Monoclonal Antibodies Prepared against *Dictyostelium* Actin: Characterization and Interactions with Actin

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ABSTRACT Three mouse monoclonal antibodies, Act I, Act II, and Act IV, against actin from the cellular slime mold *Dictyostelium discoideum*, have been made and characterized. All three antibodies are IgG1 and share the following properties: They form stable complexes with monomeric *Dictyostelium* actin, which prevents polymerization of the actin into filaments. On addition to preformed actin filaments, they cause a reduction in filament size and in the viscosity of the actin solution. They cross-react strongly with actins from the lower eucaryotes *Physarum* and *Acanthamoeba*, but not with α-actins from rabbit and human muscle or β- and γ-actins from human erythrocytes and a human B lymphoid cell line. Act II and Act IV recognize a similar antigenic determinant that is topographically distinct from that identified by Act I. In protein immunoblotting, only Act I bound strongly to *Dictyostelium* actin. Analysis of actin fragments with this technique showed that amino acids 13 to about 50 are required for Act I binding to actin. A comparison of the amino acid sequences of actins from lower eucaryotes and higher vertebrates implicates threonine 41 as a critical residue in the Act I antigenic site. The properties of Act II and Act IV suggest that they recognize antigenic sites involving the NH_2-terminal six residues.

Antibodies to actin have been difficult to obtain, and this can be attributed to the high conservation of primary sequence throughout evolution (see reference 7). To date, most antiactin sera have been produced by animals immunized with denatured actins: by SDS treatment (19), by precipitation with alum (14), by chemical cross-linking and modification with glutaraldehyde (12), or by incubation of G-actin at 4°C for at least a week (36). In general, the sera obtained have been of low titer and contain low concentrations of specific antibody. Despite these limitations, polyclonal sera have been of use as reagents for localizing actin-containing structures in cells using immunofluorescence techniques (19). The broad cross-reactivities of these sera with actins from diverse sources have allowed these studies to be carried out with various cell types and organisms (e.g., 10).

With specific monoclonal antibodies one may obtain large amounts of homogeneous antibody directed against single antigenic determinants (16). It is sometimes possible to use a weak immune response and obtain high-affinity antibodies against antigens such as actin that have traditionally proved to be poorly immunogenic. In addition, hybridoma clones producing antibodies that recognize different epitopes on the actin molecule can be selected and used to study the interaction of actin with the many proteins that appear to regulate its functional organization in cells (17, 41).

In this report, we describe the preparation and properties of three mouse monoclonal antibodies, Act I, Act II, and Act IV, against actin from the cellular slime mold, *Dictyostelium discoideum*. A preliminary report of portions of this work has been presented (30).

MATERIALS AND METHODS

Actin

Dictyostelium actin (*D.* actin)[1] was prepared by the method of Uyemura et al. (37). A similar, though scaled-down, procedure was used to prepare [^{35}S]actin from *Dictyostelium* (29).

Rabbit skeletal muscle actin was purified from acetone powders using the method of Spudich and Watt (31) with further purification by chromatography on diethylaminoethyl (DEAE) cellulose and size exclusion columns as described by Pardee and Spudich (24). Human muscle actin was isolated in the same way from an acetone powder prepared from thigh muscle tissue (provided by Dr. R. J. Rouse, Stanford University Medical Center).

Human nonmuscle actin used in these studies was an actin-rich extract of a human B lymphoblastoid cell line, prepared using the initial steps of the

[1] *Abbreviations used in this paper:* *D.* actin, *Dictyostelium* actin; ^{3}H-IAA, [^{3}H]iodoacetic acid; RIA, radioimmune assay.

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procedure of Uyemura et al. (37). Washed cells were lysed by sonication, and a high-speed supernatant fraction was prepared by centrifugation at 150,000 g for 90 min at 4°C. This was chromatographed at 4°C over a Sephadex G-150 superfine column (Pharmacia Fine Chemicals, Piscataway, NJ). Fractions containing actin as the major band revealed by SDS PAGE were used in the antibody-binding studies. The actin was >50% of the total protein in the fraction.

Actins from *Acanthamoeba castellanii*, *Physarum polycephalum*, and human erythrocytes were the generous gifts of Dr. T. D. Pollard (The Johns Hopkins University), Dr. V. T. Nachmias (University of Pennsylvania), and Dr. S. L. Schrier (Stanford University Medical Center), respectively.

D. actin was radiolabeled with [^{3}H]iodoacetic acid (^{3}H-IAA) as follows. F-actin in F buffer (3 mM triethanolamine–HCl, pH 6.5; 0.2 mM dithiothreitol; 0.5 mM ATP; 0.005% NaN_3; 100 mM KCl; 1 mM $MgCl_2$) was incubated with a 20-fold molar excess of ^{3}H-IAA (203 mCi/mmol; New England Nuclear, Boston, MA) for 9 h at 37°C, then 14 h on ice. After a 24-h dialysis at 4°C versus two changes of F buffer, the labeled actin was sedimented twice at 30 psi, 20 min in an Airfuge (Beckman Instruments, Inc., Palo Alto, CA) and finally resuspended in F buffer at 2 mg/ml. The efficiency of labeling approached 1 mol of iodoacetate per mol of actin.

Monoclonal Antibody Production

The strategy and basic methods have been described (25). *D.* actin at 5 mg/ml in F buffer was diluted to 1 mg/ml with PBS and emulsified with an equal volume of Freund's complete adjuvant. Female BALB/c mice, 4–6 wk of age, were immunized subcutaneously with 100 μl of this emulsion. At 30 and 60 d the mice were reimmunized with 50 μl of antigen similarly prepared using Freund's incomplete adjuvant. 1 wk after the third immunization, mice were bled from the tail and the sera obtained were tested for antiactin antibodies in an indirect solid-phase radioimmune assay (RIA). The mice were rested for 4 mo and then immunized intravenously on four consecutive days with 25–100 μl of *D.* actin diluted in PBS as described above. On occasion the intravenous injection failed and the antigen was then administered intraperitoneally. A similar immunization schedule was shown by Stähli et al. (32) to increase the yield of specific hybridomas against soluble antigens. On the fifth day the spleen cells from nine immunized animals were fused with cells of the P3/NSI/1-Ag4-1 myeloma line (NSI) (16) as described (25). Hybridomas were cloned using the fluorescence-activated cell sorter (Becton Dickinson Immunocytometry Systems, Mountain View, CA) as modified by Parks et al. (27).

IgG was obtained from ascitic fluids and purified by ammonium sulfate precipitation and chromatography on Sephadex G-200 and DEAE–cellulose columns. Purified immunoglobulins were stored frozen in PBS containing 0.1% sodium azide as a preservative. $F(ab')_2$ and F(ab) fragments were prepared by papain degradation (26).

C4 antibody was a gift from Dr. James L. Lessard (Children's Hospital Research Foundation, Cincinnati, OH). This mouse monoclonal IgG1 was made against actin isolated from chicken gizzard smooth muscle tissue (20).

Antigen–Antibody Binding Assays

SOLID-PHASE RIA: F-actin from *Dictyostelium* was diluted to 100–250 μg/ml in PBS N_3 (PBS buffer with 0.01% NaN_3) containing 1 mM ATP, and added to the wells of 96-well polyvinylchloride U-bottom microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) at 25 μl per well. Wells to be used as negative controls contained PBS N_3 with 1% (wt/vol) BSA at 25 μl per well. After an overnight incubation at 4°C in a moist environment, antigen was removed from the wells, saved, and stored on ice. F-actin solutions were repeatedly used in the RIA for periods of up to a month. Wells were washed twice with PBS N_3 containing 0.5% BSA. The plates were incubated with the second wash for 15 min at room temperature in order to saturate all protein-binding sites. Appropriate dilutions of monoclonal antibodies were added to the wells and incubated for 2–4 h at room temperature, after which the wells were washed three times with PBS N_3 containing 0.5% BSA. 300,000 cpm (~0.03 μg) of ^{125}I-$F(ab')_2$ rabbit anti-mouse IgG in 25 μl of PBS with 0.5% BSA were added to each well and incubated for 1–2 h at room temperature. After four washes with PBS N_3 containing 0.5% BSA, the wells were cut from the plates using a hot wire, and assayed for radioactivity in a Beckman Gamma 4000 (Beckman Instruments Inc., Mountain View, CA).

FORMATION AND ANALYSIS OF IMMUNE COMPLEXES WITH MONOMERIC ACTIN: ^{35}S-labeled F-actin was centrifuged for 20 min at 30 psi in an Airfuge. The pellet was resuspended in G buffer (3 mM triethanolamine–HCl, pH 7.5; 1 mM ATP; 0.2 mM dithiothreitol; 0.005% NaN_3) containing 0.1% (wt/vol) BSA by sonication for 10 s on ice with a Kontes Micro-ultrasonic Cell Disruptor (Kontes Glass Co., Vineland, NJ) fitted with a 4.5-in probe at a power setting of 8 (9.9 W, 25 kHz). After depolymerization of the actin on ice for 120 min, the solution was clarified by centrifugation for 60 min at 30 psi in an Airfuge. The concentration of actin monomers in the supernatant was determined by assaying an aliquot for radioactivity and spectrophotometrically using the formula of Gordon et al. (11): Concentration in mg/ml = (A 290 nm − A 320 nm)/(0.62).

To remove aggregates, we centrifuged solutions of IgG for 30 min at 30 psi in an Airfuge. IgG concentrations were determined spectrophotometrically using the formula: Concentration in mg/ml = A 280 nm × 0.627.

[^{35}S]G-actin (25 μg) was incubated overnight on ice with a 10-fold molar excess of IgG (893 μg). Control incubations contained the nonspecific IgG, X63 (16) or no added antibody. 50 μl of dextran blue 2000 (Pharmacia Fine Chemicals) at 1 mg/ml in G buffer was then added and the samples were individually applied to a 1.5 × 75-cm column of Sephadex G-150 superfine resin (Pharmacia Fine Chemicals) in G buffer. Fractions (0.7 ml) were collected and assayed for [^{35}S]actin by liquid scintillation counting and for absorbance at 650 nm, to locate the dextran blue which was used as a marker for the void volume of the column.

Viscometry

Actin assembly and disassembly were measured by high-shear viscometry performed at 25°C in a Cannon-Manning Semi-micro viscometer, size 100, with a buffer flow time of 65 s (Cannon Instrument Co., State College, PA). The specific viscosity (ηsp) of the solution was determined from the formula: ηsp = (flow time sample − flow time buffer)/(flow time buffer).

Proteolytic Cleavages of Actin

Protease from *Staphylococcus aureus* V8 (Miles Laboratories Inc., Elkhart, IN), papain (Sigma Chemical Co., St. Louis, MO), and trypsin-*N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Worthington Biochemical Corp., Freehold, NJ) were used to generate fragments from ^{3}H-labeled *D.* actin monomer in G buffer. Cleavage with V8 protease was done in the presence of 0.1% SDS for 60 min at 25°C using enzyme at 1 or 2% (wt/wt) with respect to actin which was at 500 μg/ml. Papain cleavage was for 15 or 30 min at 25°C using enzyme at 1% (wt/wt) with respect to actin. Trypsin-TPCK cleavage was for 60 min at 25°C using enzyme at 10% (wt/wt) with respect to actin. Reactions were stopped by boiling with SDS PAGE sample buffer (18).

Hydroxylamine Cleavage of Actin

Treatment of polypeptides with hydroxylamine under denaturing conditions specifically cleaves peptide bonds between asparagine and glycine residues (3). *D.* actin contains a single such bond between residues 12 and 13 (40). Actin monomer labeled with ^{3}H at Cys-373 at 500 μg/ml was incubated in 200 mM K_2CO_3; 2 M guanidine–HCl; 1 M hydroxylamine; 1 mM ATP at pH 9.0 for 4 h at 45°C. After incubation the solution was neutralized with concentrated HCl and dialyzed overnight at 4°C versus PBS N_3 containing 1 mM ATP.

SDS PAGE, Immunoblotting, and Autoradiography

The system of Laemmli (18) was used as described by Ames (1). Molecular weight markers (Pharmacia Fine Chemicals, Piscataway, NJ) included phosphorylase *b* (94 kilodalton [kD]), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

Immunoblotting was performed as described by Towbin et al. (35), except that all solutions in which the nitrocellulose was incubated contained 0.1% (vol/vol) Triton X-100 and 0.02% (wt/vol) SDS (8). Samples were electrophoresed and the gel was cut into replicate slices. One was stained for protein with Coomassie Brilliant Blue. The other slices were washed with Laemmli (18) electrode buffer without SDS. Gel slices were then lined up on nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH) and proteins were electrophoresed onto the paper using a Trans-Blot Cell (Bio-Rad Laboratories, Richmond, CA). One replicate blot was stained for protein with amido black (0.1% in 45% methanol, 10% acetic acid) and then destained in 90% methanol, 2% acetic acid (28). The other blots were sequentially incubated with solutions containing BSA (1 h at 37°C) and then either antiactin antibody or control immunoglobulin (X63) (7.5 μg/ml IgG for 14–16 h at room temperature with gentle rotation). After extensive washing with buffer and buffer containing 0.5 M NaCl, the papers were incubated for 2 h at room temperature in a solution containing 1.2×10^6 dpm/ml of ^{125}I-$F(ab')_2$ rabbit anti-mouse IgG (~0.08 μg). After five to six additional washes with buffer the nitrocellulose paper blots were air dried, then autoradiographed on Kodak X-OMAT R film (Eastman Kodak Co.,

Rochester, NY) at −70°C using a Cronex Intensifying Screen (Dupont Co., Wilmington, DE).

Coomassie Blue-stained gels containing ^{3}H-labeled proteins were prepared for autoradiographic analysis by treatment with 2,5-diphenyloxazole in DMSO according to the procedure of Bonner and Laskey (4). Treated gels were dried onto filter paper and autoradiographed as above.

Electron Microscopy

Samples placed on carbon-coated grids were negatively stained with 1% aqueous uranyl acetate (13) and were observed and photographed using a Philips EM 201 (Philips Electronic Instruments, Inc., Mahwah, NJ) at 80 kV under conventional operating conditions.

RESULTS

Monoclonal Antibodies against D. Actin[2]

The sera from mice immunized with *D.* actin showed specific binding to *D.* actin in a solid-phase RIA. When compared with sera from unimmunized mice, a specific titer of about 1 in 1,000 was detected. Specific binding to rabbit skeletal muscle actin was detected but was very weak compared to *D.* actin. Spleen cells from nine immunized mice were individually fused with NS1 myeloma cells and plated out into a total of 32 24-well plates without feeder cells. In eight plates no growth of hybrid cells was seen. Vigorous growth was seen in about one-third of the wells of the remaining plates and 140 culture supernatants were tested for antibodies against actin. Of these 29 gave binding that was greater than twice the background value. On subculture, four of the 29 retained activity, and three were subsequently cloned to give hybridomas Act I, Act II, and Act IV. All three monoclonal antibodies were IgG1 as shown by RIA using radioiodinated goat anti-mouse isotype specific IgG as the second-step reagents. As actin is known to have significant affinity for a wide range of proteins including immunoglobulin, it was important to show that the binding of the monoclonal antibodies was not a low-affinity "nonspecific" interaction. This interaction has been characterized by Fechheimer et al. (9) and probably involves the Ig constant region. The following experiments showed this was unlikely as (*a*) all three antibodies bound to *D.* actin but only very weakly to rabbit skeletal muscle actin, (*b*) control IgG1 with specificity for human leukocyte antigen molecules gave no binding to actin in the RIA, (*c*) soluble *D.* actin but not rabbit actin could completely block antibody binding to solid-phase *D.* actin in the RIA, (*d*) no inhibition of monoclonal anti-human leukocyte antigen-binding to solid-phase human leukocyte antigens by *D.* actin was detected, and (*e*) the Act antibodies, but not control IgG1, in conjunction with rabbit anti-mouse IgG and *S. aureus* bacteria quantitatively precipitated *D.* actin from solution. At this point, each hybridoma was used to generate an ascitic fluid from which the immunoglobulin was purified.

Act I Identifies a Different Epitope of D. Actin from Act II and Act IV

The topographical relationship of the antigenic determinants (epitopes) of actin bound by Act I, Act II, and Act IV was investigated by determining if two different monoclonal

[2] The hybridoma cells are available from the American Type Culture Collection Hybridoma Bank, 12301 Parklawn Drive, Rockville, MD 20852.

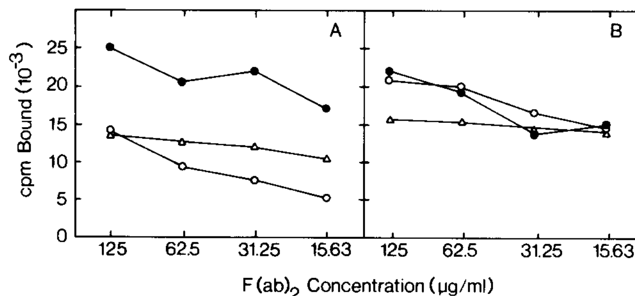


FIGURE 1 Two topographically distinct epitopes are recognized by the three monoclonal antibodies. The binding to *D.* actin of mixtures of antibodies in a solid-phase RIA under conditions of antibody saturation (25 μg/ml). In *A* the binding of Act I (○) and Act IV (△) alone are compared with Act I + Act IV (●). *B* shows the binding of Act II (○), Act IV (△), Act II + Act IV (●). $F(ab)_2$ fragments were used to reduce the size of the antibody probes and eliminate nonspecific binding of actin to the Fc region. ^{125}I-$F(ab')_2$ rabbit anti-mouse IgG was added at 250,000 dpm/well (0.6 μg).

antibodies could simultaneously bind to the same actin molecule. To reduce steric hindrance and nonspecific interaction between actin and the Fc region, $F(ab)_2$ fragments were used. Fig. 1*A* shows clearly that Act I and Act II antibodies can bind to *D.* actin at the same time. In contrast, Act II and Act IV do not show this additive effect (Fig. 1*B*). These results suggest that Act II and Act IV recognize a closely related site on the actin molecule that is topographically separate from that recognized by Act I. In similar experiments, Act I and Act IV also showed the additive binding behavior. The results of competition assays, in which dilutions of nonradioactive Act antibodies were tested for their capacity to inhibit the binding of radioactive Act antibodies to *D.* actin, led to the same conclusions.

Act I, Act II, and Act IV Bind to Monomeric Actin

Analytical size exclusion column chromatography was used to show that Act I, Act II, and Act IV bound actin monomers in solution with high affinity. These results also further substantiated the combining site specificity of the antibodies. Fig. 2 presents elution profiles of ^{35}S-labeled actin monomer preincubated with Act I antibody or with the X63 antibody as a negative control. Actin monomer alone (Fig. 2*A*) or actin monomer preincubated with X63 (Fig. 2*B*) eluted as a single broad peak within the included volume of the column. In contrast, when actin monomer was preincubated with the Act I monoclonal antibody, its elution position shifted markedly to a narrow peak between tubes 30 and 40, close to the elution position of the dextran blue (Fig. 2*C*). Similar results were obtained with Act II and Act IV. Therefore, in the presence of Act I, Act II or Act IV monomeric actin chromatographs as if it were in a high molecular weight complex, i.e., bound to the antibody. The sharpness of the complex peak and the lack of a peak due to free actin monomer show that the interaction between actin monomer and each monoclonal antibody is of high functional affinity ($\geq 10^{9}$ M^{-1}). From these experiments one cannot determine if the stability of the immune complex is purely a result of the affinity between one antibody combining site and one actin molecule, or if closed trimolecular complexes between one IgG and two actin molecules are formed in which actin–actin interactions contribute.

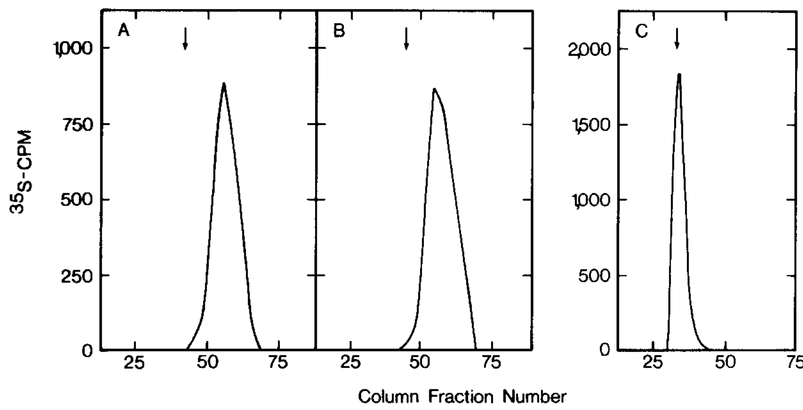


FIGURE 2 Act I antibody and *D.* actin monomers form stable complexes as detected by analytical size exclusion column chromatography. [35S]*D.* actin monomer was analyzed on a Sephadex G150 superfine column under three conditions: (*A*) Actin alone; (*B*) actin preincubated with a nonspecific IgG1 (X63); (*C*) actin preincubated with Act I IgG1. The arrow in each panel marks the excluded volume of the column determined by simultaneous chromatography of dextran blue.

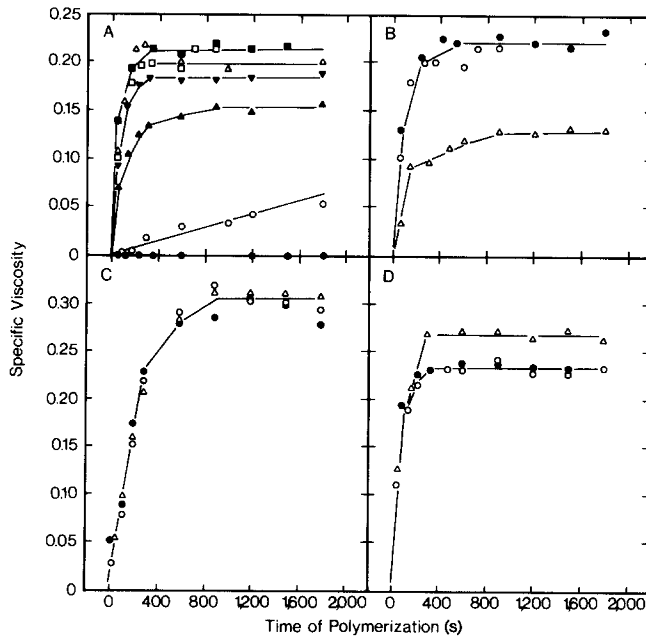


FIGURE 3 Effects of antiactin monoclonal antibodies on the polymerization of actin monomers into filaments. In these experiments, the polymerization of monomeric actins was followed with time by high-shear viscometry. Antibodies were added to the actin before addition of salts that would favor polymerization. (*A*) Act IV was added to *D.* actin at antibody/actin ratios of: 0.1 (□); 1:100 (△); 1:25 (▼); 1:10 (▲); 1:5 (○); 1:2 (●); X63 was added at an antibody/actin ratio of 1:2 (■). (*B*) Act I was added to rabbit muscle actin at antibody/actin ratios of: 1:1 (○); 1:10 (●); 1:2 (△). (*C*) C4 was added to rabbit muscle actin at antibody/actin ratios of: 0:1 (○); 1:50 (●); 1:5 (△). (*D*) C4 was added to *D.* actin at antibody/actin ratios of: 0:1 (○); 1:50 (●); 1:5 (△).

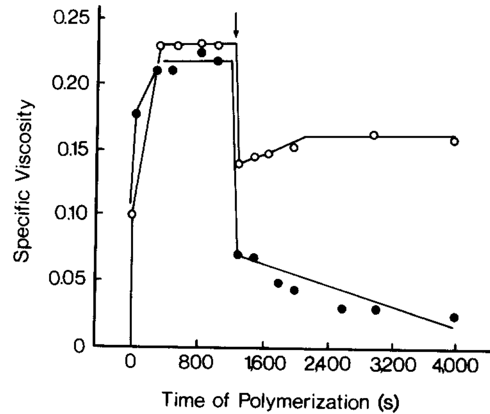


FIGURE 4 Act antibodies reduce the viscosity of *D.* actin filaments in solution. *D.* actin monomer at 250 μg/ml in G buffer was induced to assemble into filaments by the addition of salts at $t = 0$ (100 mM KCl, 1 mM $MgCl_2$ final concentrations). At the time indicated by the arrow, Act IV was added at antibody/actin molar ratios of 1:10 (○); 1:2 (●). The viscosity of the solution was monitored with time.

Act Antibodies Inhibit Formation of F Actin

As the antibodies bound strongly to monomeric actin it was possible that they would interfere with actin polymerization to form filaments. To address this question, we used high-shear viscometry, a technique that is sensitive to filament length, to measure polymerization (15, 23). A series of tubes containing actin monomer at 250 μg/ml in G buffer was prepared. Just before the addition of salts (100 mM KCl, 1 mM $MgCl_2$, final concentrations), which were used to initiate filament formation, antiactin antibodies were mixed with the actin solutions in various molar ratios, and assembly was followed with time. Fig. 3 shows the results of several of these experiments. In Fig. 3*A* the antibody used was Act IV. Both the rate and the extent of assembly decreased as the molar ratio of antibody increased. At one antibody per two actin monomers, assembly was completely inhibited. In the control tube, where X63 was mixed with actin monomer at a ratio of one per two actins, no effect on assembly of filaments was observed. Act I and Act II yielded identical results to those obtained with Act IV (data not shown).

Fig. 3*B* shows the effect of Act I on the assembly kinetics of actin from rabbit skeletal muscle. At one antibody per 10 actins there is no noticeable effect; however, assembly was markedly less complete when the ratio was one antibody per two actins. As control IgG1 (X63) did not inhibit, this result shows that the monoclonal antibodies do have a low but specific affinity for rabbit muscle actin. This weak cross-reactivity could not be detected by RIA (see Fig. 6).

The effect of the cross-reactive monoclonal antiactin C4 on assembly of *D.* actin and rabbit actin filaments was also assessed (Fig. 3*C* and *D*). Even when the antibody was present at a molar ratio of one per five actins, there was no effect on the assembly of either skeletal muscle actin (Fig. 3*C*) or *D.* actin (Fig. 3*D*). No decrease in either the rate or extent of filament formation was observed. In fact, when C4 was present at one per five actins, the extent of assembly was slightly increased over the control.

Act Antibodies Reduce the Size of Preformed Actin Filaments

High-shear viscometry was also used to assess the effect of the antiactin antibodies on preformed actin filaments (Fig. 4). When Act IV antibody was added to filaments, there was

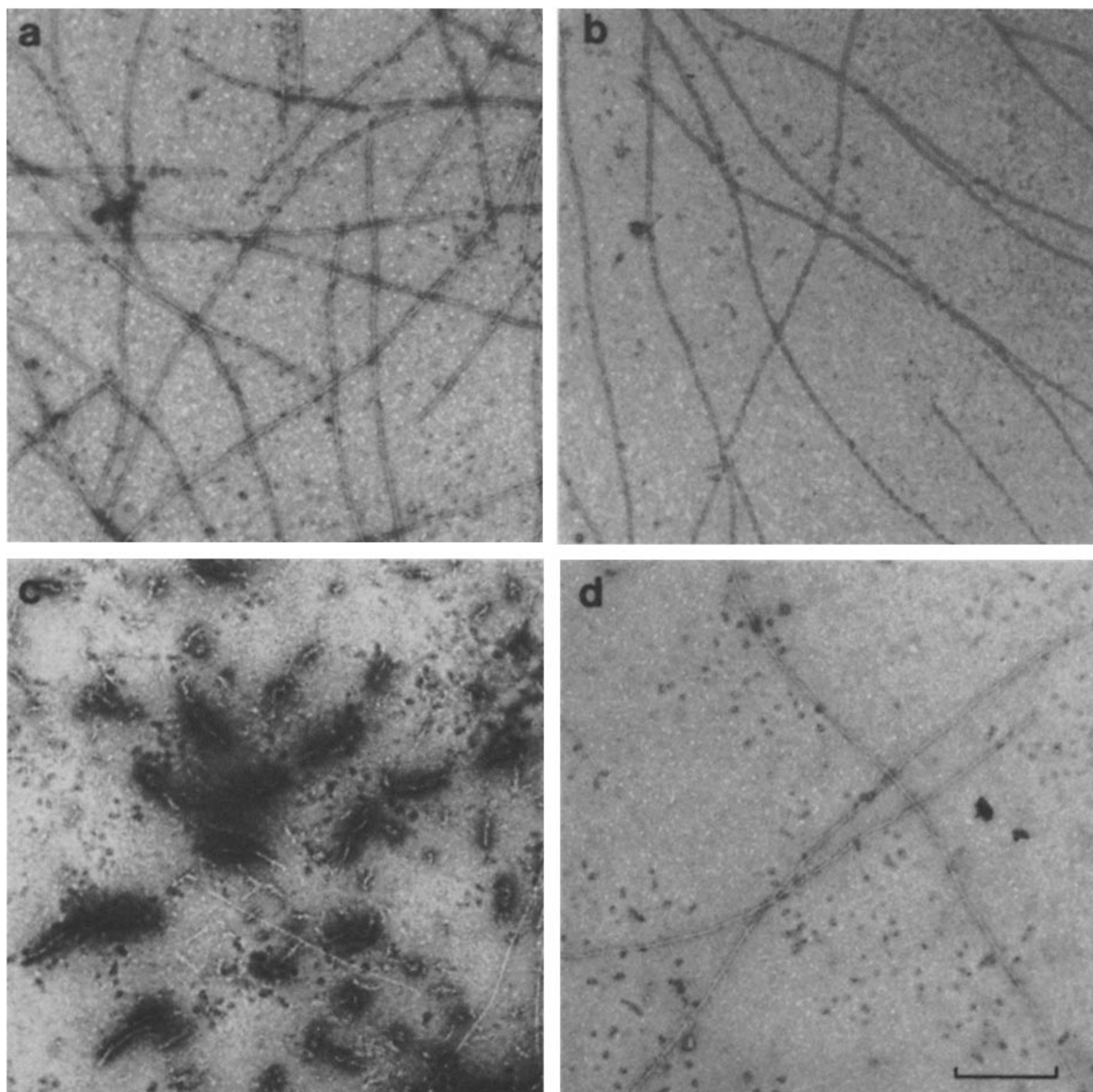


FIGURE 5 Act antibodies visibly fragment *D.* actin filaments in solution. *D.* actin monomer at 100 μg/ml in G buffer was assembled into filaments by the addition of salts (100 mM KCl, 1 mM $MgCl_2$ final concentrations). The filaments were then treated with antibodies and prepared for electron microscopy. (*a*) Control filaments; no antibody added. (*b*) X63 IgG added at an antibody/actin molar ratio of 1:2. (*c*) Act II IgG added at an antibody/actin molar ratio of 1:2. (*d*) Act II Fab added at an antibody fragment/actin molar ratio of 1:2. Bar, 0.2 μm. × 75,000.

an immediate and significant drop in the viscosity of the solution. At a molar ratio of one antibody per 10 actins the reduced viscosity level was maintained with time; at a ratio of one antibody per two actins, the viscosity continued to decrease slowly to very low levels. This suggested that antibody binding resulted in filament disruption and that, when sufficient antibody was present, all the actin could be reduced to small oligomers or monomers.

A reduction in the high-shear viscosity of a solution of actin filaments can be attributed to several factors including filament shortening, either by fragmentation or by depolymerization, and filament bundling which effectively reduces the axial ratio of the particles in solution. To try to distinguish these possibilities, we observed the effect of antibody on filaments directly by electron microscopy of negatively stained specimens (Fig. 5). In the absence of antibody (Fig. 5*a*) or in the presence of X63 (Fig. 5*b*) the actin filaments were long and smooth surfaced. When filaments were incubated briefly with the same amount of an Act antibody, they were seen to be reduced in size and had a coat of stain-excluding material that was presumably antibody. This indicated that antibody was inducing a fragmentation or depolymerization and not a bundling of actin filaments. To test the role of antibody bivalency, the experiment was also performed with the Fab fragment of Act II at a ratio of one fragment per two actins. In this case the filaments remained intact and were largely undecorated. It can therefore be concluded that for Act II, antibody bivalency is necessary for antibody-induced changes

in the size of actin filaments.

Cross-reactions of Act I, Act II, and Act IV

The RIA was used to assess the reactions of Act I, Act II, and Act IV with six different actins. Similar patterns of cross-reaction were obtained for all three antibodies and are illustrated for Act I in Fig. 6. The antibodies bind to *D.* actin, but not to rabbit or human skeletal muscle actins (Fig. 6*A*). Although at high antibody concentrations some binding to muscle actins was seen, this was no greater than the control where BSA was the plated antigen. In contrast, the antibodies bound actins from the lower eucaryotes *Physarum* and *Acanthamoeba* as strongly as they bound *D.* actin (Fig. 6*B*). Nonmuscle actins from erythrocytes and a human lymphoid cell line showed no more antibody binding than the BSA control. Therefore, Act I, Act II, and Act IV recognize epitopes shared by actins from lower eucaryotes, but not by actins from higher organisms, whether they are of the α-(skeletal muscle) or β- and γ-(nonmuscle) isotypes. In contrast, to Act I, Act II, and Act IV, the C4 monoclonal antibody (Fig. 6*C*) bound equivalently to *D.* actin and rabbit skeletal muscle actin.

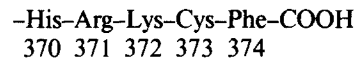
Immunoblot Analysis of Actin-Antibody Reactions

Protein immunoblotting can be a useful method for localizing epitopes inasmuch as it uses the analytical potential of SDS PAGE. Act I, Act II, and Act IV were therefore tested for reactivity against *D.* actin in the immunoblot procedure. Only Act I gave a strong specific reaction (Fig. 7). The minor reaction seen with Act IV in Fig. 7 was not considered significant inasmuch as the protein standards, phosphorylase *b* (94 kD) and carbonic anhydrase (30 kD), showed reactions of similar magnitude. The mammalian actin preparations that showed no cross-reactivity in the RIA in Fig. 6 also showed no specific reaction with Act I on immunoblotting. The reaction of Act I with *D.* actin by immunoblotting was subsequently used to localize a region on the *D.* actin molecule required for Act I binding.

Localization of a Site on Actin Required for Act I Binding

The strategy was to generate actin fragments and examine their reactivity with Act I by immunoblotting. The carboxy terminal region was specifically labeled at Cys-373 with ^{3}H-IAA, to provide a known positional marker. Fragments which retained the ^{3}H label were designated as having retained the carboxyl terminal and were therefore lacking an amino terminal segment.

^{3}H-LABELING OF ACTIN AND LOCALIZATION OF THE ^{3}H-LABEL: A 20-fold molar excess of ^{3}H-IAA over actin was used and the incorporation of radioactivity was ~5% of the total, indicating a labeling stoichiometry of close to 1:1. To determine if the label was positioned at Cys-373, a tryptic digestion of the actin was performed and the extent of digestion and release of radioactivity monitored by SDS PAGE and autoradiography. The carboxyl terminus of *D.* actin has the sequence


–His–Arg–Lys–Cys–Phe–COOH
370 371 372 373 374

(40) and –Arg and –Lys are both susceptible to trypsin cleav-

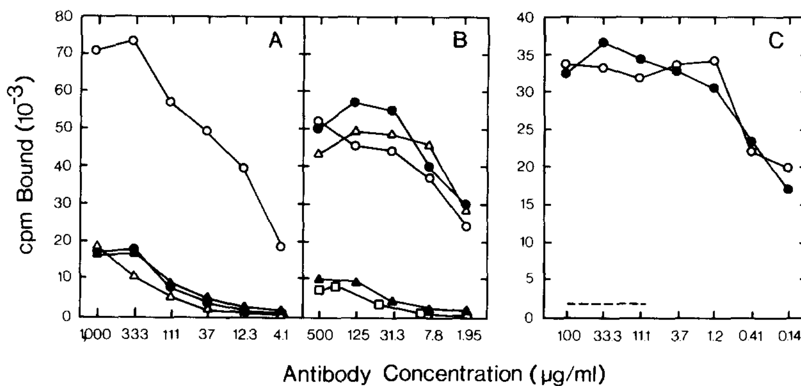


FIGURE 6 Cross-reactions of monoclonal antiactins. Solid-phase RIAs were as follows: (*A*), Act I on *D.* actin (○), rabbit skeletal muscle α-actin (●), human skeletal muscle α-actin (△), BSA (▲). (*B*), *D.* actin (○), *Physarum* actin (●), *Acanthamoeba* actin (△), human lymphocyte β- and γ-actin (▲), human erythrocyte β- and γ-actin (□). (*C*), C4 antibody on *D.* actin (○), rabbit skeletal muscle α-actin (●). Dashed line shows background binding with BSA as antigen.

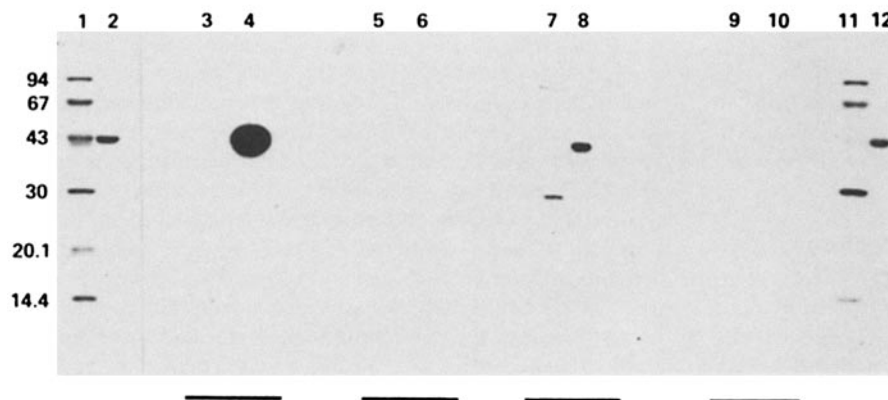


FIGURE 7 Act I but not Act II or Act IV reacts with *D.* actin in the immunoblot assay. Lanes *1* and *2*, Coomassie Blue-stained protein gel. Each subsequent pair of lanes was loaded with replicate samples. Lane *1*, molecular weight markers ($\times 10^{-3}$) as indicated; Lane *2*, *D.* actin; Lanes *3–10*, autoradiograms of immunoblots reacted with Act I (*3* and *4*), Act II (*5* and *6*), Act IV (*7* and *8*) or X63 (*9* and *10*) antibodies followed by ^{125}I-F(ab′)$_2$ rabbit anti-mouse IgG; Lanes *11* and *12*, amido black-stained nitrocellulose blot demonstrating efficient protein transfer.

age resulting in release of label at Cys-373 in a Lys–Cys–Phe tripeptide. As shown in Fig. 8, all of the radioactivity was lost on tryptic digestion although the mobility of the actin was not noticeably changed. This strongly suggests that the ^{3}H label is indeed attached to the actin at Cys-373, because only a minor cleavage, too small to be seen as a change in electrophoretic mobility, was sufficient to remove this label.

CLEAVAGE WITH PROTEASE FROM *S. AUREUS* V8: [^{3}H]*D.* actin was digested with different amounts of V8 protease from *S. aureus* and the fragments were analyzed by SDS PAGE and either Act I blotting or autoradiography to determine which fragments retained Cys-373. As shown in lanes *3* and *4* of Fig. 9*a*, a number of discrete fragments were generated from *D.* actin. Six major fragments are labeled 1–6 in Fig. 9*a*. The immunoblot (Fig. 9*a*, lanes *7* and *8*) shows that fragments 1, 2, 3, and 5 react with Act I. The ^{3}H autoradiogram shows that only fragments 4 and 6 retained the carboxyl terminus and could therefore be localized within the molecule. Cleavage from the COOH terminus did not result in the loss of Act I reactivity as evidenced by fragments 1, 2, 3, and 5. Cleavage from the NH_2 terminus resulted in the loss of Act I reactivity as evidenced by fragments 4 and 6. Fragment 4 is the largest without Act I reactivity and has an apparent molecular weight of ~25,000. Therefore, the COOH terminal 25 kD of *D.* actin are not sufficient for Act I reactivity and this implies that the NH_2 terminal 18 kD are important for Act I binding. From examination of the sequence of *D.* actin, one can postulate that the cleavage that generates fragment 4 is the Glu-Gly bond between residues 167 and 168. This assumes that the V8 protease cleaves specifically at the carboxyl side of glutamic acid residues.

CLEAVAGE WITH PAPAIN: [^{3}H]*D.* actin was treated with papain and the pattern of fragmentation was analyzed as described in the previous section. The results are in agreement with those obtained with V8 protease. The largest fragment containing the carboxyl terminus that did not react with Act I was fragment 1, of apparent molecular weight 37,000 (Fig. 9*b*). This extends the analysis made with V8 protease fragment 4 and suggests that the NH_2 terminal 6 kD of *D.* actin are required for binding of Act I in this system.

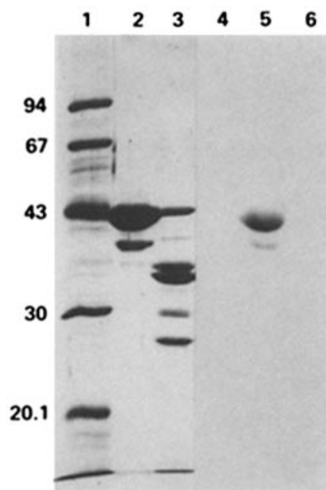


FIGURE 8 ^{3}H-IAA treatment labels *D.* actin at Cys-373. Radiolabeled *D.* actin monomer (500 μg/ml) was incubated with TPCK-trypsin (50 μg/ml) for 60 min at 25°C. The reaction was stopped by the addition of an equal volume of SDS PAGE sample buffer and boiling for 3 min. Lanes *1–3* and *4–6* are replicates, with the former showing the Coomassie Blue staining pattern of the proteins and the latter showing the autoradiogram obtained from a replicate gel slice treated with 2,5-diphenyloxazole. Lanes *1* and *4*, molecular weight markers (×10^{-3}) as indicated; Lanes *2* and *5*, untreated labeled actin; Lanes *3* and *6*, trypsin-treated labeled actin. Note the absence of radiolabeled material in lane *6*, especially at the position of actin, whereas in lane *3* there is Coomassie Blue staining material co-migratory with *D.* actin.

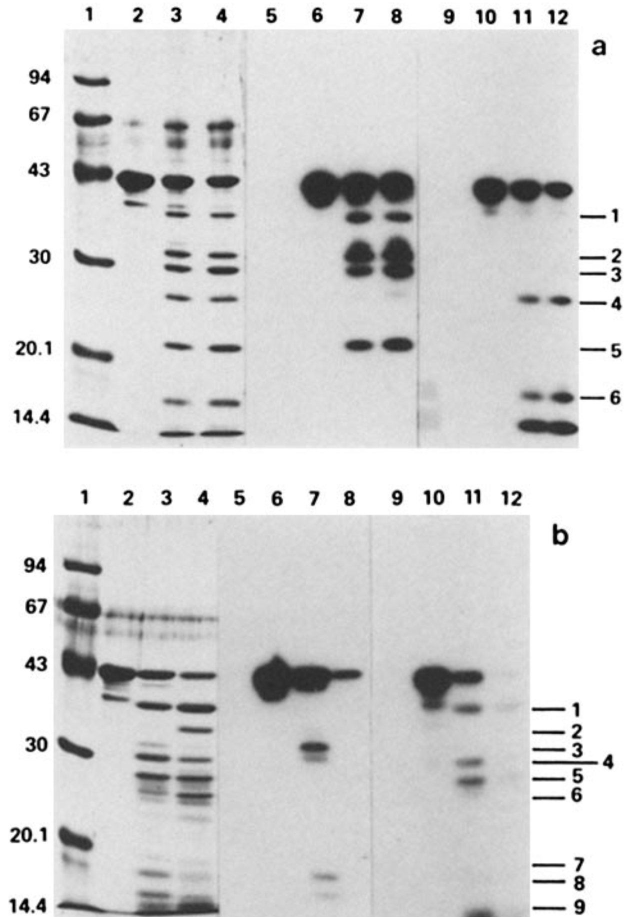


FIGURE 9 Identification of proteolytic cleavage fragments of *D.* actin that react with Act I. ^{3}H-labeled *D.* actin monomer was treated with V8 protease (*a*) or papain (*b*) and analyzed by SDS PAGE, autoradiography, and immunoblotting. Replicate sets of four lanes show Coomassie Blue staining of the gel to detect protein (lanes *1–4*), autoradiography of an immunoblot with Act I and ^{125}I-F(ab')$_2$ rabbit anti-mouse IgG (lanes 5–8), and autoradiography of the gel to detect the presence of the ^{3}H-labeled COOH-terminal region (lanes *9–12*). Lanes *1*, *5*, and *9* contain standard protein; lanes *2*, *6*, and *10* are untreated [^{3}H]*D.* actin; lanes *3*, *7*, and *11* contain [^{3}H]*D.* actin treated with 1% wt/wt V8 protease for 60 min (*a*) or with 1% wt/wt papain for 15 min (*b*); in lanes *4*, *8*, and *12* the protease treatments were 2% wt/wt V8 for 60 min (a) and 1% wt/wt papain for 30 min (*b*). The molecular weights (×10^{-3}) of protein standards are shown on the left and the positions of the major fragments are shown on the right. The use of the same numbers does not imply identity between the fragments with the same numbers in *a* and *b*.

CLEAVAGE WITH HYDROXYLAMINE: [^{3}H]*D.* actin was treated with hydroxylamine and the fragments assayed for retention of Cys-373 and for Act I binding. After treatment a new species was seen that had a mobility compatible with the removal of a small number of amino acids from the actin (Fig. 10, lane *4*). The corresponding autoradiogram in Fig. 10, lane *12* shows that this new species retains the carboxyl terminus. This is consistent with cleavage at the asparagyl–glycine bond between residues 12 and 13 as previously described (3, 34). The fragment that has lost the first 12 NH_2 terminal amino acids retains reactivity with Act I as shown in Fig. 10, lane *8*. This result shows that these residues are not necessary for Act I binding.

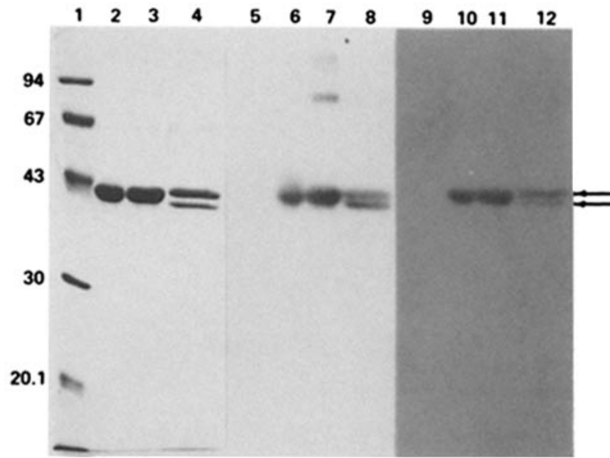


FIGURE 10 The NH_2-terminal 12 residues of *D.* actin are not required for Act I binding. ^3H-labeled *D.* actin was treated with hydroxylamine and the fragments were analyzed by SDS PAGE, autoradiography, and immunoblotting. Replicate sets of four lanes show Coomassie Blue staining for protein (lanes *1–4*), immunoblot with Act I and ^{125}I-$F(ab')_2$ rabbit anti-mouse IgG (lanes *5–8*), and autoradiogram of gel to detect the ^3H label at residue 373 in the COOH terminal region (lanes *9–12*). Lanes *1*, *5*, and *9* contain protein standards with molecular weights ($\times 10^{-3}$) as shown; lanes *2*, *6*, and *10* contain untreated actin; lanes *3*, *7*, and *11*, "mock"-treated actin, and lanes *4*, *8*, and *12*, hydroxylamine-treated actin. "Mock" treatment was identical to hydroxylamine treatment except that hydroxylamine was omitted. The upper arrow on the right indicates the position of intact actin, the lower arrow the position of the hydroxylamine-generated fragment of an apparent molecular weight of 42,000.

Summary of Localization of the Act I Binding Region on D. Actin

Fig. 11 summarizes the analysis of actin fragments for Act I binding. Cleavage of actin with V8 protease results in the formation of a 25-kD fragment of actin that has retained the carboxyl terminus, but has lost the ability to bind Act I antibody. Cleavage of actin with papain produces a fragment with similar properties, but with a molecular weight of 37,000. These results suggest that the amino acids necessary for antibody recognition of the actin reside in the amino terminal 50 (approximately) residues. However, the hydroxylamine result indicates that the initial 12 amino terminal residues are not required for antibody recognition of the actin. In summary, amino acids from 13 through ~50 in the *D.* actin sequence are required for binding of the Act I antibody.

The amino acid sequences of actins were compared to identify substitutions that correlated with the species specificity of Act I (6, 22, 38, 39, 40). In particular, a search was made for residues that were shared by actins from the lower eucaryotes and substituted in mammalian actins. Within the sequence 13–50, there is a single residue that satisfies this criterion. At position 41 the three lower eucaryotes have threonine whereas mammalian actins have glutamine. This suggests that threonine 41 is a critical residue for formation of the Act I antigenic site. As Act I reacts strongly in immunoblotting, it is probable that threonine 41 interacts directly with the Act I combining site. However, the other possibility that the substitution at position 41 causes a conformational and antigenic change, at a topographically distinct site that binds Act I, is not ruled out.

DISCUSSION

Hyperimmunization of BALB/c mice with *D.* actin results in a significant immune response that was utilized to make three monoclonal antibodies, Act I, Act II, and Act IV. The specificity of these antibodies reflects that of the polyclonal sera in that they were of high affinity for *D.* actin and of low affinity for rabbit skeletal muscle actin. All three antibodies bound similarly to actins of three lower eucaryotic organisms, *Dictyostelium*, *Physarum*, and *Acanthamoeba*, but did not bind to α-, β-, or γ-actins from human or rabbit. This species specificity correlates with the extent of amino acid sequence homology between the various actins (6, 22, 38, 39, 40). Lessard et al. (20) prepared two murine monoclonal antibodies, C4 and B4, against chicken gizzard actin. The C4 antibody appears to be broadly specific, whereas the B4 antibody reacts strongly only with muscle actins. Caceres et al. (5) described two monoclonal antibodies against quail breast actin (QAB1, QAB2), which do not discriminate between vertebrate actins. Thus highly specific and broadly specific monoclonal antibodies can be prepared against actins from diverse sources.

The three antibodies, Act I, Act II, and Act IV identify two topographically distinct epitopes of the *D.* actin molecule; one recognized by Act I and the other by Act II and Act IV. Analysis of *D.* actin fragments by immunoblotting showed that amino acids 13 to about 50 are required for Act I binding and that the threonine residue at position 41 is critically involved in forming the Act I epitope.

The effects the three antibodies have upon the properties of *D.* actin have so far been indistinguishable. They form stable complexes with monomeric actin which prevents the actin from polymerizing into filaments. Conversely, addition of the antibodies to preformed actin filaments causes them to become smaller. When a stoichiometric amount of antibody is added (*a*) to monomeric actin, polymerization is completely inhibited, and (*b*) to filamentous actin, the viscosity is reduced to the level of monomeric actin. These results show that the energy gained by forming complexes between antibody and actin is greater than that gained by forming actin filaments. The antibodies could interfere with filament formation either by covering or conformationally altering a site involved in actin–actin interaction, or by steric hindrance when bound to a site not involved in actin–actin interaction. The latter is a serious possibility inasmuch as an antibody molecule is about

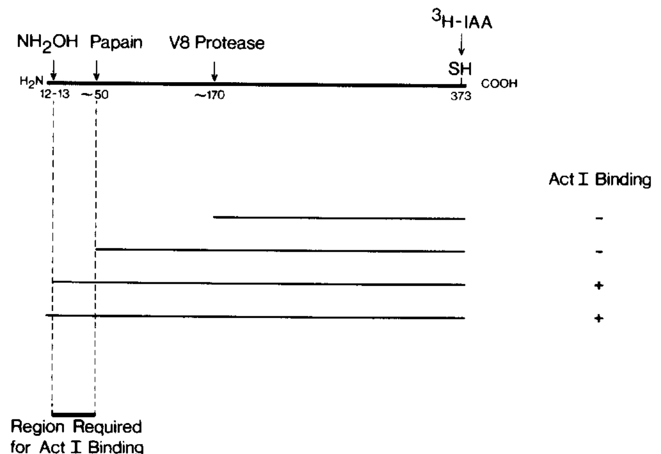


FIGURE 11 Summary of the results of the experiments to localize the region on *D.* actin required for Act I antibody binding.

three times the size of an actin monomer. However, the results with the C4 antibody (Fig. 3*C* and *D*) show that not all monoclonal antibodies will inhibit actin filament assembly. The mechanism by which the antibodies depolymerize filaments is not known. It could involve removal of the actin monomer pool or direct binding to the filaments resulting in fragmentation within the filament or removal of monomers from the ends. A cooperative effect between the two combining sites of the antibody appears to be involved as Fab fragments of Act II do not affect filament integrity.

A variety of circumstantial evidence suggest that the epitopes recognized by Act II and Act IV are formed by residues in the NH_2 terminal region of the actin molecule. Combining the results of crystallographic studies with a study of proteolytic susceptibility Mornet and Ue (21) concluded that actin consists of a small NH_2-terminal domain of ~80 residues linked to a larger COOH-terminal domain that contains the rest of the molecule. Within the actin filament the small domains are turned "inward" and the larger domains are turned "outward" (33). This is consistent with the conclusion of Benjamin et al. (2) that antibodies against the NH_2-terminal part of monomeric actin do not bind actin filaments whereas antibodies against the COOH-terminal region do. In keeping with this picture, and knowing that Act I, Act II, and Act IV bind monomer and destroy filaments, one would postulate that all three antibodies are against epitopes of the smaller NH_2-terminal domain. For Act I, there is independent evidence from the immunoblotting analysis that this is correct. If one assumes it is also true for Act II and Act IV, then what residues could be involved in their target epitopes? Within the actin sequence, only residues at positions 2, 5, 6, 41, 234, 295, 297, 306, 317, and 360 fulfill the criterion of being shared by *Dictyostelium*, *Physarum*, and *Acanthamoeba* actins and distinct from mammalian actins. Clearly, it is only the residues at positions 2, 5, 6, and 41 that could be in a smaller NH_2-terminal domain. Inasmuch as Act II and Act IV are against a topographically different epitope from Act I, involvement of threonine 41 is excluded. One is thus left with glycine 2, valine 5, and glutamine 6 as the most likely residues to be involved in the epitopes recognized by Act II and Act IV.

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