Identification by Monoclonal Antibodies and Characterization of Human Platelet Caldesmon

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Abstract. Actin-based gels were prepared from clarified high-salt extracts of human platelets by dialysis against physiological salt buffers. The gel was partially solubilized with 0.3 M KCl. Mice were immunized with the 0.3 M KCl extract of the actin gel, and hybridomas were produced by fusion of spleen cells with myeloma cells. Three hybridomas were generated that secrete antibodies against an 80-kD protein. These monoclonal antibodies stained stress fibers in cultured cells and cross-reacted with proteins in several tissue types, including smooth muscle. The cross-reacting protein in chicken gizzard smooth muscle had an apparent molecular weight of 140,000 and was demonstrated to be caldesmon, a calmodulin and actin-binding protein (Sobue, K., Y. Muramoto, M. Fujita, and S. Kakiuchi, *Proc. Natl. Acad. Sci. USA,* 78:5652–5655). No proteins of molecular weight greater than 80 kD were detectable in platelets by immunoblotting using the monoclonal antibodies. The 80-kD protein is heat stable and was purified using modifications of the procedure reported by Bretscher for the rapid purification of smooth muscle caldesmon (Bretscher, A., 1985, *J. Biol. Chem.*, 259:12873–12880). The 80-kD protein bound to calmodulin–Sepharose in a Ca^{++}-dependent manner and sedimented with actin filaments, but did not greatly increase the viscosity of F-actin solutions. The actin-binding activity was inhibited by calmodulin in the presence of calcium. Except for the molecular weight difference, the 80-kD platelet protein appears functionally similar to 140-kD smooth muscle caldesmon. We propose that the 80-kD protein is platelet caldesmon.

CALDESMON is a calmodulin- and actin-binding protein originally identified in and purified from chicken gizzard smooth muscle (20). The purified gizzard protein contains two peptides of 138,000 and 140,000 mol wt. There is conflicting evidence as to whether the protein is a dimer. The actin-binding activity is regulated by calmodulin. In the presence of micromolar calcium, calcium–calmodulin forms a complex with caldesmon. This complex binds poorly to actin filaments. Reducing the calcium level below 1 μM results in the release of calmodulin and restores the actin-binding activity of caldesmon (4, 20). Purified caldesmon inhibits the "superprecipitation" of actin and myosin (17, 21) and also inhibits the actin-activated ATPase activity of smooth muscle myosin. This inhibitory activity of caldesmon may depend upon its phosphorylation state (17). Kakiuchi and co-workers have developed a model in which they propose that calmodulin and caldesmon act as a regulatory system for the interaction of actin and myosin (20, 21). Caldesmon is also reported to be a potent actin filament cross-linker, and at high molar ratios will organize actin filaments into bundles (3). Caldesmon is not specific to smooth muscle, since antibodies raised against chicken gizzard caldesmon detect cross-reacting peptides in immunoblots of nonmuscle cells, which include cultured cells and platelets (4, 12, 18, 19). Some of the peptides detected in these nonmuscle tissues have molecular weights lower than 140,000. In immunofluorescence studies, anti-caldesmon polyclonal antibodies localize caldesmon on the stress fibers of cultured cells (4, 19).

We have been interested in the structure and regulation of the actin cytoskeleton in human platelets and have studied the gelation of actin in platelet extracts. These gels contain actin, myosin, alpha-actinin, and several minor proteins. Monoclonal antibodies have been raised to some of the minor components of these gels. We report particularly on three hybridomas that have been generated which produce monoclonal antibodies that recognize a previously unidentified 80-kD platelet protein. These antibodies cross-react with material from sources other than platelets and with purified chicken gizzard caldesmon. Immunofluorescence studies on cultured cells localize the 80-kD protein in stress fibers. The platelet protein has been purified and shown to have actin-binding activity that is regulated by calmodulin. It appears to be a nonmuscle caldesmon of a size smaller than the smooth muscle protein.

Materials and Methods

Platelet Actin Gel Production and Fractionation

Fresh or outdated platelets were obtained from the local blood center and were rotated at 18°C until used. Platelet high salt extracts, 0.6 M KCl, were prepared using a method similar to that reported previously (5). In some experiments, to ensure activation, platelets were treated wtih 10 μM of Ca^{2+} ionophore,

A23187, before sonication. The high salt platelet extract was dialyzed overnight against 100 vol of 100 mM KCl, 10 mM imidazole-HCl, 1 mM EGTA, 1 mM NaN_3, pH 7.5, at 4°C. An actin gel formed during dialysis and was collected by centrifugation at 8,000 *g*, then washed three times with the dialysis buffer. The washed gel was extracted twice for 15 min with 10 vol of 2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.1 mM NaN_3, pH 7.5. This removed most of the alpha-actinin and some actin (10). The alpha-actinin–depleted gel was recovered by centrifugation and extracted twice for 15 min with 5 vol of 0.3 M KCl, 20 mM Tris-HCl, 2 mM EGTA, 1 mM NaN_3, pH 7.5. The supernatants were recovered by centrifugation and the gel residue, largely actomyosin, was discarded.

Monoclonal Antibody Production

Monoclonal antibodies were produced using the methods reported for the production of monoclonal antibodies to platelet gelsolin (11). The mice were immunized with ~20 μg of 0.3 M KCl extract protein per injection. After five injections, a high titer response, positive by immunoblotting at greater than a 1:2,000 dilution, was detected against several proteins from the platelet contractile gel.

The animal was killed, the spleen was removed, and splenocytes were fused with A653 myeloma cells as described previously (11). Hybridomas were screened by ELISA assay and by immunoblotting against the 0.3 M KCl supernatant. ELISA and immunoblot positive hybridomas were screened further by immunofluorescence methods described below. Cloned hybridomas were re-tested by all three methods. Larger quantities of monoclonal antibodies were obtained by injection of 10^8 hybridoma cells into the peritoneal cavity of pristane-primed mice and subsequent collection of ascites fluid. One monoclonal, 23H2, was adapted to and grown in defined media to obtain antibody free of contaminating mouse proteins (11). The 23H2 antibody was used in most of the experiments reported here, but the results obtained with all of the anti-80-kD antibodies were similar. We have not yet localized the epitope(s).

Immunoblotting Procedure

Immunoblots were performed by a modification of the method of Towbin et al. (25). Briefly, proteins separated by electrophoresis in 8 or 10% SDS polyacrylamide gels were electrophoretically transferred to nitrocellulose sheets. The nitrocellulose sheets were blocked for 24 h in Dulbecco's phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++}, that contained 5% calf serum, 1% gelatin, 1% bovine serum albumin (BSA), and 10 mM NaN_3. The sheets were then blotted with the first antibody, either culture supernatants or diluted ascites fluid. After washing and incubation with peroxidase-conjugated goat anti-mouse IgG (CooperBiomedical, Inc., Malvern, PA), the immunoblots were extensively washed with PBS that contained 0.1% Triton X-100 and developed with 0.5% 4-chloro-1-naphthol and 0.02% H_2O_2. Occasionally immunoblots were done using polyclonal antibodies made in rabbits; peroxidase-conjugated goat anti-rabbit IgG (CooperBiomedical, Inc.) was used in the second incubation step.

Cell Culture

Cell lines were maintained in Dulbecco's modified Eagle's medium that contained 10% fetal calf serum and penicillin/streptomycin. Media used for hybridomas contained 0.1 mM 2-mercaptoethanol and 10% hybridoma-screened fetal calf serum. Hybridomas were grown in air that contained 5% CO_2; other lines were kept in 7% CO_2. Swiss 3T3 cells were obtained from the American Type Culture Collection; human foreskin fibroblasts were a gift from Dr. David Via (Baylor College of Medicine). Bovine aorta smooth muscle cells were a gift from Dr. Suzanne Eskin (Baylor College of Medicine). For immunofluorescence studies, cells were grown to ~50% confluency on glass coverslips.

Indirect Immunofluorescence

Immunofluorescence staining of cells on coverslips was done using a method similar to that published by Carron et al. (6). Cells on coverslips were rinsed in Dulbecco's PBS without Ca^{++} and Mg^{++}, then fixed in 3% formaldehyde, 1% dimethyl sulfoxide in this PBS, for 20 min at room temperature. Coverslips were rinsed in PBS, then extracted and permeabilized in acetone at −20°C for 6–7 min. Coverslips were rinsed again in PBS and incubated in the first antibody, usually hybridoma culture supernatant, at 37°C for 1 h. After rinsing in PBS, the coverslips were incubated in fluorescently tagged goat anti-mouse IgG (CooperBiomedical, Inc.). Some experiments included labeling with fluorescein-tagged phalloidin (a gift of Dr. T. Wieland, Max-Planck-Institut für Medizinische, Heidelberg, West Germany). This was either included in the second antibody incubation or a third incubation of 30 min was performed with phalloidin at a concentration of 10 μg/ml. Tetramethylrhodamine isothiocyanate–conjugated second antibody was used in the double-label studies. The coverslips were mounted and examined as described (6). Final magnifications are as indicated.

Immunofluorescence staining of platelets was done on platelets spread on glass coverslips. Platelets were obtained as platelet-rich plasma, diluted with Dulbecco's PBS, then allowed to spread on glass coverslips for 1–15 min. The platelets were then processed for indirect immunofluorescence as described previously (6). Use of polylysine-coated coverslips did not affect the degree of attachment and spreading. Calf serum at a concentration of 10% was included in the antibody staining reagents to minimize background.

Protein Purification

Actin was isolated from rabbit skeletal muscle by the method of Spudich and Watt (24) and was further purified by gel-filtration on Sephadex G-150 (14).

Chicken gizzard caldesmon was purified using the method described by Bretscher (3). The platelet 80-kD protein was purified using a modification of this procedure. Platelets were obtained from the local blood center and washed as described previously (5). Washed platelets were resuspended in 6 vol of extraction buffer, 0.3 M KCl, 5 mM EGTA, 2 mM $MgCl_2$, 0.5 mM diisopropylfluorophosphate, 50 mM imidazole-HCl, pH 6.9, then sonicated in 30-s bursts, with cooling on ice in between, for a total of 6 min. The lysate was heated, with constant stirring, in a boiling water bath until the temperature of the lysate reached 90°C, then was held at this temperature for 5 min. After cooling on ice, the lysate was clarified at 50,000 *g* for 30 min at 4°C. The heat-soluble extract was then fractionated with $(NH_4)_2SO_4$. The 30–50% pellet contained the 80-kD protein, as determined by gel electrophoresis and immunoblot. This pellet was resuspended in 2 ml of 30 mM NaCl, 1 mM EGTA, 0.1 mM dithiothreitol (DTT),[1] 10 mM imidazole-HCl, 0.1 mM NaN_3, pH 7.0 and dialyzed against the same buffer. This material was loaded onto a 5 ml DEAE-Sephacel column equilibrated with the above buffer and eluted with a 30–500 mM NaCl gradient. The protein content was determined, and fractions were analyzed by 8% SDS polyacrylamide gel electrophoresis. Fractions that contained the 80-kD protein were pooled and dialyzed against 100 mM KCl, 0.1 mM EGTA, 0.1 mM DTT, 0.1 mM NaN_3, 10 mM Tris-HCl, pH 7.5. The 80-kD protein could also be purified from the 30–50% $(NH_4)_2SO_4$ pellet by affinity chromatography on calmodulin–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). The 30–50% pellet was resuspended in 100 mM KCl, 0.1 mM EGTA, 0.1 mM DTT, 0.1 mM NaN_3, 10 mM Tris HCl, pH 7.5. $CaCl_2$ was added to the dialyzed material to a concentration of 0.2 mM and applied to a 5 ml calmodulin–Sepharose column. The column was washed with 200 mM KCl, 0.2 mM $CaCl_2$, 0.1 mM DTT, 0.1 mM NaN_3, 10 mM Tris-HCl, pH 7.5 and eluted with 1 mM EGTA in the same buffer. The fractions were analyzed on 10% SDS polyacrylamide gel electrophoresis.

Low-shear Viscometry

Falling-ball viscometry, a low-shear viscometric method, was done following the method of MacLean-Fletcher and Pollard (15). The 140- and 80-kD caldesmons used in the assay were dialyzed against polymerization buffer (100 mM KCl, 5 mM NaPipes, 1 mM ATP, 1 mM $MgCl_2$, pH 7.0), then mixed with F-actin at a final actin concentration of 12 μM. The samples were drawn up into 100-μl capillary tubes and incubated at room temperature for 1 h. The apparent viscosity was measured by timing a 0.64-mm stainless steel ball falling 2 cm through the sample at an 80° inclination. The apparent viscosity is expressed as the ratio of the viscosity of the sample to that of the actin control.

Sedimentation

Sedimentation assays were used to examine the interaction of actin with caldesmon and the 80-kD protein. Proteins in polymerization buffer were mixed and incubated in centrifuge tubes at room temperature for 1 h. The final concentrations of protein were as follows: actin, 12 μM; 80-kD protein, calmodulin, and 140-kD caldesmon, as given in figure legends. The samples were then centrifuged in an airfuge at 30 psi for 30 min at room temperature, conditions that sediment F-actin. After centrifugation, the supernatant was carefully aspirated off the pellet, and the pellet was resuspended in a volume of sample buffer equal to the original volume of the sample. Both supernatants and pellets were analyzed on 8% SDS polyacrylamide gels. The amount of caldesmon protein present in the pellet and supernatant was quantitated by densitometric scanning of the Coomassie Blue–stained gels.

1. *Abbreviations used in this paper:* DTT, dithiothreitol; MLCK, myosin light chain kinase.

Miscellaneous

SDS polyacrylamide gel electrophoresis was done according to Laemmli (13). Whole cell and tissue samples were prepared for electrophoresis by homogenization in boiling Laemmli sample buffer followed by heating to 95°C for 5 min. Protein determinations were done using a modification of the method of Bradford (2).

Results

Preparation of Platelet Actin Gels

Fig. 1 shows the SDS polyacrylamide gel electrophoresis profiles from different steps in the preparation of actin gels from platelets. These gels were formed by dialyzing extracts from activated or unactivated platelets against physiological salt buffers. Unactivated platelet extracts produced gels with alpha-actinin and actin as the only major components (Fig. 1, lane *3*). If platelets were activated before sonication, the gels also contained myosin as a major component, along with a 140-kD protein (Fig. 1, lane *6*), a proteolytic fragment of myosin, identified using anti-myosin monoclonal antibodies (data not shown). The myosin-containing gels from activated platelets contracted during gelation. The smooth, translucent appearance of this contractile gel was different from the flocculent appearance of the unactivated gels. Both types of gel also contained several minor protein components.

Specific components of the contractile gel could be removed by extraction of the gel with different salts. A low-salt buffer that contained Ca^{++} was used to extract most of the alpha-actinin and some of the actin (Fig. 1, lane *7*). This Ca^{++} extract was used to purify alpha-actinin and platelet actin as previously reported (10). Extraction of the alpha-actinin depleted gel with 0.3 M KCl buffer that contained EGTA removed most of the 140-kD myosin fragment, some myosin, and a few minor components (Fig. 1, lane *8*). The remaining gel residue contained actin, some myosin, and several minor components.

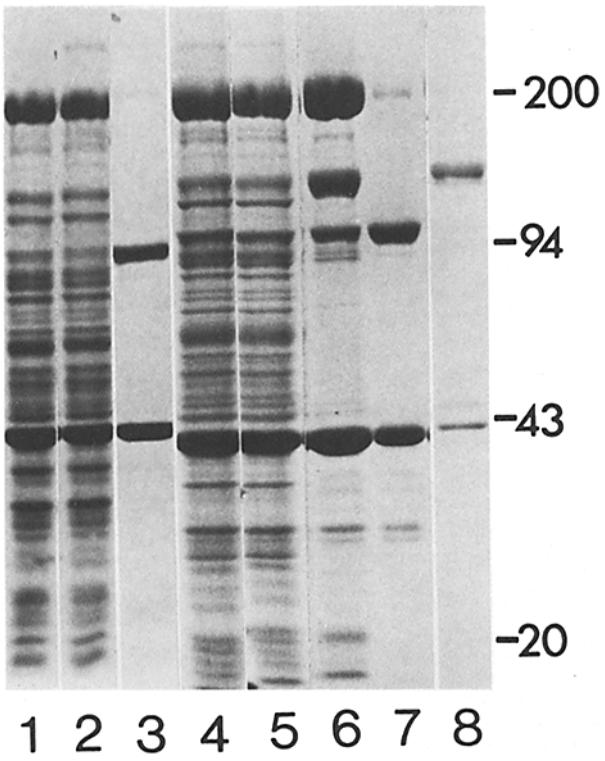

Figure 1. SDS polyacrylamide gel electrophoresis of platelets and platelet fractions. Samples from various steps in the preparation of platelet actin gel extracts were analyzed by 8% SDS polyacrylamide gel electrophoresis. Lanes *1* and *4*, whole platelets; lane *2*, supernatant above unactivated platelet gel; lane *3*, unactivated platelet gel; lane *5*, supernatant above activated platelet contractile gel; lane *6*, contractile gel; lane *7*, Ca^{++} extract of contractile gel; lane *8*, 0.3 M KCl/EGTA extract of contractile gel. The molecular weights of standards, in kilodaltons, are shown at right. The standards used were: phosphorylase b, 94,000; BSA, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lysozyme, 14,300.

Monoclonal Antibody Characterization

The 0.3 M KCl/1 mM EGTA extract of the activated, contractile gel was used to immunize mice. After 3 mo of immunization, sera exhibited a strong response against several platelet proteins when used at a dilution of 1:2,000 in immunoblots. Spleen cells were used to prepare hybridomas by fusion with A653 myeloma cells. Antibody-producing hybridomas were screened by ELISA, immunoblot, and indirect immunofluorescence. Most of the monoclonal antibodies were to either a 260-kD protein, presumably platelet actin-binding protein, or to myosin. Three antibodies, 6D2, 8B5, and 23H2 recognized an unidentified 80-kD protein (Fig. 2). The three anti-80-kD antibodies have given identical results on immunoblots and immunofluorescence staining experiments.

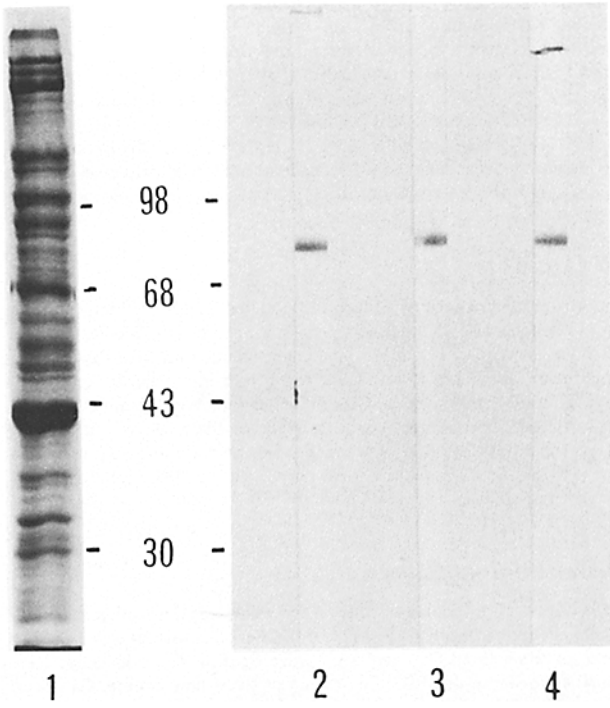

Figure 2. Comparison of immunoblots performed with the three monoclonal antibodies to 80-kD protein. After electrophoresis on 8% SDS polyacrylamide gels, whole platelet protein was transferred to nitrocellulose, and immunoblots were done using culture supernatants from the three hybridomas. Lane *1*, Coomassie Blue–stained gel; lane *2*, 6D2; lane *3*, 8B5; lane *4*, 23H2. Molecular weights are shown in kilodaltons.

Characterization of Monoclonal Antibodies by Indirect Immunofluorescence

All of the hybridomas were screened by indirect immunofluorescence using culture supernatant in the first incubation step. Positive clones were selected for expansion. The anti-80-kD antibodies stained the stress fibers in human cells (Fig. 3*A*) and cross-reacted with non-human cells, including mouse 3T3 cells (Fig. 3*B*) and bovine smooth muscle cells (Fig. 3*C*). The antibodies stained platelets, but little detail was visible (data not shown). The stress fibers were brightly stained in all instances, and the staining pattern appeared somewhat periodic, similar to that seen with anti-myosin antibodies (Fig. 3*D*). Double-label studies, not shown, indicated that the localization of the 80-kD protein in the cell was very similar to that of actin. The staining patterns seen with the three different anti-80-kD antibodies were indistinguishable; examples of all three are shown in Fig. 3.

Identification and Initial Characterization of the 80-kD Protein

Immunoblot analysis was done on samples from the various steps in the preparation of the platelet contractile gel. The 80-kD protein was present in whole platelets, in clarified platelet extract, and was enriched in the contractile gel (Fig. 4, lanes *1*, *2*, and *4*). In some preparations two closely spaced bands were resolved. The 80-kD protein was not present in low ionic strength-Ca^{++} extracts of the gel (Fig. 4, lane *5*), presumably because calmodulin was not present, but was removed by the 0.3 M KCl-EGTA extraction step (Fig. 4, lane *6*).

To examine the cross-reactivity and tissue distribution of the 80-kD protein, rat and mouse tissue samples and two cell lines, one human and one mouse, were tested by immunoblotting. A mixture of all three anti-80-kD monoclonal antibodies was used in the immunoblots to maximize the detection of cross-reacting proteins that were present in aorta, liver, spleen, uterus, and both types of fibroblasts (Fig. 5). The cross-reacting species in aorta, liver, spleen, and fibroblasts had a molecular weight of ~80,000, similar to the platelet peptide (Fig. 5, lanes *7–10*). The smooth muscle samples (Fig. 5, lanes *3*, *4*, *5*, and *6*) contained two sizes of peptides; the molecular weight of the major one was 140,000, and the minor one was 80,000. Uterus samples from mouse and rat contained both peptides. Chicken gizzard smooth muscle also contained a cross-reacting 140-kD peptide (Fig. 5, lane *5*). The stained gel of the whole tissue contained a prominent band at 140 kD (Fig. 6, lane *1*). All three anti-80-kD antibodies cross-reacted with the chicken gizzard protein with approximately the same intensity.

At least three proteins of ~140,000 mol wt have been purified from chicken gizzard; vinculin (8, 9), myosin light chain kinase (MLCK) (1, 7), and caldesmon (3, 20). Immunoblots were done with chicken gizzard protein using antibodies to vinculin and MLCK to determine if they were recognized by the anti-80-kD antibody. Anti–smooth muscle vinculin was the gift of Dr. Joann Otto (Purdue University, West Lafayette, IN), and anti-MLCK was the gift of Dr. Vincent Guerriero (Baylor College of Medicine). The results, shown in Fig. 6, demonstrate that vinculin and MLCK are of lower molecular weights than the 140,000-mol-wt peptide recognized by the anti-80-kD antibody.

Purification of Gizzard 140-kD Caldesmon and the Platelet 80-kD Protein

140-kD caldesmon was purified according to the published procedures of Bretscher (3). The result is shown in Fig. 7, lane *8*. All three anti-80-kD antibodies react strongly with purified gizzard caldesmon (see Fig. 7, lanes *8* and *9*). The platelet 80-kD protein was purified using a modification of the same

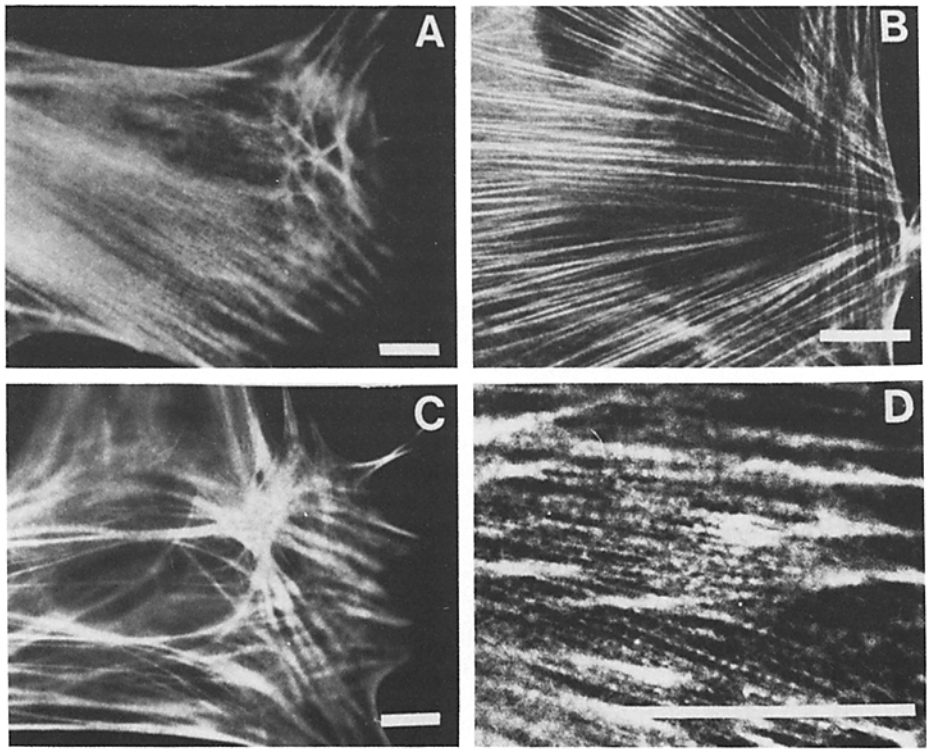

Figure 3. Immunofluorescence staining of cultured cells using anti-80-kD monoclonal antibodies. Various cell lines were grown on glass coverslips and processed for immunofluorescence as described in Materials and Methods. Fluorescein isothiocyanate–conjugated goat anti–mouse IgG was used as the second antibody. (*A*) Anti-80-kD, 23H2, staining of human foreskin fibroblasts; (*B*) anti-80-kD staining of mouse 3T3 cells; (*C*) anti-80-kD staining of cultured bovine smooth muscle cells with 8B5; (*D*) high magnification of stress fibers from human foreskin fibroblasts stained with anti-80-kD antibody 6D2. The scale bar in each panel equals 10 μm.

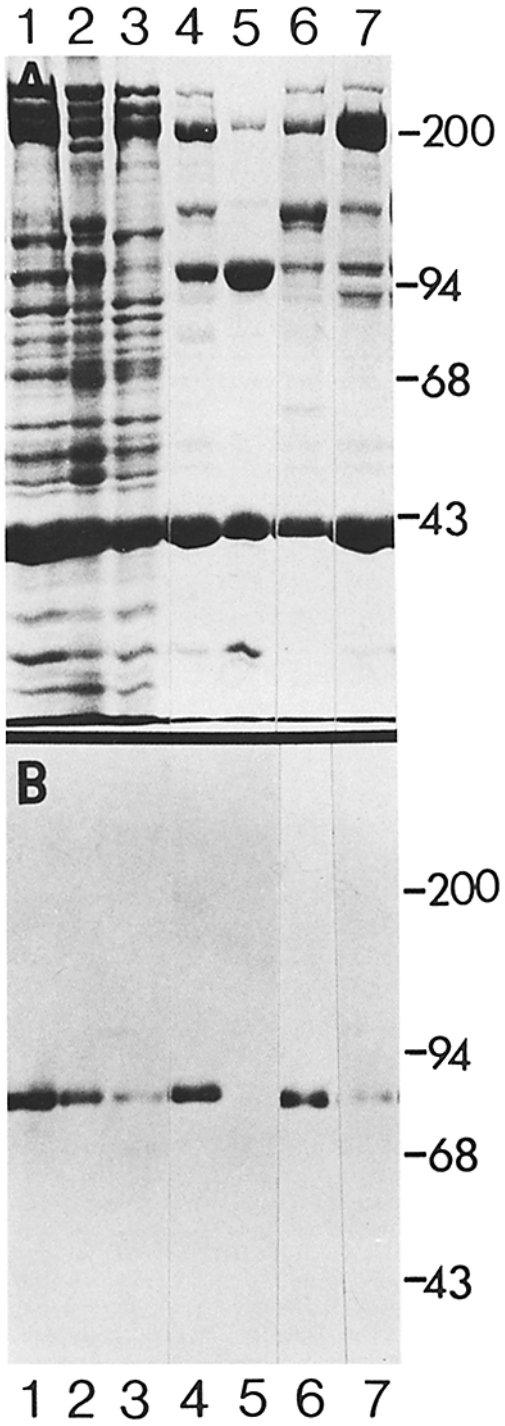

Figure 4. Distribution of 80-kD protein in platelet fractions. Samples from various stages in the preparation of contractile gel extracts were separated by electrophoresis on duplicate 8% SDS polyacrylamide gels. One of the gels was stained with Coomassie Blue (*A*) and the other was transferred to nitrocellulose and immunoblotted with monoclonal antibody 23H2 (*B*). Lane *1*, whole platelets; lane *2*, clarified platelet lysate; lane *3*, supernatant over the contractile gel; lane *4*, contractile gel; lane *5*, Ca^{++} extract of the contractile gel; lane *6*, 0.3 M KCl/EGTA extract of the contractile gel; lane 7, actomyosin residue. Molecular weights are shown in kilodaltons at right.

caldesmon isolation procedure (3). The heat-stable protein fraction of lysed platelets contained an 80-kD protein as a major cross-reacting component (Fig. 7 *B*, lane *4*); little cross-reacting 80-kD protein was found with the heat-precipitated material (Fig. 7 *B*, lane *3*). The major component of the heat-stable fraction was a 33–36-kD protein with the characteristics of platelet tropomyosin (Fig. 7 *A*, lane *4*). The 80-kD protein precipitated with 30–50% $(NH_4)_2SO_4$ (Fig. 7 *A*, lane *6*) along with major unidentified components with molecular weights of 72,000 and 62,000 that showed no cross-reactivity.

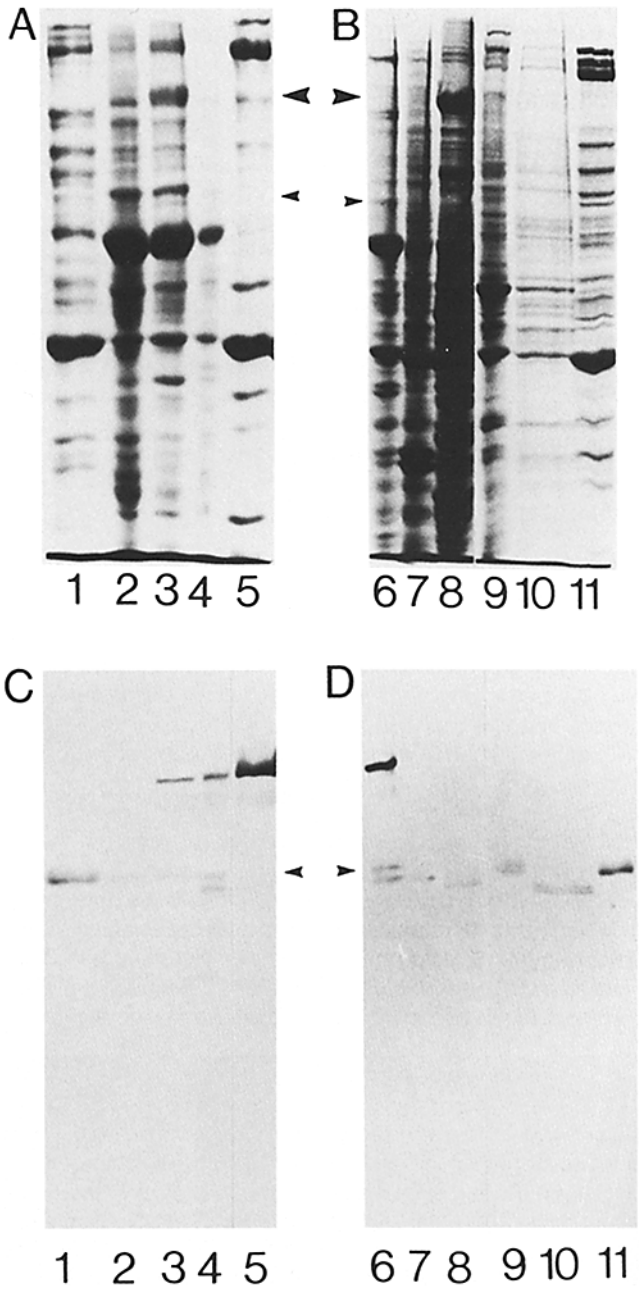

Figure 5. Distribution of 80-kD protein and cross-reacting peptides in cells and tissues. Rat tissue samples, cultured cells, and chicken gizzard homogenate were separated by electrophoresis on duplicate 10% SDS polyacrylamide gels. One gel was stained with Coomassie Blue (*A*), and one was transferred to nitrocellulose and immunoblotted with a mixture of the three anti-80-kD antibodies (*B*). Lane *1*, platelet lysate; lane *2*, rat aorta; lane *3*, mouse aorta; lane *4*, mouse uterus; lane *5*, chicken gizzard; lane *6*, rat uterus; lane *7*, rat spleen; lane *8*, rat liver; lane *9*, WI38 cell lysate; lane *10*, 3T3 mouse cell lysate; lane *11*, whole platelet lysate (control). Large arrowheads, ~140,000 mol wt; small arrowheads, ~80,000 mol wt.

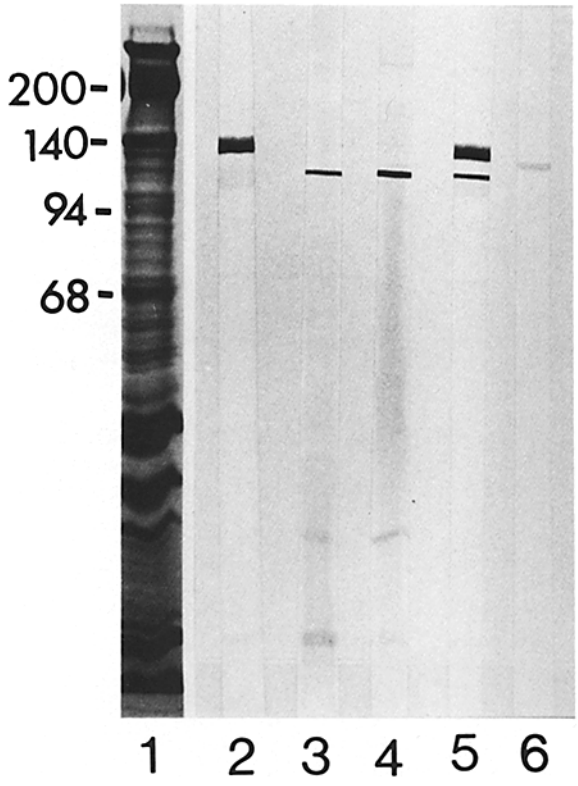

Figure 6. The 140-kD chicken gizzard protein is of different molecular weight than vinculin or MLCK. Immunoblots were performed on nitrocellulose transfers of whole gizzard homogenate. 8% SDS polyacrylamide gels were used. Lane *1*, Coomassie Blue–stained gel of whole gizzard; lane *2*, immunoblot using anti-80-kD antibody 23H2; lane *3*, immunoblot using anti–platelet vinculin; lane *4*, immunoblot using anti–gizzard vinculin; lane *5*, immunoblot using both 23H2 and anti–platelet vinculin; lane *6*, immunoblot with anti-MLCK. Molecular weights are shown in kilodaltons at left.

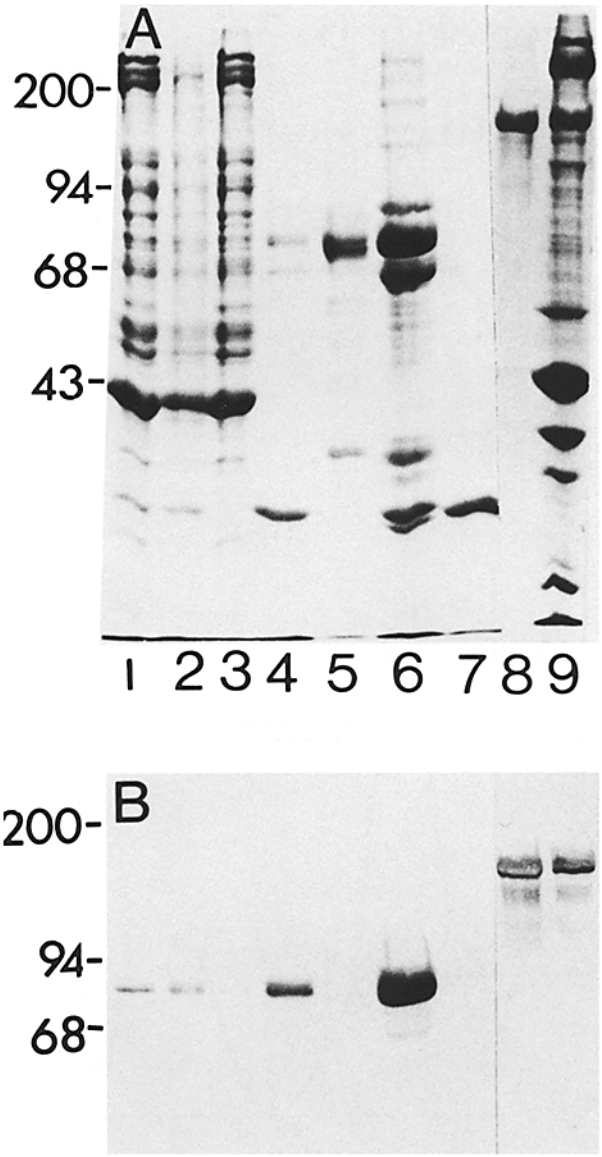

Figure 7. Immunoblot analysis of purification steps for platelet 80-kD protein. A heat-stable protein fraction was prepared from whole lysed platelets and was fractionated with $(NH_4)_2SO_4$. (*A*) Coomassie Blue–stained gel; (*B*) immunoblot using 23H2 antibody of gel in panel *A*. Lane *1*, whole platelet lysate; lane *2*, heated whole platelet lysate; lane *3*, heat-insoluble pellet; lane *4*, heat-stable extract; lane *5*, 30% $(NH_4)_2SO_4$ precipitate; lane *6*, 30–50% $(NH_4)_2SO_4$ precipitate; lane *7*, 50–65% $(NH_4)_2SO_4$ precipitate; lane *8*, purified caldesmon; lane *9*, whole gizzard homogenate. Molecular weights are shown in kilodaltons at left.

The 80-kD protein elutes from DEAE-Sephacel at 150–200 mM NaCl, approximately in fractions 43–50, at ~95% purity (Fig. 8 and inset). The protein could be purified initially or purified further by affinity chromatography on calmodulin–Sepharose. Pooled DEAE fractions, or alternatively the resuspended 30–50% $(NH_4)_2SO_4$ pellet, were dialyzed against 0.1 M NaCl, 20 mM Tris-HCl, 0.1 mM DTT, 0.1 mM EGTA, pH 7.5. $CaCl_2$ was added to 0.2 mM and the sample was loaded onto calmodulin–Sepharose equilibrated with 0.1 M NaCl, 20 mM Tris-HCl, 0.1 mM DTT, 0.2 mM $CaCl_2$. After washing with 0.2 M NaCl in the same buffer, bound protein was eluted with 0.2 M NaCl, 20 mM Tris-HCl, 0.1 mM DTT, 1 mM EGTA. Fig. 9 shows an elution profile for a purification from the entire heat-stable extract. Contaminants and some of the 80-kD protein are eluted with 0.2 M NaCl, but the majority of the 80-kD protein is eluted with EGTA. The purified protein gives two bands, visible at low protein loads, on SDS gels in approximately equimolar ratio; both are recognized by the antibody. The purified protein is fairly stable at 0°C and can be stored for at least 3 wk and retain activity.

Using polyclonal antibodies to 140-kD caldesmon, Bretscher and Lynch (4) have detected 80-kD protein(s) in heat stable extracts. In addition, Kakiuchi and co-workers have reported the detection of 150,000-mol-wt caldesmon-like proteins in platelets (12). These results suggest the 80-kD protein may be a proteolytic fragment. However, we have been unable to detect cross-reacting platelet proteins of greater than 80,000 mol wt even in samples of freshly drawn, gel-filtered platelets (16) treated with several proteolysis inhibitors. To test directly for proteolysis during platelet lysate preparation, purified 140-kD caldesmon was radioiodinated and used as a protease target. ^{125}I-Caldesmon was added to the whole platelet suspension (Fig. 10, lane *2*) before lysis by sonication or to a separate sample after sonication (Fig. 10, lane *3*). Neither sample showed significant degradation com-

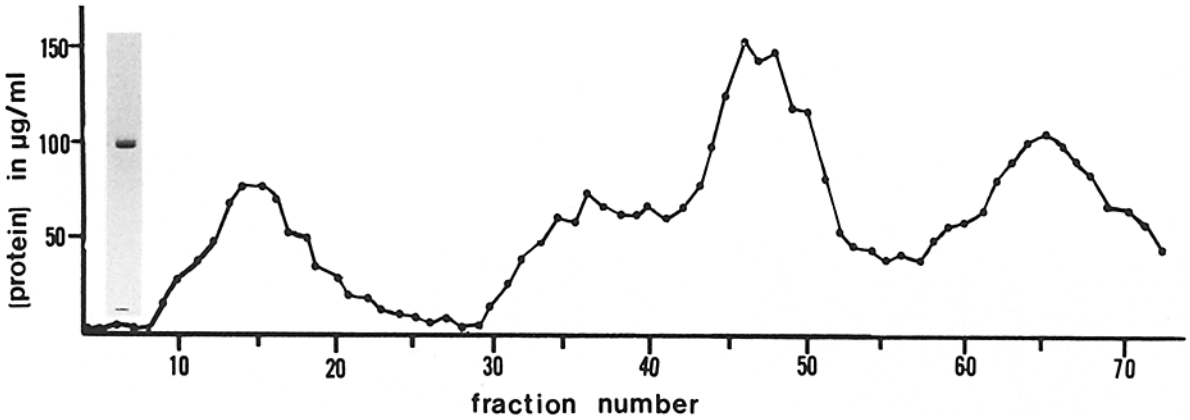

Figure 8. DEAE chromatography of the 30–50% $(NH_4)_2SO_4$ fraction from the heat-stable platelet extract. The 30–50% $(NH_4)_2SO_4$ fraction from a heat-stable platelet extract was chromatographed on DEAE-Sephacel. The protein content of each fraction was determined and analyzed by SDS polyacrylamide gel electrophoresis. 80-kD caldesmon elutes at 150–200 mM NaCl in fractions 42–52. The inset at the left is the SDS gel of the peak fraction, number 45.

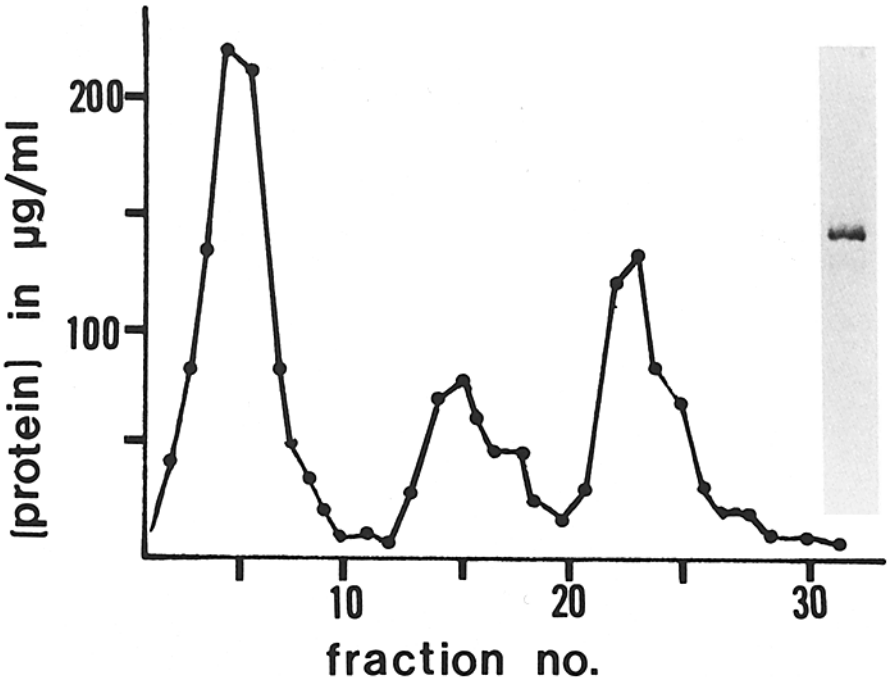

Figure 9. Affinity chromatography of 80-kD protein on calmodulin–Sepharose. An entire heat-stable extract was chromatographed on a 5-ml calmodulin–Sepharose affinity column. The column was eluted with the starting buffer, with 200 mM KCl plus 0.2 mM $CaCl_2$ and with 200 mM KCl plus 1 mM EGTA. The buffers were changed at fractions 9 and 19, respectively. The protein profile of the fractions is shown. The 80-kD protein elutes with EGTA. The inset at right is the peak fraction, 23, as it appears on a 10% SDS gel.

pared with the starting material (Fig. 10, lane *9*). Similarly, there was no increase in degradation after further processing of the lysates (Fig. 10, lanes *5* and *6*). The size of the endogenous peptide detected by immunoblotting identically handled samples that did not contain ^{125}I-caldesmon was 80,000 mol wt (data not shown). We conclude that the 80-kD protein is not a breakdown product of a larger platelet caldesmon and that 80,000 mol wt is the native size of platelet caldesmon.

Comparison of the Actin-binding Activities of 140-kD Caldesmon and the 80-kD Protein

The two purified proteins were used in a viscosity assay to determine if the actin cross-linking activities of 80-kD caldesmon were similar to those of 140-kD caldesmon. Samples were prepared by mixing either 80- or 140-kD caldesmon with F-actin at molar ratios ranging from 1:200 to 1:15, then incubating for 1 h at 20°C before measuring the viscosity. Fig. 11 shows the results. Both proteins increased the viscosity of F-actin, but the gizzard protein had a greater effect than the 80-kD platelet protein at all concentrations tested. In addition, the actin cross-linked with gizzard caldesmon appeared to undergo a marked reduction in viscosity at high molar ratios, consistent with the bundling of actin filaments that has been reported for gizzard caldesmon (3). At the concentrations used, the 80-kD caldesmon did not decrease the viscosity markedly at high molar ratios.

Centrifugation experiments show that both proteins sediment with F-actin (Fig. 12, lanes *4* and *8*). At a molar ratio of caldesmon to actin of 1:20, both proteins bind to the actin filaments, and we detect little caldesmon that is not associated with the actin pellet. Inclusion of Ca^{++} had no effect on the binding of either protein to actin filaments. Neither the 80- nor the 140-kD protein sediments alone (Fig. 12, lanes *5* and *9*) under conditions that will pellet F-actin alone (Fig. 12, lane *2*).

The binding of 80-kD caldesmon or 140-kD caldesmon to

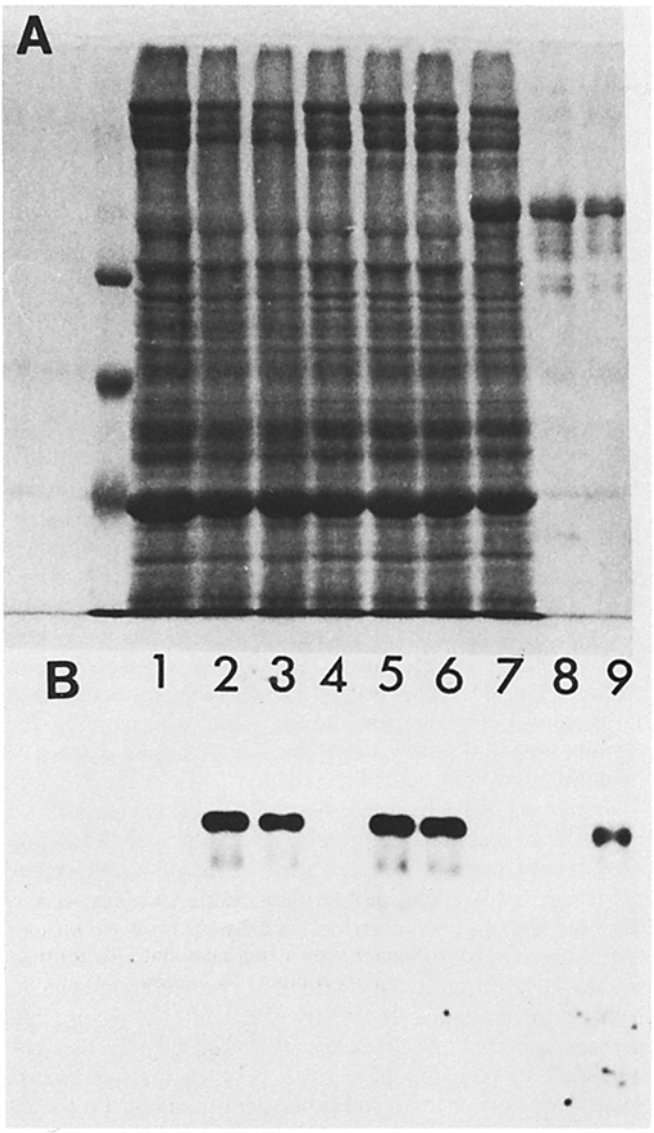

Figure 10. Incubation of radio-iodinated 140-kD caldesmon with platelet lysates. ^{125}I-labeled chicken gizzard caldesmon was added to duplicate samples of washed platelets, either before or after sonication and then incubated for 2 h at 4°C. Samples were prepared for electrophoresis, run on 8% SDS gels, stained, dried, and autoradiographed. (*A*) Coomassie Blue–stained gel; (*B*) autoradiogram of gel in *A*. Lane *1*, whole platelets (control); lane *2*, whole platelet lysate with ^{125}I-caldesmon added before sonication; lane *3*, whole platelet lysate with ^{125}I-caldesmon added after sonication; lane *4*, control sample after KCl addition and 2-h extraction; lane *5*, same sample as in lane *2* after KCl addition and extraction; lane *6*, same sample as in lane *3* after KCl addition and extraction; lane *7*, same sample as in lane *1* with 1 μg gizzard caldesmon added; lane *8*, gizzard caldesmon; lane *9*, unlabeled gizzard caldesmon, ~5 μg, with ~0.1 μCi, <50 ng, of ^{125}I-caldesmon added.

F-actin is inhibited in the presence of calcium and calmodulin (Fig. 13). Calmodulin will inhibit the binding of 80-kD protein and 140-kD caldesmon to actin filaments, but a large excess of calmodulin relative to caldesmon is required for complete inhibition of the actin-binding activity. At a two- to fourfold molar ratio of calmodulin to caldesmon, only ~50% of the caldesmon is inhibited from binding to actin filaments and

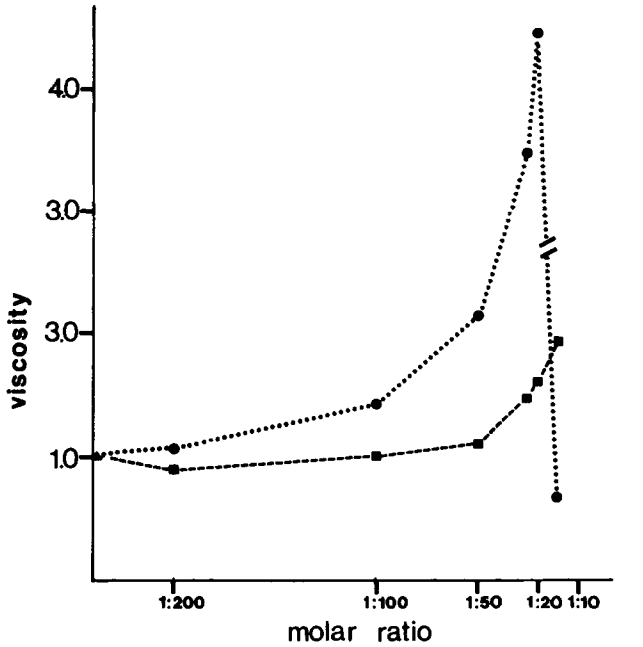

Figure 11. Low-shear viscometry of actin plus 80- or 140-kD caldesmon. 140- or 80-kD caldesmon was mixed with F-actin in polymerizing buffer, incubated, and the viscosity measured. The results are expressed as the ratio of the viscosity of the sample to the value for the actin control. The circles are the data for 140-kD caldesmon; the squares are the data for 80-kD caldesmon.

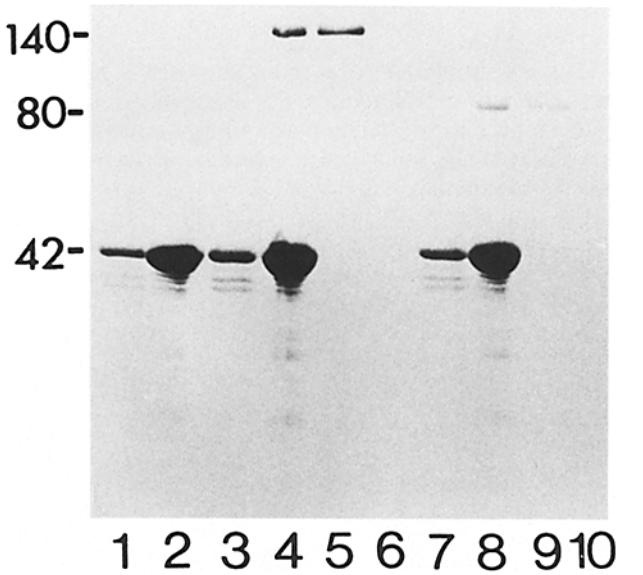

Figure 12. Sedimentation analysis of the interaction of 140- and 80-kD caldesmon with actin. F-actin was mixed with 140 or 80-kD caldesmon and incubated for 1 h before centrifugation in an airfuge for 30 min at 30 psi to sediment the actin filaments. The supernatants and pellets were analyzed on 10% SDS polyacrylamide gels. The final concentration of protein in the samples was as follows: actin, 12 μM; 80- or 140-kD caldesmon, 0.6 μM. The even-numbered lanes are the pellets, and the odd-numbered lanes are the corresponding supernatants. Lanes *1* and *2*, actin only; lanes *3* and *4*, actin with 140-kD caldesmon; lanes *5* and *6*, 140-kD caldesmon alone; lanes *7* and *8*, actin with 80-kD caldesmon; lanes *9* and *10*, 80-kD caldesmon alone.

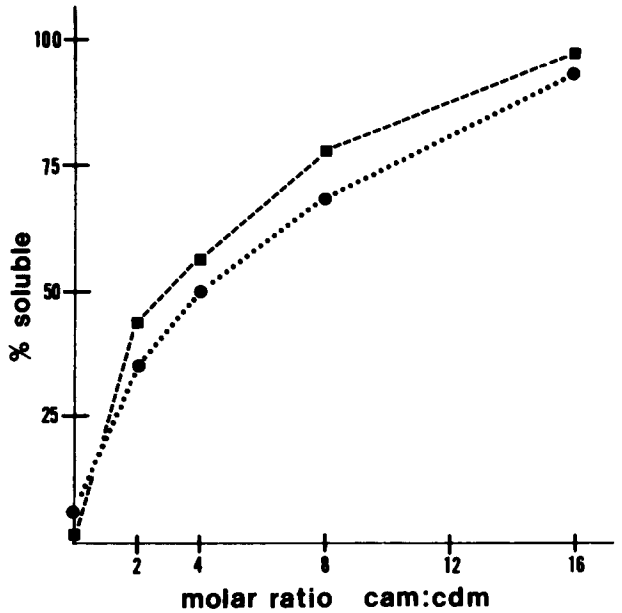

Figure 13. Inhibition of 140- and 80-kD caldesmon binding to actin by calmodulin. Samples of 80- and 140-kD caldesmon were mixed with actin, in the presence of increasing amounts of calmodulin. All samples were run in the presence of calcium. The samples were incubated for 1 h, sedimented, and analyzed on 12% SDS gels. The amount of 140- or 80-kD caldesmon remaining in the supernatant was quantitated by densitometric scanning. The data are plotted as the percent of 80-kD caldesmon (■) or 140-kD caldesmon (●) soluble relative to the molar ratio of calmodulin to caldesmon in the sample. The actin concentration was 12 μM. The caldesmon 140 or 80 kD concentration was 0.5 μM, and the calmodulin concentration varied from 1 μM to 8 μM. *cam*, calmodulin; *cdm*, caldesmon.

found in the supernatant over the actin pellet. A ratio of at least 8–10 mol of calmodulin per mole of caldesmon is required for complete inhibition of binding to actin filaments. Approximately the same molar excess is required by both types of caldesmon.

Discussion

We have generated hybridomas and produced monoclonal antibodies to a previously unknown 80,000-mol-wt cytoskeletal protein from human platelets. This protein is a minor component of the platelet contractile actin gel, but was enriched in the 0.3 M KCl supernatant used to immunize mice for hybridoma production. Most of the antibodies produced from this fusion were against known platelet cytoskeletal proteins (Dingus, J., S. Hwo, and J. Bryan, unpublished results). Three were against the same 80,000-mol-wt protein. These anti-80-kD antibodies cross-reacted with proteins of the same size in nonmuscle tissues, but recognized caldesmon, a protein of higher molecular weight in smooth muscle. The immunofluorescence localization of the protein on stress fibers in cultured cells was identical to that reported for caldesmon (4, 19).

We have purified the 80-kD platelet protein and compared its properties to those of chicken gizzard caldesmon. Both proteins are relatively heat stable and can be purified from heat-stable cell extracts. The proteins have similar salt precipitation and DEAE binding behavior and both will bind to calmodulin–Sepharose in the presence of calcium and are eluted with EGTA. Additionally, both proteins bind to actin filaments and the binding can be inhibited by calmodulin. In the presence of calcium and calmodulin, there is reduced binding of caldesmon and the 80-kD protein to actin filaments, although complete inhibition of binding requires a significant excess of calmodulin, which suggests the affinity of caldesmon for actin is significantly greater than for calcium–calmodulin. Calcium, or calmodulin in EGTA-containing buffers, have no effect on the actin-binding activity of either protein. Based on the functional similarities of the two proteins, and on the monoclonal antibody cross-reactivity, we have concluded that the 80-kD protein is the native form of platelet caldesmon.

The obvious differences between the two caldesmons are the molecular weights and the cross-linking activities. We find no evidence to suggest that 80-kD platelet caldesmon is actually a breakdown product of a larger caldesmon in platelets. Our antibodies do not detect any larger proteins in platelets and 140-kD caldesmon, added to the platelet lysates, is not degraded during our handling procedures. It is not clear how to reconcile our results with those of Kakiuchi and co-workers (12), who have reported on a 150,000-mol-wt caldesmon in platelets. One possibility is that the polyclonal antibodies used by these workers were not monospecific. Alternatively, the platelet preparations used may have had varying degrees of contamination with white blood cells.

Bretscher (3) has reported that the 140-kD caldesmon is a potent actin cross-linking protein that can induce the formation of very large bundles of actin filaments visible in the phase microscope. This ability of caldesmon to cross-link is disputed and has been described as an artifact due to protein aggregation (23). We detect little cross-linking activity, defined by low shear viscosity, with the 80-kD caldesmon, but do confirm the cross-linking activity of the 140-kD gizzard caldesmon reported by Bretscher. It is conceivable that the difference in cross-linking activity between the two caldesmons is due either to an additional actin-binding site or to a site for self-association on the 140-kD protein that is missing on the 80-kD caldesmon. This would allow the 140-kD species to directly cross-link filaments or to associate then cross-link filaments. Clearly more detailed structural studies will be required to resolve these questions and determine if these proteins are derived from separate genes.

The function of caldesmon in cells is under study. Caldesmon is one of the few actin-binding proteins that may be regulated directly by calmodulin (4, 20). Caldesmon can interfere with the interaction of actin and myosin (17, 21). Kakiuchi and co-workers have proposed that caldesmon is a regulatory protein that controls contraction by a "flip-flop" mechanism (20–22). This model proposes that the interaction of actin and myosin is regulated by caldesmon, calmodulin, and Ca^{++}. Under low calcium conditions, actin filaments would have caldesmon bound. When intracellular calcium levels rise above micromolar, the caldesmon actin-binding activity would be inhibited by the binding of calmodulin to caldesmon and allow myosin binding. These conditions also allow activation of MLCK and phosphorylation of myosin. The effect of 80-kD caldesmon on the actomyosin interaction is unknown, but clearly it could have the same putative regulatory functions as 140-kD caldesmon since it binds to

actin filaments and is regulated by calmodulin. One puzzling fact for both proteins is the large excess of calmodulin required for the inhibition of the actin-binding activity. The affinity of both proteins for calmodulin appears to be low and the binding to calmodulin can be disrupted at fairly low salt concentrations, unlike prototype calmodulin binding interactions. The existence of two sizes is interesting but further complicates the picture. The low cellular concentration and the observation that one form does not effectively cross-link filaments suggests a regulatory rather than a structural role for these molecules.

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References

1. Adelstein, R. S., and C. B. Klee. 1981. Purification and characterization of smooth muscle light chain kinase. *J. Biol. Chem.* 256:7501–7509.

2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

3. Bretscher, A. 1984. Smooth muscle caldesmon. Rapid purification and F-actin crosslinking properties. *J. Biol. Chem.* 259:12873–12880.

4. Bretscher, A., and W. Lynch. 1985. Identification and localization of immunoreactive forms of caldesmon in smooth and non-muscle cells: a comparison with the distributions of tropomyosin and alpha-actinin. *J. Cell Biol.* 100:1656–1663.

5. Bryan, J. 1986. Isolation and characterization of platelet gelsolin. *Methods Enzymol.* In press.

6. Carron, C. P., S. Hwo, J. Dingus, D. M. Benson, I. Meza, and J. Bryan. 1986. A re-evaluation of cytoplasmic gelsolin localization. *J. Cell Biol.* 102:237–245.

7. Dabrowska, R., and D. J. Hartshorne. 1978. A Ca^{++} and modulator-dependent myosin light chain kinase from non-muscle cells. *Biophys. Biomed. Res. Commun.* 85:1352–1359.

8. Feramisco, J. R., and K. Burridge. 1980. A rapid purification of alpha-actinin, filamin, and a 130,000 dalton protein from smooth muscle. *J. Biol. Chem.* 255:1194–1199.

9. Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193–205.

10. Hou, D. C., J. Dingus, G. C. Rosenfeld, and J. Bryan. 1983. Actin gelation in platelet extracts. *J. Cell Biol.* 97(5, Pt. 2):276a. (Abstr.)

11. Hwo, S., and J. Bryan. 1986. Immuno-identification of Ca^{2+}-induced conformational changes in human gelsolin and brevin. *J. Cell Biol.* 102:227–236.

12. Kakiuchi, R., M. Inui, K. Morimoto, K. Kanda, K. Sobue, and S. Kakiuchi. 1983. Caldesmon, a calmodulin-binding protein, is present in aorta, uterus, and platelets. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 154:351–356.

13. Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

14. MacLean-Fletcher, S., and T. D. Pollard. 1980. Identification of a factor in conventional muscle actin preparations which inhibits actin filament self-association. *Biophys. Biomed. Res. Commun.* 96:18–27.

15. MacLean-Fletcher, S., and T. D. Pollard. 1980. Viscometric analysis of the gelation of *Acanthamoeba*. Extraction and purification of two gelation factors. *J. Cell Biol.* 85:414–428.

16. Nachmias, V. T. 1980. Cytoskeleton of human platelets at rest and after spreading. *J. Cell Biol.* 86:795–802.

17. Ngai, P. K., and M. P. Walsh. 1984. Inhibition of smooth muscle actin-activated myosin Mg^{++}-ATPase activity by caldesmon. *J. Biol. Chem.* 259:13656–13659.

18. Ngai, P. K., and M. P. Walsh. 1985. Detection of caldesmon in muscle and non-muscle tissues of the chicken using polyclonal antibodies. *Biophys. Biomed. Res. Commun.* 127:533–539.

19. Owada, M. K., A. Hakura, K. Ida, I. Yahara, K. Sobue, and S. Kakiuchi. 1984. Occurrence of caldesmon (a calmodulin-binding protein) in cultured cells: comparison of normal and transformed cells. *Proc. Natl. Acad. Sci. USA.* 81:3133–3137.

20. Sobue, K., Y. Muramoto, M. Fujita, and S. Kakiuchi. 1981. Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. *Proc. Natl. Acad. Sci. USA.* 78:5652–5655.

21. Sobue, K., K. Morimoto, M. Inui, K. Kanda, and S. Kakiuchi. 1982. Control of actin-myosin interaction of gizzard smooth muscle by calmodulin- and caldesmon-linked flip-flop mechanism. *Biomed. Res.* 3:188–196.

22. Sobue, K., K. Morimoto, K. Kanda, K. Maruyama, and S. Kakiuchi. 1982. Reconstitution of Ca^{++}-sensitive gelation of actin filaments with filamin, caldesmon, and calmodulin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 138:289–292.

23. Sobue, K., K. Takahashi, T. Tanaka, K. Kanda, N. Ashino, S. Kakiuchi, and K. Maruyama. 1985. Crosslinking of actin filaments is caused by caldesmon aggregates but not by its dimers. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 182:201–204.

24. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.* 246:4866–4871.

25. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4355.