. Lanes: 1 and 7, molecular size standards; 2–6, 100 μg of rat brain cortex cytosol, rat brain cerebellar cytosol, rat heart cytosol, rat brain cortex membranes, and rat brain cerebellum, respectively; 8 and 9, protein stains and biotinylated calmodulin-binding patterns with purified calcineurin (5 μg) and calmodulin-stimulated phosphodiesterase (1.0 μg). If 5 mM EGTA was included in the incubation and wash buffers, no binding of biotinylated calmodulin was detected (EGTA buffer blot).

ment of calcineurin. When 1.0 μg of calmodulin-stimulated phosphodiesterase was electroblotted, a band corresponding to the 61-kDa subunit was visible, as was a known proteolytic fragment of 60-kDa (lane 9). When 5 mM EGTA was included in the incubation with biotinylated calmodulin, no binding was observed, indicating that the binding was dependent on Ca^{2+}. When a partially purified sample from bovine brain was analyzed before and after chromatography on calmodulin-Sepharose, only material prior to chromatography reacted (Fig. 4), indicating that the protein band recognized was a calmodulin-binding protein. In this experiment, only 6–8 μg of crude material was applied to a miniature $NaDodSO_4$/polyacrylamide gel and 10–15 ng of binding protein was detected using the avidin-peroxidase system. Thus, this method is sensitive and applicable to analysis of material in limited quantity.

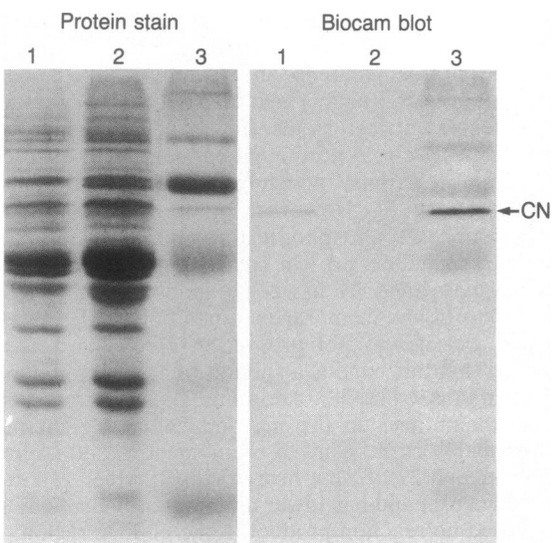

FIG. 4. Binding of biotinylated calmodulin in partially purified fractions before and after chromatography on calmodulin-Sepharose. A preparation of partially purified bovine brain calcineurin (DEAE Bio-Gel, peak II fraction; see ref. 4) was exhaustively treated with calmodulin-Sepharose to deplete the fraction of calmodulin-binding proteins and samples of the fraction before (lane 1, 6 μg) and after (lane 2, 8 μg) chromatography were electrophoresed on a 13% $NaDodSO_4$/polyacrylamide miniature gel (0.5 mm). Lane 3, colored protein standards (Bethesda Research Laboratories) with 50 ng of purified calcineurin (arrow) as an internal calmodulin-binding protein standard. After incubation with biotinylated calmodulin and avidin-peroxidase, the reaction product was visualized as described.

Calcineurin was subjected to isoelectric focusing in the presence of 6 M urea in the first dimension, followed by $NaDodSO_4$/PAGE in the second dimension (Fig. 5). The 61-kDa catalytic subunit appeared as at least three separate spots after protein staining, with isoelectric points in the range of 5.4 to 5.7. When the same sample was electroblotted after two-dimensional electrophoresis, incubated with 25 μg of biotinylated calmodulin, and detected using avidin-alkaline phosphatase, several additional 61-kDa spots that bound calmodulin were detected, with isoelectric values of 5.4 to 6.0. Isoelectric focusing of calcineurin in native gels has suggested that several isozymes can be detected in the absence of urea (unpublished observations). These results suggest that the catalytic 61-kDa subunit of calcineurin, which binds calmodulin, may exist *in vivo* in multiple forms that exhibited altered charge properties. The ability to use the two-dimensional procedure emphasizes the usefulness of this method for detection and comparison of similarly sized calmodulin-binding subunits.

DISCUSSION

The results presented in this study demonstrate that biotinylated calmodulin can serve as a simple and sensitive probe for the detection and quantification of calmodulin-binding proteins on both protein electroblots and slot blots. Functionally, the biologic activity of this calmodulin derivative is well preserved with 85–90% the potency of native calmodulin for activation of the protein phosphatase. Its dependency on Ca^{2+} and antagonism by known inhibitors (trifluoperazine, melittin) also suggests preservation of its biochemical properties.

Slot blots, unlike dot blots, produced uniform bands of immobilized proteins, which were then scanned and quantified by using an appropriate densitometer. We were able to detect 2 ng of immobilized calcineurin using alkaline phosphatase, which is sufficiently sensitive for most applications of this assay. Similar to the findings of Leary *et al.* (16), we found that avidin-alkaline phosphatase was more sensitive than avidin-peroxidase. Additional advantages of slot blots are that standards can be run on the same filter and processed in parallel with tissue samples, or, alternatively, strips can be cut from a single slotted sheet and incubated under different conditions such as with inhibitors or agents that may otherwise affect protein binding.

The chief advantages of the biotinylated calmodulin overlays are in its low cost, speed of detection (1 day), sensitivity (nanogram range), and remarkably low background. More importantly, only proteins that comigrated with known

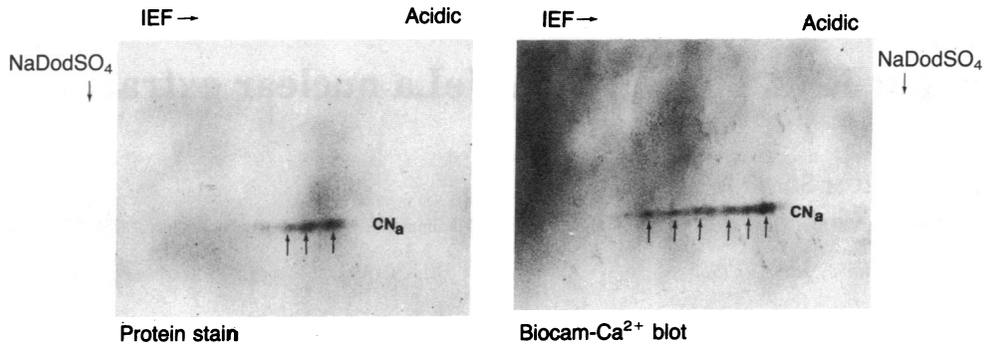

FIG. 5. Detection of biotinylated calmodulin binding to calcineurin subunits after two-dimensional gel electrophoresis. Purified bovine brain calcineurin (5.0 μg) was subjected to isoelectric focusing (IEF) followed by 12% $NaDodSO_4$/PAGE. The 61-kDa subunit of calcineurin (CN_a) separated into three distinct spots after Coomassie blue staining (small arrows on protein stain). An identical sample was transferred to nitrocellulose paper, incubated with 25 μg of biotinylated calmodulin (Biocam-Ca^{2+} blot), and the calmodulin binding was detected by avidin-alkaline phosphatase methods. The photograph in the figure is a magnification of a portion of the gel.

calmodulin-binding proteins were reactive and fractions depleted of calmodulin-binding proteins were unreactive. In agreement with previous investigations using the ^{125}I-calmodulin overlay procedure, the major binding proteins in brain appear to be the protein kinase (subunits of 52 and 62 kDa), the protein phosphatase (61-kDa subunit), and higher molecular weight proteins such as spectrin (220 kDa) p75 protein (30) (75 kDa), and caldesmon. In agreement with Flanagan *et al.* (21), cerebellar extracts appeared to lack the 52-kDa (kinase subunit) postsynaptic density protein when analyzed by the present method. Since the method can be scaled down to accommodate microgram quantities of protein (5–10 μg), it may provide a convenient and versatile approach for the analysis of small quantities of tissues.

Another useful application of this method might be the rigorous identification of calmodulin-binding subunits in combination with two-dimensional electrophoresis (24). Indeed, the 61-kDa subunit of purified calcineurin, which appears as a single protein band on one-dimensional $NaDodSO_4$/PAGE, may consist of several closely related molecules that differ only slightly in charge, but all of which bind biotinylated calmodulin. A similar two-dimensional gel pattern was reported by Klee and Haiech (32), with several Coomassie blue-stained spots associated with the 61-kDa subunit. These results suggest several (isozymic) species of the catalytic subunit and do not result from treatment with 6 M urea, since native gel isoelectric focusing of calcineurin also showed multiple focused bands of calcineurin. Whether the apparent isozymes of calcineurin are the result of posttranslational differences, such as methylation (33), glycosylation, or differences in the primary protein structure and whether they exhibit unique catalytic or regulatory properties is not known.

It is clear that this approach may be suitable to the study of other protein–binding protein interactions. Proteins whose structures are functionally stable to $NaDodSO_4$/PAGE and renaturation conditions and for which interactions with biotinylated substrate/effector are of high affinity might well be detected by using the strategy outlined in this report.

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