Detection of Actin-binding Proteins in Human Platelets by
125I-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 125I-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 125I-G-actin from rabbit skeletal muscle that was radiolabeled with 125I 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 125I-G-actin binds to several proteins in human platelets, platelet extracts, and the particulate fraction. Control experiments demonstrate that the 125I-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 125I-G-actin to 90°C for 3 min eliminates all binding. Prominent 125I-G-actin-binding activities were present at Mr 90,000 and 40,000. The binding to the 90,000 Mr protein appears to be at least partially Ca" sensitive, whereas the binding to the 40,000 Mr protein does not. 125I-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 radiiodinated 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 125I-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 Mr 90,000 and 40,000. At least one of these, the 90,000 Mr protein, requires Ca" for maximal binding. Both the 90,000 and 40,000 Mr proteins are present in the high-speed supernate and the particulate fraction. The 90,000 Mr protein is identical to a 90,000 Mr 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 pHe 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 MgCl2, 0.2 mM dithioerythritol (DTE), 5.0 mM EGTA, 5.0 mM CaCl2, and 10 μg/ml leupeptin. All subsequent procedures were performed at 4°C. The platelet lysates were 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 CaCl₂, and 1.0 mM NaCl. Actin was radiiodinated 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 ¹²⁵I using chloramine-T (8).

**Gel Overlay Technique Using ¹²⁵I-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 CaCl₂, 0.2 mM ATP, 1.0 mM MgCl₂, 0.05% NaN₃, 0.1% gelatin, 5.0 mM NaH₂PO₄, 5.0 mM Na₂HPO₄, 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 ¹²⁵I-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 ¹²⁵I-G-actin to Platelet Proteins**

Fig. 1 shows the Coomassie Blue-staining pattern and corresponding autoradiograph of the ¹²⁵I-G-actin probe and rabbit skeletal muscle actin. The results demonstrate that the ¹²⁵I-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 ¹²⁵I-G-actin binding activities are present in all three platelet fractions. Two major binding activities are detected at Mr 90,000 and ~40,000. In addition, we can clearly identify eight minor bands at Mr of 75,000, 70,000, 65,000, 58,000, 48,000, 33,000, 28,000, and 20,000. Because the concentration of ¹²⁵I-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 ¹²⁵I-G-actin to microgram amounts of DNase I after electrophoresis, although we have demonstrated previously, using immunoprecipitation, that the ¹²⁵I-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 Mr, 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 ¹²⁵I-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 Mr 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 Mr band is the only obvious component; longer exposures bring out the minor components but saturate the film in the 40,000 Mr 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.

**Specificity of the ¹²⁵I-G-actin Binding**

We have performed three types of experiments to establish the specificity of binding of ¹²⁵I-G-actin in the overlay technique. First, the ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-G-actin is diluted with the unlabeled actin before incubating the mixture with the gel. We conclude that the unlabeled actin and ¹²⁵I-G-actin compete for binding to the proteins on the gels.

Finally, the binding of the ¹²⁵I-G-actin is not affected by...
high salt. Fig. 4 shows the effect of incubating the $^{125}\text{I}-\text{G}$-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 $^{125}\text{I}-\text{G}$-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 $^{125}\text{I}-\text{G}$-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 that the amounts of binding of $^{125}\text{I}-\text{G}$-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 $^{125}\text{I}-\text{G}$-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 $^{125}\text{I}-\text{G}$-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 $^{125}\text{I}-\text{G}$-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 $^{125}\text{I}-\text{G}$-actin probe be in a native form. The bound actin can be fixed in situ and visualized by autoradiography. The method is
limited to the identification of monomeric proteins that can bind G-actin and are reenable 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 previously used 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 renaturing 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 $M_r$ protein to 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. 74:3530-3534.

2. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to $^{125}$I-containing colchicine. Biochem. J. 133:259-258.

3. Bradish, S. 1980. Removal of SDS from proteins for immunnochemical analysis: a simple method utilizing ultracentrifugation in sucrose-density gradients containing non-ionic detergent. 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. Carless, P., F. Markey, I. Bilkjad, 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. Gitterman, J., R. Ju, 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 aortic microvessels. J. Biol. Chem. 250:5069-5070.

8. Hunter, W. M., and P. C. Greenwood. 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature (Lond.) 194:495-496.

9. Ibenberg, G., U. Arbi, and T. P. Pollard. 1980. An actin-binding protein from Acanthamoeba regulates actin filament polymerization and interactions. Nature (Lond.) 288:435-459.

10. Kane, R. E. 1975. Preparation and purification of polymerized actin from sea urchin eggs. Cell Biol. 86:305-316.

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. 255:7407-7413.

13. Lacks, S. A., S. S. Springhorn, and A. L. Rosenthal. 1979. Effects of the composition of sodium dodecyl sulfate preparations on the renaturation of enzymes after polyacrylamide gel electrophoresis. Anal. Biochem. 100:357-363.

14. Lassizumi, 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. Marron, R. E., and R. P. Dettin. 1980. Renaturation and localization of enzymes in polyacrylamide gels: studies with UDP-glucose pyrophosphorylase of Bathycromulium. 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 synthesize F-actin. J. Biol. Chem. 252:399-402.

18. Renator, J., J. Reiser, and G. R. Stark. 1979. Transfer of proteins from gels to diazo-benzylpyrroloxyethyl paper and detection with anti sera: a method for studying antibody specificity and antigen structure. Proc. Natl. Acad. Sci. U. S. A. 76:3116-3120.

19. Scholmeijer, 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. Shibata, Y., H. Shibata, M. Gallo, P. Davies, I. Paxton, 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. Soumier, E. M. 1957. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 15:503-517.

23. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-tropomyosin complex with actin and the protolytic fragments of myosin. J. Biol. Chem. 246:4866-4871.

24. Tsuchimori, H., T. Stehelin, 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. 72:4430-4434.

26. Wang, K., and J. Bryan. 1980. Calcium-mediated regulation of actin assembly in human platelets. Eur. J. Cell Biol. 22:899-902.

27. Wang, L. L., and J. Bryan. 1980. Calcium-mediated regulation of actin assembly in human platelet extracts. J. Cell Biol. 87:25.2164f (Abst.).

28. Weber, K., and D. J. Kane. 1981. Reversible denaturation of enzymes by sodium dodecyl sulfate. J. Biol. Chem. 256:4504-4509.

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. 255:9404-9500.