Detection of Actin-binding Proteins in Human Platelets by ^{125}I-Actin Overlay of Polyacrylamide Gels

MICHAEL C. SNABES, A. E. BOYD III, and JOSEPH BRYAN
Departments of Medicine and Cell Biology, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT Actin-binding proteins have been identified in human platelets with a gel-overlay technique that uses ^{125}I-G-actin. Platelet proteins were separated on SDS polyacrylamide gels using the buffer system of Laemmli (1970, *Nature* [*Lond.*] 227:680–685). The proteins were fixed in the gels with methanol–acetic acid, the SDS was washed out, and the proteins were renatured. The gels were incubated with ^{125}I-G-actin from rabbit skeletal muscle that was radiolabeled with ^{125}I according to the method of Bolton and Hunter (1973, *Biochem. J.* 133:529–538) and has been shown to retain biological activity. After nonspecifically bound radioactivity was washed out, gels were dried and processed for autoradiography. The ^{125}I-G-actin binds to several proteins in human platelets, platelet extracts, and the particulate fraction. Control experiments demonstrate that the ^{125}I-G-actin can be displaced by use of increasing amounts of unlabeled actin, that the binding is stable to 0.6 M NaCl, and that preheating the ^{125}I-G-actin to 90°C for 3 min eliminates all binding. Prominent ^{125}I-G-actin-binding activities were present at M_r 90,000 and 40,000. The binding to the 90,000 M_r protein appears to be at least partially Ca^{++} sensitive, whereas the binding to the 40,000 M_r protein does not. ^{125}I-G-actin bound to proteins in the SDS gels can be fixed *in situ* and compared directly with the stained gel. This technique should prove generally useful in identification and purification of some actin-binding proteins from cells and tissues.

A number of actin-associated proteins have been identified and purified from both muscle and nonmuscle cells. These range from the troponins and tropomyosins, isolated from thin filaments, which are involved in the Ca^{++}-regulated contraction of striated muscle, to the identification, in nonmuscle cells, of proteins that cross-link (7, 17, 20, 25), bundle (4, 10,11), depolymerize (29), and cap (9) the ends of actin filaments. Most of these actin-associated proteins have been studied by use of liquid chemical methods. One approach employed recently to localize calmodulin-binding proteins (6) involves the specific interaction of radioiodinated calmodulin with proteins separated on polyacrylamide gels. This method derives from recent studies with nucleic acids (1, 22) and antibodies (18, 24) and a larger body of work on the renaturation of proteins after solubilization in SDS (3, 12, 13, 16, 28). In this report we apply a gel-overlay method using ^{125}I-G-actin to study the interactions between G-actin and associated proteins in human platelets. The advantage of the approach is that individual actin-binding proteins can be identified by their molecular weight, using microgram amounts of cells or tissues. The potential disadvantages are that actin-binding proteins that have more than one subunit or that are difficult to renature under the conditions used are not detected easily. Human platelets contain several actin-binding proteins that are detected by the overlay method, including major components at M_r 90,000 and 40,000. At least one of these, the 90,000 M_r protein, requires Ca^{++} for maximal binding. Both the 90,000 and 40,000 M_r proteins are present in the high-speed supernate and the particulate fraction. The 90,000 M_r protein is identical to a 90,000 M_r molecule previously identified by Wang and Bryan (26, 27).

MATERIALS AND METHODS

Preparation of Platelet Extracts and Particulate Fractions

Fresh human platelets were centrifuged at 1,000 *g* for 10 min at room temperature to separate the residual erythrocytes and leukocytes. The platelets in the supernate were then separated from the serum by centrifugation at 20,000 *g* for 20 min. The platelets were washed in phosphate-buffered saline at pH 6.5 (5) and lysed by sonication in 5 ml of buffer A containing 10 mM PIPES, 0.34 M sucrose, 0.2 mM ATP, 0.1 mM $MgCl_2$, 0.2 mM dithioerythritol (DTE), 5.0 mM EGTA, 5.0 mM $CaCl_2$, and 10 μg/ml leupeptin. All subsequent procedures were performed at 4°C. The platelet lysate was centrifuged at 250,000 *g* for 60 min. The supernate was removed and the pellet was resuspended in a Dounce homogenizer in modified buffer A containing no calcium and with a final EGTA concentration of 1.0 mM. The particulate fraction was washed three times in this buffer.

Radioiodination of Rabbit Skeletal Muscle Actin

Actin was isolated by the method of Spudich and Watt (23) and purified

further on a Sephacryl S-200 column in 2.0 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 1.0 mM NaN_3. Actin was radioiodinated as described previously (21) according to the method of Bolton and Hunter (2). The final specific activity of actin used in the overlay experiments averaged 10 μCi/μg. Molecular weight markers were labeled with ^{125}I using chloramine-T (8).

Gel Overlay Technique Using ^{125}I-Actin

Slab gel electrophoresis was performed using the buffer system of Laemmli (14). 0.75-mm-thick gels were fixed at room temperature in 40% methanol, 10% acetic acid for 30 min, then washed in 10% ethanol at room temperature for 12 h. The gels were transferred to buffer B containing 0.2 mM $CaCl_2$, 0.2 mM ATP, 1.0 mM $MgCl_2$, 0.05% NaN_3, 0.1% gelatin, 5.0 mM NaH_2PO_4, 5.0 mM Na_2HPO_4, 0.2 M NaCl, pH 7.4. After 90–120 min in buffer B, the gels were sealed into polyethylene bags with ~1 μCi/ml of ^{125}I-actin. After 12–16 h at 4°C, nonspecific radioactivity was removed by washing the gels for 2 d with at least five changes of buffer B (250 ml per wash). The gels were then dried and subjected to autoradiography.

RESULTS

Binding of ^{125}I-G-actin to Platelet Proteins

Fig. 1 shows the Coomassie Blue–staining pattern and corresponding autoradiograph of the ^{125}I-G-actin probe and rabbit skeletal muscle actin. The results demonstrate that the ^{125}I-G-actin migrates as a single band that is coincident with the unlabeled actin standard.

Fig. 2 shows the stained protein pattern from whole human platelets, the soluble platelet fraction, and the washed particulate fraction. Fig. 2 also shows that ^{125}I-G-actin binding activities are present in all three platelet fractions. Two major binding activities are detected at M_r 90,000 and ~40,000. In addition, we can clearly identify eight minor bands at M_r of 75,000, 70,000, 65,000, 58,000, 48,000, 33,000, 28,000, and 20,000. Because the concentration of ^{125}I-G-actin is ~2 nM, the actin-binding affinities of these proteins must be high. The intensities of individual bands, for any given exposure of the autoradiographs, are difficult to relate to the actual quantity of the binding proteins because we have no information on either the relative binding constants or the degree of renaturation of the individual proteins. We have been unsuccessful, for example, in detecting binding of ^{125}I-G-actin to microgram amounts of DNase I after electrophoresis, although we have demonstrated previously, using immunoprecipitation, that the ^{125}I-G-actin will bind the native enzyme (21). On the other hand, on the basis of Coomassie Blue stain, the 90,000 and 40,000 M_r proteins are minor components in the platelet extract, probably present in nanogram amounts on the gel, but are labeled intensely. Several additional points can be made: We detect no binding of the ^{125}I-G-actin to muscle actin after electrophoresis. This point is obscured somewhat in Fig. 2, because the amounts of actin present in lanes *h*, *i*, and *j* are markedly different. This difference in local protein load seriously distorts the shape and actual position of the 40,000 M_r band and gives the particulate protein a slightly different apparent mobility. We have not seen two proteins in the total platelet preparations. Finally, the apparent intensities of the bands are a function of the time of exposure to the dried gel. On short exposures the 90,000 M_r band is the only obvious component; longer exposures bring out the minor components but saturate the film in the 90,000 M_r region. In some of the following experiments we have reduced the exposure times to avoid film saturation at the expense of losing some of the minor components.

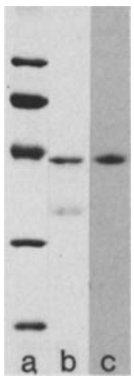

FIGURE 1 10% SDS polyacrylamide gel of ^{125}I-G-actin from rabbit skeletal muscle. Lane *a*, Coomassie Blue–staining pattern of M_r standards: phosphorylase *b*, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; and trypsin inhibitor, 20,100. Lane *b*, Coomassie Blue–staining pattern of 5 μg of rabbit skeletal muscle actin, 1000 cpm of ^{125}I-G-actin, and 4 μg of DNase I. Lane *c*, autoradiograph of lane *b*.

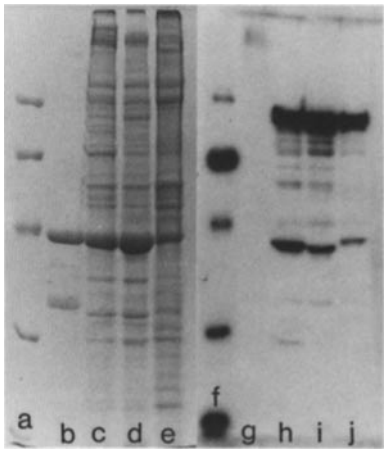

FIGURE 2 Coomassie Blue–staining pattern and autoradiograph of the ^{125}I-G-actin overlay of human platelet fractions. 10% SDS gels were run in the Laemmli buffer system. Lanes *a–e* are stained with Coomassie Blue. Lane *a* contains the chloramine-T-iodinated standards as in Fig. 1 *a*, lane *b*, 5 μg of skeletal muscle actin and 4 μg of DNase I; lane *c*, 27 μg of whole platelet; lane *d*, 28 μg of platelet extract; lane *e*, 27 μg of a washed particulate fraction of platelets. Lanes *f–j* are the autoradiograph from a companion gel run in parallel and used in the overlay procedure.

Specificity of the ^{125}I-G-actin Binding

We have performed three types of experiments to establish the specificity of binding of ^{125}I-G-actin in the overlay technique. First, the ^{125}I-G-actin must be intact because heating to 90°C for 3 min totally eliminates binding of the tracer to the gel (data not shown).

Secondly, the ^{125}I-G-actin can be displaced by unlabeled actin. Fig. 3 shows the effect of preincubating the gel with two different concentrations of unlabeled actin before addition of ^{125}I-G-actin. Preincubation of the gel with a 10-fold excess of unlabeled actin markedly decreases the amount of binding, whereas a 50-fold excess of unlabeled actin almost completely eliminates the binding. Similar results are obtained if the ^{125}I-G-actin is diluted with the unlabeled actin before incubating the mixture with the gel. We conclude that the unlabeled actin and ^{125}I-G-actin compete for binding to the proteins on the gels.

Finally, the binding of the ^{125}I-G-actin is not affected by

high salt. Fig. 4 shows the effect of incubating the ^{125}I-G-actin and the gel with 0.6 M NaCl in buffer B. There is no apparent reduction in the labeling of proteins in the gel; some of the binding components actually appear to be more heavily labeled. We have also performed the overlay with 70 mM NaCl in buffer B and again see no marked difference.

Effect of Calcium on the Binding Activity

Fig. 5 shows the results of incubating the ^{125}I-G-actin with the gel in the presence of Ca^{++} or EGTA. In the presence of EGTA, there is a clear decrease in the binding of ^{125}I-G-actin to the 90,000 M_r protein but no effect on the binding to the 40,000 M_r protein. Densitometry of the x-ray film indicates

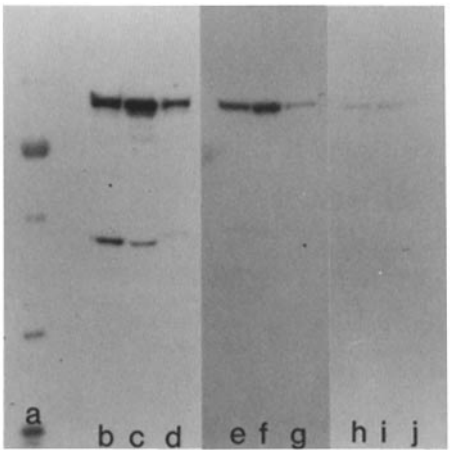

FIGURE 3 Displacement of ^{125}I-G-actin binding by unlabeled actin. Lane *a*, chloramine-T-iodinated M_r standards as in Fig. 1. Lanes *b–d* are the platelet fractions incubated with only ^{125}I-G-actin. Lanes *e–g* were preincubated with 1.0 μg/ml and *h–j* were preincubated with 5.0 μg/ml unlabeled rabbit skeletal muscle actin in buffer B for 8 h before the addition of 1.0 μCi/ml ^{125}I-G-actin. After a 12-h incubation with ^{125}I-G-actin, the gels were processed as described in Materials and Methods. The platelet fractions are identical to those given in Fig. 2, but the exposure time has been reduced to avoid saturation of the x-ray film in the 90,000 M_r region. The exposure time for the three panels is the same.

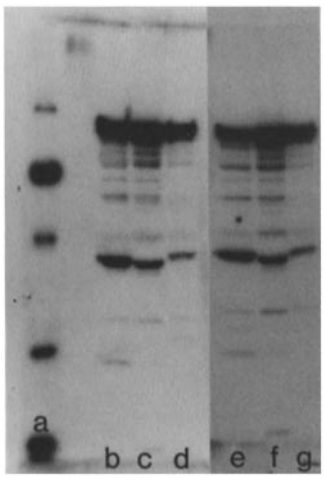

FIGURE 4 Effect of high salt on ^{125}I-G-actin binding to platelet proteins. The order of lanes is the same as described in Fig. 2. Lane *a*, chloramine-T-iodinated M_r standards as in Fig. 1. Lanes *b–d* were incubated in the 0.2 M NaCl buffer; lanes *e–g* were incubated in 0.6 M NaCl.

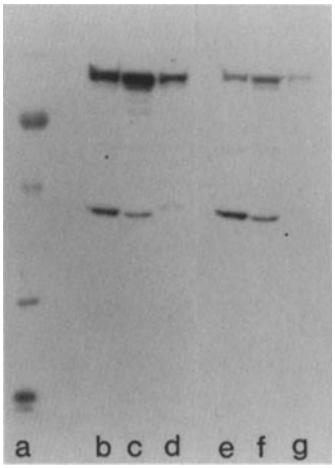

FIGURE 5 The effect of calcium removal on ^{125}I-G-actin binding to platelet proteins. Lane *a*, chloramine-T-iodinated M_r standards as in Fig. 1. Lanes *b–d* are from a gel incubated with 0.2 mM Ca^{++} present throughout the overlay procedure. Lanes *e–g* are from a companion gel that had 1.0 mM EGTA present during the incubation with the ^{125}I-G-actin. Equivalent amounts of platelet extract were run on both sets of gels, and autoradiograms were exposed for equal periods of time. The total exposure time has been reduced to minimize saturation of the film in the 90,000 M_r region.

that the amounts of binding of ^{125}I-G-actin to the 40,000 M_r protein in the presence and in the absence of Ca^{++} are within 10% of each other, but the amount of binding to the 90,000 M_r protein is reduced four to five times in the absence of Ca^{++}. Alternatively, we have performed the binding with Ca^{++} present, then washed with 1.0 mM EGTA in the buffer. The results are essentially the same as those in Fig. 5, indicating that the binding is reversible. Finally, we have recovered the unbound ^{125}I-G-actin after these incubations and tested its ability to bind to DNase I, using a DNase I binding/immunoprecipitation assay (21). The results indicate that the EGTA incubation does not affect the subsequent binding of the ^{125}I-G-actin to DNase I.

Effect of Fixation and Staining after the Overlay

Fig. 6 demonstrates that the bound iodinated actin can be fixed *in situ* by use of the routine Coomassie Blue procedure for SDS slab gels. This particular gel was carried through the overlay procedure, washed for an additional 5 min in distilled water to remove some of the gelatin, then fixed and stained with 0.25% Coomassie Blue in 50% ethanol and 10% acetic acid. After 6–12 h the gel was destained by diffusion in 10% acetic acid before drying for autoradiography. The results eliminate any ambiguity in the possible identification of the 40,000 M_r protein with actin and directly compare the whole platelet preparation with the partially purified 90,000 M_r, Ca^{++}-sensitive, actin-associated protein described by Wang and Bryan (26, 27).

DISCUSSION

These results show that ^{125}I-G-actin can be used to detect specific actin-binding proteins in platelets after separation by SDS gel electrophoresis. This binding can be displaced by unlabeled actin, is stable in high salt, and requires that the ^{125}I-G-actin probe be in a native form. The bound actin can be fixed *in situ* and visualized by autoradiography. The method is

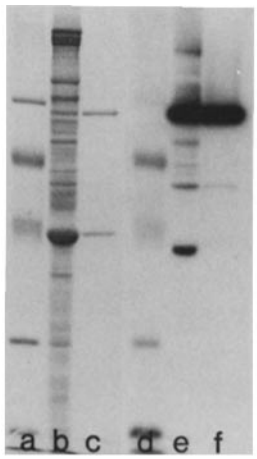

FIGURE 6 A direct comparison of the stained protein pattern with the autoradiograph. The gel was overlaid with iodinated actin, then fixed and stained as described in the text. Lanes *a* and *d* are the chloramine-T-iodinated markers described in Fig. 1. Lanes *b* and *e*: 25 μg of whole platelet proteins. Lanes *c* and *f*: ~8 μg of a fraction from the EGTA eluate of a DNase I-agarose column (26, 27). Lanes *a*–*c* are the stained proteins; lanes *d*–*f* are the autoradiogram.

limited to the identification of monomeric proteins that can bind G-actin and are renaturable under the experimental conditions used. We do not detect binding to either the high molecular weight, actin-binding protein (19) or myosin, both of which are present in platelets. We have not tried to preincubate gels with myosin light chains, for example, to reconstitute active myosin. The concentration at which the ^{125}I-actin is used in the overlay (~100 ng/ml) is below the critical concentration for actin assembly, and we presume that the ^{125}I-G-actin is in the monomeric form. We have been unsuccessful in renaturing DNase I, a protein known to bind to G-actin, which we have used previously to characterize the ^{125}I-G-actin (21). Other workers have reported the renaturation of the enzymatic activity of DNase I on SDS polyacrylamide gels (12).

There are obvious extensions of the ^{125}I-G-actin overlay method that may obviate some of the limitations. These include using actin oligomers, attempting to reconstitute multimeric binding complexes, and improving the renaturation conditions. In addition, our preliminary results (Dingus, Snabes, and Bryan, unpublished observations) indicate that the overlay is directly applicable to two-dimensional gels. We are currently using the overlay method to follow the major platelet-binding proteins during purification and to identify membrane-associated actin-binding proteins in platelets and other cell types.

The major binding activity at M_r 90,000 appears to be Ca^{++} sensitive. Wang and Bryan (26, 27) have previously reported on the partial purification of this protein by use of the Ca^{++} sensitivity and binding to DNase I–actin columns. Fig. 6 demonstrates that the overlay identifies this protein and others. Markey et al. (15) have also reported on the binding of 90,000 and 68,000 M_r platelet proteins to DNase I–actin columns. The 68,000 M_r activity could correspond to the ^{125}I-G-actin binding activities identified in this study at either 65,000 or 70,000 M_r. This is the first identification of a prominent 40,000 M_r actin-binding activity. Although it is possible that the 40,000 M_r actin-binding activity is a proteolytic fragment of the larger 90,000 M_r molecule, there are several reasons to think that this is not the case. We have done the overlay on extracts of platelets prepared with and without leupeptin and with and without EGTA to minimize proteolysis. In these experiments, the 40,000 M_r protein is always observed. In addition, the 40,000 M_r activity is found in samples prepared by pipetting whole platelets directly into SDS. This binding activity migrates slightly ahead of actin and we detect no binding to purified actin alone. We conclude that the 40,000 M_r activity is not a proteolytic fragment of the 90,000 M_r protein and is also not attributable to binding to actin itself.

We thank Dr. Lei-Lei Wang for gifts of platelets and platelet extract.

This work was supported by National Institutes of Health grants GM 26091 to J. Bryan and AM23033 and a grant from the Juvenile Diabetes Foundation to A. E. Boyd.

Received for publication 16 April 1981, and in revised form 1 June 1981.

REFERENCES

1. Alwine, J. C., D. J. Kemp, and G. R. Stark. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci. U. S. A.* 74:5350–5354.
2. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to ^{125}I-containing acylating agents. *Biochem. J.* 133:529–538.
3. Bradhi, S. 1980. Removal of SDS from proteins for immunochemical analysis: a simple method utilizing ultracentrifugation in sucrose-density gradients containing non-ionic detergents. *J. Biochem. Biophys. Methods* 2:79–90.
4. Bryan, J., and R. E. Kane. 1978. Separation and interaction of the major components of sea urchin actin gel. *J. Mol. Biol.* 125:207–224.
5. Carlsson, L., F. Markey, I. Blikstad, T. Persson, and U. Lindberg. 1979. Reorganization of actin in platelets stimulated by thrombin as measured by the DNAse I inhibition assay. *Proc. Natl. Acad. Sci. U. S. A.* 76:6376–6380.
6. Glenney, J. R., Jr., and K. Weber. 1980. Calmodulin-binding proteins of the microfilaments present in isolated brush borders and microvilli of intestinal epithelial cells. *J. Biol. Chem.* 255:10551–10554.
7. Hartwig, J. H., and T. P. Stossel. 1975. Isolation and properties of actin, myosin, and a new actin-binding protein of rabbit alveolar macrophages. *J. Biol. Chem.* 250:5696–5705.
8. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.).* 194:495–496.
9. Isenberg, G., U. Aebi, and T. P. Pollard. 1980. An actin-binding protein from *Acanthamoeba* regulates actin filament polymerization and interactions. *Nature (Lond.)* 288:455–459.
10. Kane, R. E. 1975. Preparation and purification of polymerized actin from sea urchin eggs. *J. Cell Biol.* 66:305–316.
11. Kane, R. E. 1976. Actin polymerization and interaction with other proteins in temperature-induced gelation of sea urchin egg extracts. *J. Cell Biol.* 71:704–714.
12. Lacks, S. A., and S. S. Springhorn. 1980. Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. *J. Biol. Chem.* 255:7467–7473.
13. Lacks, S. A., S. S. Springhorn, and A. L. Rosenthal. 1979. Effect of the composition of sodium dodecyl sulfate preparations on the renaturation of enzymes after polyacrylamide gel electrophoresis. *Anal. Biochem.* 100:357–363.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.)* 227:680–685.
15. Markey, F., T. Persson, and U. Lindberg. 1981. Characterization of platelet extracts before and after stimulation with respect to the possible role of profilactin as microfilament precursor. *Cell.* 23:145–153.
16. Marrow, R. E., and R. P. Dottin. 1980. Renaturation and localization of enzymes in polyacrylamide gels: studies with UDP glucose pyrophosphorylase of *Dictyostelium. Proc. Natl. Acad. Sci. U. S. A.* 77:730–734.
17. Maruta, H., and E. D. Korn. 1977. Purification from *Acanthamoeba castellanii* of proteins that induce gelation and syneresis of F-actin. *J. Biol. Chem.* 252:399–402.
18. Renart, J., J. Reiser, and G. R. Stark. 1979. Transfer of proteins from gels to diazobenzyloxymethyl paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc. Natl. Acad. Sci. U. S. A.* 76:3116–3120.
19. Schollmeyer, J.V., G. H. R. Rao, and J. G. White. 1978. An actin-binding protein in human platelets. *Am. J. Pathol.* 93:433–445.
20. Shizuta, Y., H. Shizuta, M. Gallo, P. Davies, I. Pastan, and M. Lewis. 1976. Purification and properties of filamin, an actin-binding protein from chicken gizzard. *J. Biol. Chem.* 251:6562–6567.
21. Snabes, M. C., A. E. Boyd III, R. L. Pardue, and J. Bryan. 1981. A DNase I binding/immunoprecipitation assay for actin. *J. Biol. Chem.* 256:6291–6295.
22. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517.
23. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle conpraction. I. Biochemical studies of the interaction of the troponin-tropomyosin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
24. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76:4350–4354.
25. Wang, K., J. F. Ash, and S. J. Singer. 1975. Filamin, a new high-molecular weight protein found in smooth muscle and non-muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* 72:4483–4486.
26. Wang, L. L., and J. Bryan. 1980. Calcium-mediated regulation of actin assembly in human platelets. *Eur. J. Cell Biol.* 22:C927A (Abstr.).
27. Wang, L. L., and J. Bryan. 1980. Calcium-mediated regulation of actin assembly in human platelet extracts. *J. Cell Biol.* 87(2, Pt. 2):1647*a* (Abstr.).
28. Weber, K., and D. J. Kuter. 1971. Reversible denaturation of enzymes by sodium dodecyl sulfate. *J. Biol. Chem.* 246:4504–4509.
29. Yin, H. L., K. S. Zaner, and T. P. Stossel. 1980. Ca^{++} control of actin gelation: interaction of gelsolin with actin filaments and regulation of actin gelation. *J. Biol. Chem.* 255:9494–9500.