Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 3780–3784, June 1982
Cell Biology

A major calmodulin-binding protein common to various vertebrate tissues

(avian erythrocytes/immunoprecipitation)

H. CLIVE PALFREY*, WERNER SCHIEBLER, AND PAUL GREENGARD

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

Contributed by Paul Greengard, March 22, 1982

ABSTRACT **A major calmodulin-binding protein (CaM-BP) of M_r 240,000 was demonstrated in various rat tissues by using a ^{125}I-labeled CaM gel overlay technique. This protein (designated p240) was detected in the particulate fraction and to a lesser extent in the cytosol of all tissues studied. Binding of CaM to p240 was completely dependent on Ca^{2+}. A second, exclusively soluble, CaM-BP (M_r 115,000) common to several tissues and a number of other CaM-BPs with a more restricted tissue distribution were also observed by using this technique. CaM binding to p240 occurred in high amounts in plasma membranes from avian erythrocytes but was absent from mammalian erythrocyte membranes. Antibodies prepared against turkey erythrocyte p240 (anti-Tp240) cross-reacted with p240 in other tissues. Identity between the proteins recognized by anti-Tp240 and CaM was confirmed by demonstrating that ^{125}I-labeled CaM could bind to p240 specifically immunoprecipitated from either rat brain or turkey erythrocytes by anti-Tp240. The p240 may be related to a previously described actin-binding protein and may represent a major site of action of CaM on the cytoskeleton.**

Calmodulin (CaM), the ubiquitous Ca^{2+}-binding protein, is responsible for mediating several physiological responses associated with increases in intracellular Ca^{2+} levels. This protein has been shown to bind to and activate a number of enzymes of defined function, such as cyclic nucleotide phosphodiesterases (1, 2), Ca^{2+}, Mg^{2+} ATPases (3–5), a specific class of protein kinases (6–8), and a protein phosphatase that appears to be identical with calcineurin (9, 10). Several reports also suggest that CaM interacts with certain cytoskeletal elements—for example, microtubules (11, 12), brush border core proteins (13), and spectrin (14). These observations are of considerable interest because Ca^{2+} is widely believed to control various aspects of cell shape, motility, and translocation of intracellular organelles, and CaM may be intimately involved in these phenomena (15).

During the course of studies on the purification of CaM-dependent protein kinase from two sources, avian erythrocyte and *Torpedo* electroplaque, we noted the presence of a high molecular weight species that appeared to adsorb specifically to CaM affinity matrices. The presence of this protein in two such divergent tissues led us to investigate the possibility that the protein might have a more widespread distribution. For this purpose we used the recently developed ^{125}I-labeled CaM (^{125}I-CaM) gel overlay technique (13, 16) and have demonstrated the existence of a M_r 240,000 CaM-binding protein (CaM-BP), designated p240, common to avian erythrocytes and a number of mammalian tissues.

METHODS

Preparation of ^{125}I-CaM and ^{125}I-CaM Gel Overlay. CaM was isolated from bovine brain by the method of Grand *et al.* (17) and iodinated with the Bolton–Hunter reagent (New England Nuclear) as described by Chafouleas *et al.* (18) to a specific activity of 110 Ci/mmol (1 Ci = 3.7×10^{10} becquerels). Those authors have demonstrated that CaM iodinated by this technique retains full biological activity.

The gel overlay technique was similar to that of Carlin *et al.* (16). Briefly, $NaDodSO_4$/polyacrylamide (6% or 5–10% gradient) gels (19) were fixed, equilibrated in buffer A (150 mM NaCl/25 mM Tris·HCl, pH 7.3/0.1% NaN_3/0.5% gelatin) with either 1 mM $CaCl_2$ or 1 mM EGTA, and then incubated in sealed plastic bags with ^{125}I-CaM (20 nM; 2.2 μCi/ml, diluted in buffer A) for 3 hr. The gels were then washed (1–2 hr each) with five changes of buffer A, stained, destained, and autoradiographed. For quantitation of relative amounts of ^{125}I-CaM bound, pieces were excised from the dried gel and assayed by γ-emission spectrometry. The amount of ^{125}I-CaM bound to the p240 region of gels containing turkey erythrocyte membrane proteins was found to be linearly proportional to the amount of protein applied (2–40 μg). All ^{125}I-CaM binding experiments were conducted within this range. Molecular weights of CaM-BPs were estimated by reference to molecular weight markers (19) run on the same gel.

Preparation of Tissues. Adult rat tissues were dissected out, washed in physiological saline, and homogenized in 10 vol of 25 mM Tris·HCl, pH 7.3/1 mM EDTA/5 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride in a Teflon/glass homogenizer. After a low-speed spin (1,000 × *g* for 5 min) to eliminate debris, the supernatant was centrifuged at 150,000 × *g* for 45 min to obtain crude particulate and cytosol fractions. These were analyzed for protein concentration (20) and solubilized in a $NaDodSO_4$ stop buffer (19) prior to electrophoresis.

Membranes from avian and mammalian erythrocytes were prepared as described (21). Extraction of turkey erythrocyte membranes with 0.5% Nonidet P-40 (NP-40), 15 mM lithium diiodosalicylate, or low-ionic strength dialysis was by the procedure of Beam *et al.* (21). Nonextractable material was pelleted by centrifugation at 50,000 × *g* for 20 min.

Preparation of Antibodies to Turkey Erythrocyte p240 and Antibody Overlay. Turkey erythrocyte membranes were prepared as above and their proteins were separated on $NaDodSO_4$/4% polyacrylamide slab gels (20 cm long, 1.2 mm thick; 23 mg of protein per 10 gels). Proteins were localized by immersing the gels in 4 M Na acetate for 30 min (22), and the band corresponding to p240 was excised and stacked onto a 5-mm-thick $NaDodSO_4$/4% polyacrylamide gel. After reelectrophoresis the region containing p240 (approximately 100 μg) was visualized

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: SAC, *Staphylococcus aureus* cells; p240, protein of M_r 240,000; anti-Tp240, antibody against turkey erythrocyte p240; CaM, calmodulin; CaM-BP, calmodulin-binding protein; NP-40, Nonidet P-40.

* Present address: Dept. of Pharmacological and Physiological Sciences, Univ. of Chicago, 920 E. 58th St., Chicago, IL 60637.

as above and excised, and the gel strip was washed in physiological saline to decrease its $NaDodSO_4$ content. It was then homogenized with a Teflon/glass homogenizer in 3 ml of the same buffer, mixed with 3 ml of complete Freund's adjuvant, and emulsified by repeated passage through a 24-gauge syringe needle. Rabbits were injected intradermally with 1 ml of this material per injection site. Two booster injections of material without Freund's adjuvant were given after 1 and 2 weeks, and blood was collected 2 weeks later. Preimmune serum was obtained from the same rabbits prior to antigen injection.

The antibody overlay technique was similar to that described above for ^{125}I-CaM binding and followed the original method of Burridge (23). Gels were first incubated in antibody to turkey erythrocyte p240 (anti-Tp240; 1:10 in buffer A) for 3 hr, washed four times (1–2 hr each) in buffer A, and then incubated in ^{125}I-labeled protein A (^{125}I-protein A; Amersham; 0.5–1 μCi/ml in buffer A) for a further 2 hr. After a second series of washes in buffer A, gels were either dried directly or were stained and destained before autoradiography. All experiments were conducted within the range of protein concentrations in which anti-Tp240–^{125}I-protein A binding was found to be linear.

Immunoprecipitation. The immunoprecipitation technique was modified slightly from Ivarie and Jones (24) and Goelz *et al.* (25). Briefly, dialyzed extract (30–40 μg of protein) of turkey erythrocyte membranes (21), or synaptosomal lysate (80–150 μg of protein) prepared as described (26), was incubated at 4°C in NET buffer (25) in the presence of 0.1% $NaDodSO_4$ for 5 min (final volume, 120 μl). Then 40 μl of NET containing 25 mg of bovine serum albumin per ml and 2% NP-40 was added, followed by 10 μl of a 10% solution of freshly prepared *Staphylococcus aureus* cells (SAC; Pansorbin, Calbiochem) in NET/0.25% NP-40. Incubation was continued for 5 min, followed by sedimentation at 12,000 × *g*. The "precleared" supernatant was transferred to a second tube and incubated with 5 μl of anti-Tp240 (or preimmune serum) for 20 min. An additional 10 μl of SAC was added and 5 min later the SAC–IgG precipitate was collected as above. This pellet was washed twice with NET/0.25% NP-40 and then solubilized in 100 μl of $NaDodSO_4$ stop solution. Gel electrophoresis was carried out in $NaDodSO_4$/7.5% polyacrylamide gels and the products were analyzed by staining or by ^{125}I-CaM gel overlay as described above.

RESULTS

^{125}I-CaM Binding to Erythrocyte Membranes and to Various Rat Tissues. Membranes prepared from erythrocytes of five species were compared for their ability to bind ^{125}I-CaM, by using the gel overlay technique. The results shown in Fig. 1 illustrate the high levels of ^{125}I-CaM binding to a protein of M_r 240,000 present in avian erythrocyte membranes (turkey, goose, and duck). In contrast, mammalian (human and rat) erythrocyte membranes were devoid of such binding. The interaction of ^{125}I-CaM with p240 was totally dependent on Ca^{2+}, being eliminated in the presence of EGTA. In addition, Ca^{2+}-dependent binding was reversed by subsequent washing of the gels in EGTA-containing solutions or by including trifluoperazine (0.1 mM) in the binding buffer (data not shown).

A survey of CaM-BPs in crude particulate and cytosol fractions of several rat tissues was made with the same technique (Fig. 2). It is apparent that ^{125}I-CaM binding to a protein of M_r 240,000 is widespread in the tissues examined, with much of the activity concentrated in the particulate fraction. Although this technique cannot yield an absolute quantitative measure, the relative amount of binding to the p240 region in different tissues was remarkably similar when normalized for protein concentration. However, skeletal muscle clearly contained significantly less binding activity in this region of the gel. As indicated in Fig. 1, rat erythrocytes contained no p240 CaM-BP; thus, these results were not compromised by the presence of erythrocytes in the various tissues studied. The high amount of ^{125}I-CaM binding observed in turkey erythrocyte membranes relative to rat tissue particulate samples may be partially due to the fact that the latter preparations were crude compared to the turkey preparation.

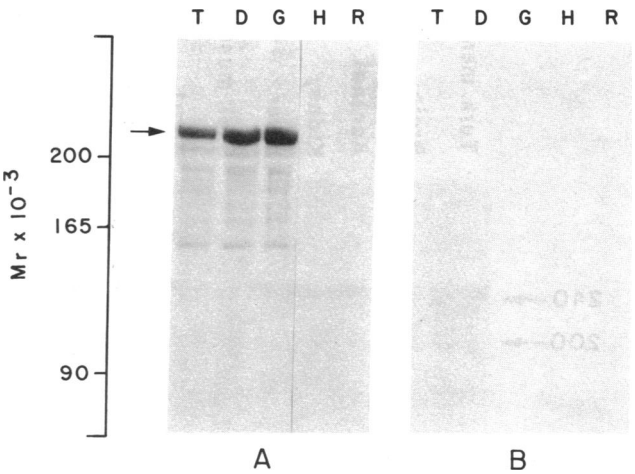

FIG. 1. Autoradiograms of $NaDodSO_4$/6% polyacrylamide gels showing binding of ^{125}I-CaM to proteins from various erythrocyte membranes (30 μg of protein per lane). T, turkey; D, duck; G, goose; H, human; R, rat. Gels were incubated with ^{125}I-CaM in the presence of 1 mM Ca^{2+} (*A*) or 1 mM EGTA (*B*). Arrow indicates p240.

Other ^{125}I-CaM-BPs in various tissues were noted; e.g., in brain, evidently a rich source of these proteins, several major CaM-BPs of lower molecular weight (150,000, 137,000, 73,500, 60,500, 45,000, and 30,000) were found. In detailed studies of the subcellular distribution of CaM-BPs in rat brain, we have found that these proteins are not restricted to any one compartment but occur in several fractions, including the cytosol (Fig. 2*B*; unpublished data). By way of comparison, Carlin *et al.* (figure 1 of ref. 16) in their examination of postsynaptic densities from canine brain found CaM-BPs of M_r 165,000, 150,000, 76,000, 60,000, and 51,000. Of particular interest was a CaM-BP of M_r 115,000 that we found in the cytosolic but not the particulate fraction in brain and all other rat tissues surveyed (Fig. 2*B*). In addition, a protein of M_r 45,000 which appeared to comigrate with one of the prominent brain CaM-BPs was also visible in adrenal, skeletal muscle, and spleen cytosol fractions. Brain, adrenal, liver, heart, spleen, and lung cytosol also showed binding in the M_r 30,000 region. The most prominent CaM-BP in skeletal muscle was a cytosolic protein of M_r 83,000 (Fig. 2*B*) that was absent from other tissues. In almost all cases, binding of the iodinated ligand was eliminated in the presence of EGTA (data not shown in Fig. 2, but see Fig. 1*B*). However, binding of small amounts of ^{125}I-CaM to certain proteins such as skeletal and cardiac muscle myosin (Fig. 2*A*, M_r 200,000) was not abolished by EGTA. This binding is probably nonspecific, although Ca^{2+}-independent binding of CaM in certain instances may be significant (cf. ref. 13). Although a complete phylogenetic survey has yet to be made, we have also observed the presence of a M_r 240,000 CaM-BP in frog erythrocytes and *Torpedo* electric organ but have been unable to detect this protein in homogenates of various organs of the invertebrate species *Aplysia californica* (unpublished data). Bartelt *et al.* (27) recently presented evidence for a similar protein in dogfish erythrocytes.

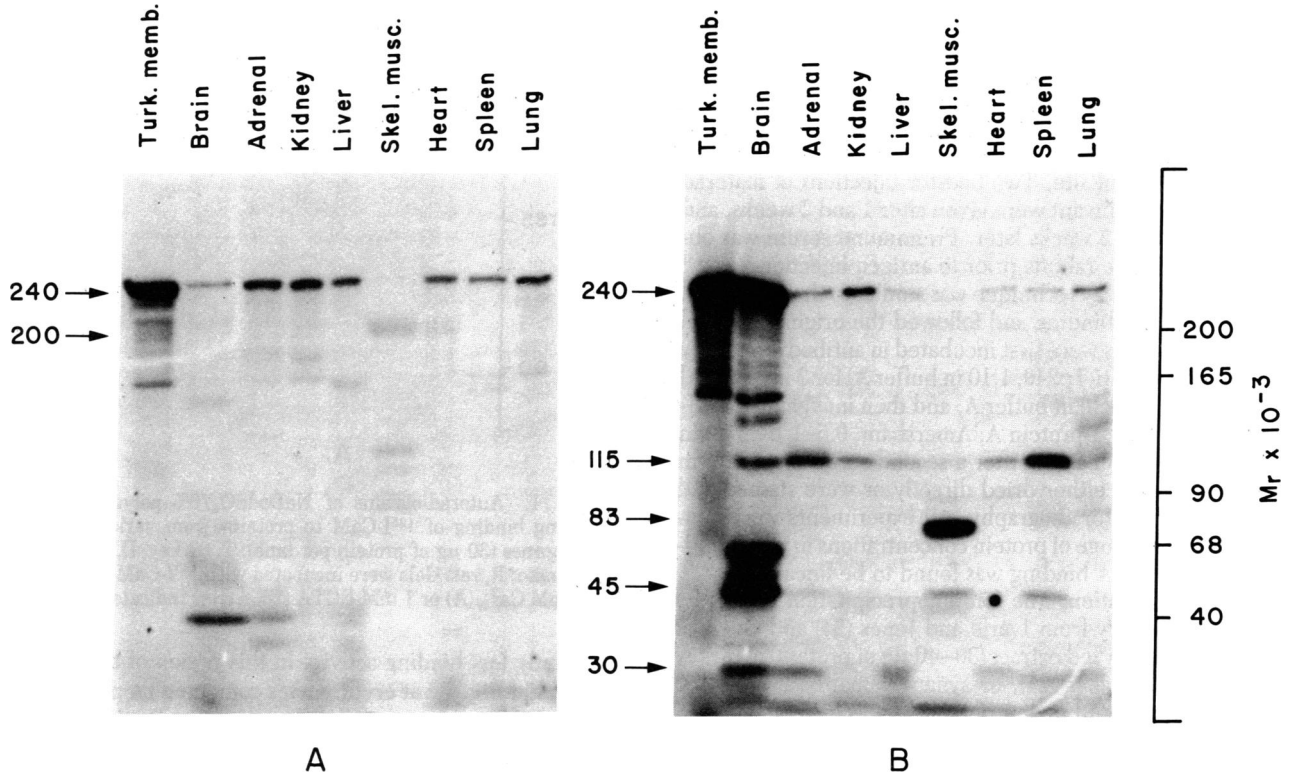

FIG. 2. Autoradiograms of $NaDodSo_4$/5–10% polyacrylamide gels showing binding of ^{125}I-CaM, in the presence of 1 mM Ca^{2+}, to rat tissue particulate (*A*) and cytosol (*B*) proteins. Each sample of rat tissue was loaded at a protein concentration of 100 μg except for brain pellet (30 μg) and supernatant (150 μg). For turkey erythrocyte membranes (Turk. memb.), the load was 30 μg. Proteins indicated by arrows at specific molecular weights (shown $\times 10^{-3}$) are discussed in detail in the text.

Anti-Tp240 Binding to Erythrocyte Membranes and to Various Rat Tissues. In addition to the major CaM-BP in various tissues appearing to have the same molecular weight on $NaDodSO_4$/polyacrylamide gels, peptide fragments, generated by thermolysin, of the protein from different sources showed extensive homology (data not shown). Therefore, it became of interest to evaluate the possibility that this protein would display immunological similarity in all the tissues under study. To this end, an antibody to the prominent p240 of turkey erythrocytes was developed and the abilities of various tissues to recognize anti-Tp240 antiserum were compared.

In erythrocyte membrane samples, anti-Tp240 binding occurred about equally in the p240 region of the three avian species tested but was absent from mammalian erythrocyte ghosts (data not shown). Positive immunological reactions were also obtained in both particulate (Fig. 3) and cytosol (data not shown) fractions from those rat tissues that had been analyzed for ^{125}I-CaM binding. Moreover, the relative amounts of anti-Tp240 bound to both erythrocyte and rat tissue samples corresponded well qualitatively with ^{125}I-CaM binding, supporting the idea that the proteins responsible for the two activities were identical. The anti-Tp240 antibodies also interacted with a series of proteins of $M_r < 240{,}000$ (Fig. 3). These were more prevalent in the particulate fraction and appeared to be similar in the several tissues studied; they may represent proteolytic breakdown products of p240.

Immunoprecipitation of M_r 240,000 CaM-BP. In order to examine the possible identity of the immunoreactive p240 with the CaM-BP, the ability of anti-Tp240 immunoprecipitates to bind ^{125}I-CaM was tested. Low ionic strength extracts of turkey erythrocyte membranes that were found to contain substantial amounts of p240 CaM-BP (see below) were preincubated in 0.1% $NaDodSO_4$ and then were incubated with antibody preparation or preimmune serum and precipitated with SAC. Under these conditions, a p240 was immunoprecipitated from the ex-

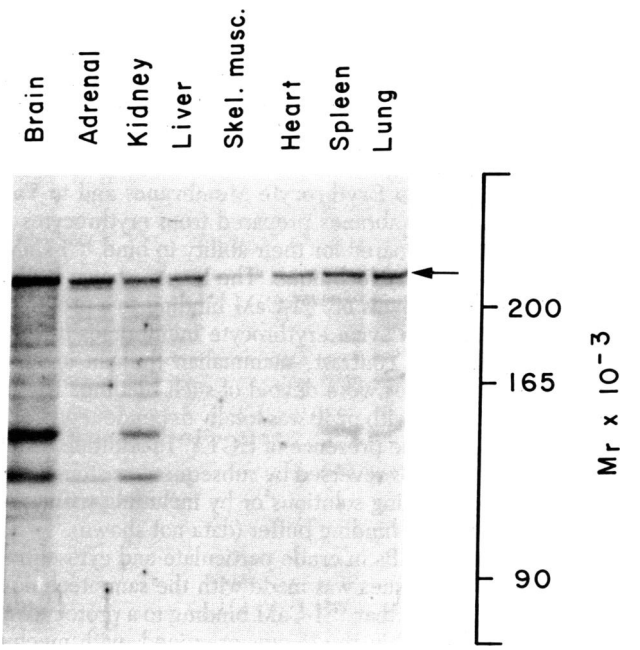

FIG. 3. Autoradiogram of a $NaDodSO_4$/6% polyacrylamide gel showing anti-Tp240–^{125}I-protein A binding to rat tissue particulate proteins. All samples were loaded at 100 μg of protein per lane. The arrow indicates the position of the p240 immunoreactive band of avian erythrocyte membranes (not shown).

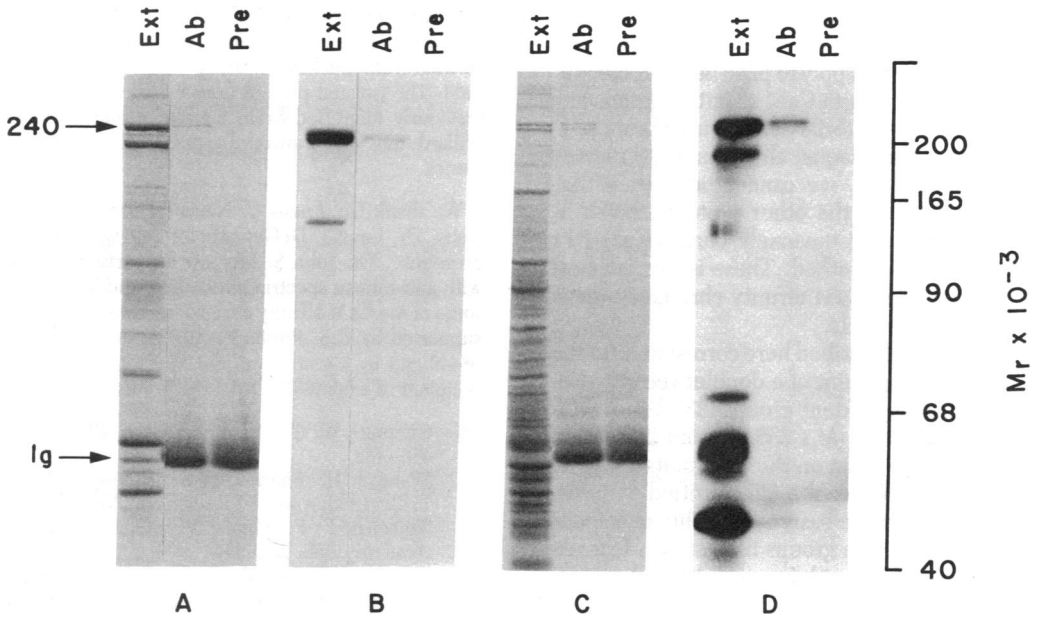

FIG. 4. Immunoprecipitation, by anti-Tp240, of proteins from turkey erythrocyte extracts (*A* and *B*) and synaptosomal lysates (*C* and *D*). Immunoprecipitated proteins were analyzed on $NaDodSO_4$/7.5% polyacrylamide gels for protein (*A* and *C*) or by autoradiography after ^{125}I-CaM overlay (*B* and *D*). Ext, total extract; Ab, anti-Tp240 immunoprecipitate; Pre, preimmune control; Ig, position of immunoglobulin.

tract by anti-Tp240 but not by preimmune serum (Fig. 4*A*). After gel electrophoresis, the immunoprecipitate was found to bind ^{125}I-CaM (Fig. 4*B*), confirming that a protein recognized by anti-Tp240 also bound CaM. Preincubation in 0.1% $NaDodSO_4$ was found to be essential to obtaining maximal specificity in the assay, but this specificity was obtained at the expense of a decrease in the quantity of antigen precipitated by the antibody (cf. ref. 25).

Similar experiments carried out with synaptosomal lysates also led to the immunoprecipitation of a M_r 240,000 CaM-BP (Fig. 4 *C* and *D*). In this case, preincubation in 0.1% $NaDodSO_4$ was also essential to solubilize the sample. Traces of a protein of M_r 235,000 were precipitated along with the p240 CaM-BP (Fig. 4*C*), a phenomenon that could be abolished by preincubation of the lysate in 0.5% $NaDodSO_4$. This is probably indicative of a relatively tight interaction between p240 and the M_r 235,000 protein (see *Discussion*), but the possibility of a direct but weaker immunological interaction between anti-Tp240 and p235 cannot be excluded.

Extraction of ^{125}I-CaM-BP and Anti-Tp240 Binding Activities from Turkey Erythrocyte Membranes. The above results clearly demonstrated that a component of the anti-Tp240 antiserum recognized the p240 CaM-BP. Further evidence that these two activities resided on the same molecule came from experiments in which the extractability of ^{125}I-CaM-BP and anti-Tp240 binding protein from turkey erythrocyte membranes was compared. The three extraction procedures used were chosen for their ability to solubilize certain proteins differentially from the membrane (cf. ref. 21). Good agreement between the amounts of the two M_r 240,000 binding components released from the membrane under these three conditions was obtained (Fig. 5). These results suggest that a single protein is responsible for the majority of both binding activities.

DISCUSSION

CaM is now considered to be a ubiquitous intracellular receptor for Ca^{2+}, mediating several of the second-messenger activities of this ion. The various CaM-BPs thus represent a next level of response in the sequence from Ca^{2+} increase in the cytoplasm to physiological effect. It is evident from the present work that the p240 represents a major binding site for CaM in most tissues. Our results suggest that this protein is homologous, and may be highly conserved, in different tissues and species, based on its ability to be recognized by an antibody directed against the protein from turkey erythrocytes (anti-Tp240). Immunoprecipitation experiments confirmed that the p240 band recognized by the antibody was the same protein that bound CaM (Fig. 4).

The ^{125}I-CaM gel overlay technique used here appears to be a useful method for identifying CaM-BPs in crude tissue extracts. Thus far, only specialized preparations such as postsynaptic densities (16) and intestinal brush borders (13) have been

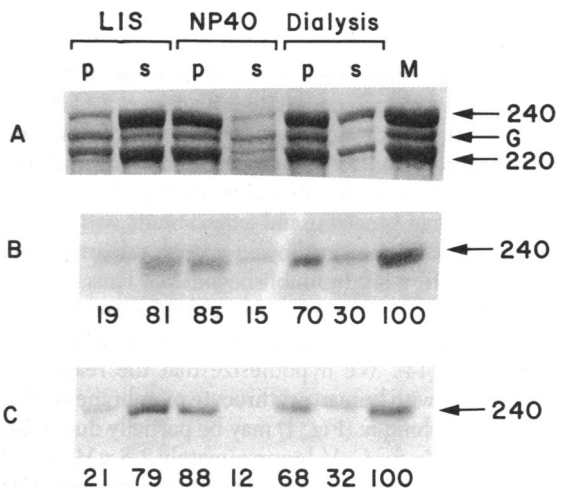

FIG. 5. Extraction, by three procedures, of protein (*A*), ^{125}I-CaM-BP (*B*), and anti-Tp240–^{125}I-protein A-reactive material (*C*) from turkey erythrocyte membranes. s, Supernatant; p, pellet; M, intact membranes. Numbers in *B* and *C* refer to the percentage radioactive material found in each lane. Only the relevant high molecular weight region of this $NaDodSO_4$/6% polyacrylamide gel is shown. LIS, lithium diiodosalicylate. In *A*, G indicates the position of the turkey erythrocyte protein goblin (21).

investigated in detail with this method. It is not yet clear if artifactual binding of CaM to certain proteins could take place in this procedure. This is unlikely to apply to p240 because specific interaction of the native protein with CaM affinity columns has been shown to take place with material derived from brain (ref. 28; see below), *Torpedo* electroplaque, and avian erythrocytes (unpublished results). However, we cannot be certain that binding of ^{125}I-CaM to some of the other proteins shown in Fig. 2 is physiologically relevant. Conversely, certain CaM-BPs might be missed by using this method. These issues can best be addressed by application of CaM affinity chromatographic techniques to native tissue extracts.

It is likely that the protein described here corresponds to the higher molecular weight component of a doublet recently isolated from brain by two independent groups (28, 29). Davies and Klee (28) purified a doublet (M_rs 235,000 and 230,000 in their gel system) from bovine brain on the basis of its ability to bind CaM; Shimo-Oka and Watanabe (29) purified a similar doublet from porcine brain on the basis of its ability to activate actomyosin ATPase activity. Both groups have shown that this protein doublet interacts directly with F-actin, although it is not known whether this activity resides in the p240 or the M_r 235,000 subunit. Interestingly, our immunoprecipitation experiments on synaptic proteins solubilized at low $NaDodSO_4$ concentration did lead to sedimentation of a second protein of M_r 235,000 (Fig. 4) which could only be dissociated at higher $NaDodSO_4$ concentrations. Kakiuchi *et al.* (30) have also purified a p240 from bovine brain by using CaM affinity chromatography in the presence of 6 M urea. Under these conditions, no evidence for the M_r 235,000 species was found. It is clear that p240 is a major constituent of brain [estimates of 2–4% (28) and 3% (30) of total brain protein have been given; see Fig. 4*C* where these proteins are conspicuously stained] and, as indicated here, this may be true for other tissues. The widespread distribution of p240 and the possibility that it interacts with F-actin suggest that it may play a major role in the modulation of cytoskeletal structure and function by intracellular Ca^{2+}.

The M_r 240,000 CaM-BP in turkey erythrocyte membranes comigrated with a gel band that stained heavily with Coomassie blue and that has previously been termed "turkey spectrin" (19, 21) on the basis of a mobility similar to that of human erythrocyte spectrin α on $NaDodSO_4$ gels. However, it is apparent that these two proteins differ markedly in some of their properties. Human spectrin α does not bind either ^{125}I-CaM or anti-Tp240 under the conditions used here. Nevertheless, the apparent association of p240 with stoichiometric amounts of a M_r 220,000 protein in extraction experiments (Fig. 5) resembles the behavior of human spectrin, and experiments with antibodies to purified human spectrin showed a weak crossreaction with avian erythrocyte p240 (unpublished data). Thus, some relationship between the two proteins cannot be excluded. It has been reported that human spectrin does bind CaM under certain conditions (14). We hypothesize that the reason for our negative results with human erythrocyte membranes in the ^{125}I-CaM overlay technique (Fig. 1) may be partially due to the low affinity of spectrin for CaM [approximately 2.8 μM (14)]. It remains to be determined whether the human spectrin–CaM interaction is physiologically significant.

Further work is necessary to define the function and localization of p240 in various tissues and in different physiological states. The availability of an antibody to this protein should aid greatly in this undertaking.

Note Added in Proof. After this paper was communicated, an article by Glenney *et al.* (31) appeared, showing that a M_r 240,000 component purified from chicken intestinal brush borders and brain could bind CaM. The isolated protein from brain corresponded to a subunit of a previously identified brain doublet termed "fodrin" by Levine and Willard (32), who showed the presence of similar proteins in several tissues.

We thank Dr. Angus C. Nairn for providing the CaM used in this study, Dr. Louis J. DeGennaro for help with the immunoprecipitation procedure, Dr. John S. Morrow for participating in the experiments with anti-human spectrin antibodies, and Yvonne Lai for carrying out some of the CaM affinity chromatography experiments. This work was supported by U.S. Public Health Service Grants MH-17387 and NS-08440 and by a grant from the McKnight Foundation. W.S. was the recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

1. Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun.* **29**, 478–482.
2. Wang, J. H., Sharma, R. K., Huang, C. Y., Chau, V. & Chock, P. B. (1980) *Ann. N.Y. Acad. Sci.* **356**, 190–204.
3. Vincenzi, F. F., Hinds, T. R. & Raess, B. U. (1980) *Ann. N.Y. Acad. Sci.* **356**, 232–244.
4. Caroni, P. & Carafoli, E. (1981) *J. Biol. Chem.* **256**, 3263–3270.
5. Pershadsingh, H. A., Landt, M. & McDonald, J. M. (1980) *J. Biol. Chem.* **255**, 8983–8986.
6. Schulman, H. & Greengard, P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5432–5436.
7. Adelstein, R. & Eisenberg, E. (1980) *Annu. Rev. Biochem.* **49**, 921–956.
8. Cohen, P., Picton, C. & Klee, C. B. (1979) *FEBS Lett.* **104**, 24–29.
9. Klee, C. B., Crouch, T. H. & Krinks, M. H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6270–6273.
10. Stewart, A. A., Ingebritsen, T. S., Manalan, A., Klee, C. B. & Cohen, P. (1982) *FEBS Lett.* **137**, 80–84.
11. Marcum, J. M., Dedman, J. R., Brinkley, B. R. & Means, A. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3771–3775.
12. Sobue, K., Fujita, M., Muramoto, K. & Kakiuchi, S. (1981) *FEBS Lett.* **132**, 137–140.
13. Glenney, J. & Weber, K. (1980) *J. Biol. Chem.* **255**, 10551–10554.
14. Sobue, K., Muramoto, Y., Fujita, M. & Kakiuchi, S. (1981) *Biochem. Biophys. Res. Commun.* **100**, 1063–1070.
15. Means, A. R., Tash, J. S. & Chafouleas, J. G. (1982) *Physiol. Rev.* **62**, 1–39.
16. Carlin, R. K., Grab, D. J. & Siekevitz, P. (1981) *J. Cell Biol.* **89**, 449–455.
17. Grand, R. J., Perry, S. V. & Weeks, R. A. (1979) *Biochem. J.* **177**, 521–529.
18. Chafouleas, J. G., Dedman, J. R., Munjaal, R. P. & Means, A. R. (1979) *J. Biol. Chem.* **254**, 10262–10267.
19. Alper, S. L., Palfrey, H. C., DeRiemer, S. & Greengard, P. (1980) *J. Biol. Chem.* **255**, 11029–11039.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
21. Beam, K. G., Alper, S. L., Palade, G. & Greengard, P. (1979) *J. Cell Biol.* **83**, 1–15.
22. Higgins, R. C. & Dahmus, M. E. (1979) *Anal. Biochem.* **93**, 257–260.
23. Burridge, K. (1978) *Methods Enzymol.* **50**, 54–64.
24. Ivarie, R. D. & Jones, P. P. (1979) *Anal. Biochem.* **97**, 24–35.
25. Goelz, S. E., Nestler, E. J., Chehrazi, B. & Greengard, P. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2130–2134.
26. Huttner, W. B. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5402–5406.
27. Bartelt, D. C., Carlin, R. K., Scheele, G. A. & Cohen, W. D. (1981) *J. Cell Biol.* **91**, 339 (abstr.).
28. Davies, P. J. A. & Klee, C. B. (1981) *Biochem. Intl.* **3**, 203–212.
29. Shimo-Oka, T. & Watanabe, Y. (1981) *J. Biochem.* **90**, 1297–1307.
30. Kakiuchi, S., Sobue, K. & Fujita, M. (1981) *FEBS Lett.* **132**, 144–148.
31. Glenney, J. R., Glenney, P., Osborn, M. & Weber, K. (1982) *Cell* **28**, 843–854.
32. Levine, J. & Willard, M. (1981) *J. Cell Biol.* **90**, 631–643.