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 NH2-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 [35S]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; 3H-IAA, [3H]iodoacetic acid; RIA, radioimmune assay.
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 *Xenathamoeba castellanii, Physarum polycephalum*, and human erythrocytes were the generous gifts of Dr. T. D. Pollard (The Johns Hopkins University), Dr. V. T. Nachmani (University of Pennsylvania), and Dr. S. L. Schier (Stanford University Medical Center), respectively.

D. actin was radiolabeled with [*H]*iodoacetic acid ([*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% NaN3; 100 mM KCl; 1 mM MgCl2) was incubated with a 20-fold molar excess of [*H*-IAA (203 mCi/mmol; New England Nuclear, Boston, MA) for 9 h at 37°C, then 1 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 antiantibodies in an indirect immunofluorescence assay (IFA). The mice were bled in 4-mo intervals 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 used in studies by Stahl et al. (32) to increase the yield of specific hybridomas against soluble antigens. On the fifth day the spleens from nine immunized animals were fused with cells of the P3NS1/1-AG4-1 plasmacytoma 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. (Fla*) and (Fla*) 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 *Dictostelium* was diluted to 100–250 µg/ml in PBS N3 (PBS buffer with 0.01% NaN3) 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 N3 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 N3 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 N3 containing 0.5% BSA, 300,000 cpm (≈0.03 µg) of [*125I*-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 N3 containing 0.5% BSA, the wells were cut from the plates with a hot wire, and assayed for radioactivity in a Beckman Gamma counter. 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 a-lactalbumin (14.4 kD).

**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 [*25I]* at Cys-373 at 500 µg/ml was incubated in 200 mM KCl, 2 mM [gamma]-HCl, 1 mM hydroxyamine, 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 N3 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 a-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 0.002% (wt/vol) SDS (8). Samples were electrophoresed and the gel was cut into replicate slices. One was stained for protein with Coomasie Brilliant Blue. The other slices were washed with Laemmli (18) electrode buffer without SDS. Gel slices were then lined up on a 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 antiantibody 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 [*125I*-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.,
aqueous uranyl acetate on 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

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 radiiodinated goat anti-mouse isotype specific IgG as the second-stage 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-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 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. 1A 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. 1B). 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 35S-labeled actin monomer preincubated with Act I antibody or with the X63 antibody as a negative control. Actin monomer alone (Fig. 2A) or actin monomer preincubated with X63 (Fig. 2B) eluted as a single broad peak within the included volume of the column. In contrast, when actin monomer was preincubated with the Act I monomeric 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. 2C). 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 (≥109 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.

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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 MgCl₂, 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 \( \mu \)g/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 \( \mu \)m. x 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. 5a) or in the presence of X63 (Fig. 5b) 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. 6A). 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. 6B). 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 organisms, 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. 6C) 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 'H-IAA, to provide a known positional marker. Fragments which retained the 'H label were designated as having retained the carboxy terminal and were therefore lacking an amino terminal segment.

**3H-LABELING OF ACTIN AND LOCALIZATION OF THE 3H-LABEL:** A 20-fold molar excess of '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

\[
\text{His} - \text{Arg} - \text{Lys} - \text{Cys} - \text{Phe} - \text{COOH}
\]

(40) and -Arg and -Lys are both susceptible to trypsin cleav-
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. 9a, a number of discrete fragments were generated from $D$. actin. Six major fragments are labeled 1–6 in Fig. 9a. The immunoblot (Fig. 9a, 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 4 and 6. Fragment 4 is the largest without Act I reactivity and has an apparent molecular weight of $\sim 25,000$. 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. 9b). 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.

**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 8** $^3$H-IAA treatment labels $D$. actin at Cys-373. Radiolabeled $D$. actin monomer (500 $\mu$g/ml) was incubated with TPCK-trypsin (50 $\mu$g/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 ($\times 10^5$) 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 ($\times 10^3$) 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.

**Figure 10** Identification of proteolytic cleavage fragments of $D$. actin that retain Cys-373. Radiolabeled $D$. actin monomer (500 $\mu$g/ml) was incubated with TPCK-trypsin (50 $\mu$g/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 ($\times 10^5$) 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.
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.
that all three antibodies are against epitopes of the smaller with this picture, and knowing that ActI, ActII, and ActIV part of monomeric actin don’t bind actin filaments whereas turned “outward” (33). This is consistent with the conclusion domains are turned “inward” and the larger domains are 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 NH2-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 NH2-terminal domain. For Act I, there is independent evidence from the immunoblots 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 NH2-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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