Isolation and Partial Characterization of Human Platelet Vinculin

G. C. ROSENFELD,* D. C. HOU,‡ J. DINGUS,‡ I. MEZA,§ and J. BRYAN‡
*Department of Pharmacology, The University of Texas Medical School of Houston, Texas 77025; ‡Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030; and §Department of Cell Biology, Centro del Instituto Politecnico Nacional, Mexico City, Mexico

ABSTRACT A 130,000 M_r protein was isolated from human platelets by sequential DEAE-Sephacel and Sepharose Cl-4B chromatography. Low shear viscometric measurements showed that the enriched protein after DEAE-Sephacel chromatography inhibited actin polymerization. This effect was somewhat greater in the presence of EGTA than in the presence of calcium. Further purification by Sepharose Cl-4B chromatography resulted in a complete loss of this inhibitory effect. Studies with fluorescent actin detected no nucleation or "+" end capping activity in either the DEAE-Sephacel- or Sepharose Cl-4B-purified vinculin. Antibodies raised in mice against the 130,000-mol-wt protein were shown to cross-react with chicken gizzard vinculin and a similar molecular weight protein was detected in WI38 cells and, Madin-Darby canine kidney cells. Lysis experiments with the Madin-Darby canine kidney cells indicated that most of the vinculin was soluble in Triton X-100, although some was found associated with the insoluble cytoskeletal residue. By immunofluorescence, vinculin in WI38 cells was localized to adhesion plaques as described by others. Discrete localization in platelets was also detected and appeared to depend on their state of adhesion and spreading. The results of these experiments suggest that human platelets contain a protein similar to vinculin. It is not clear if platelet vinculin is associated with structures analogous to adhesion plaques found in other cell types. The data indicate that the previously reported effects of nonmuscle vinculins on actin polymerization may be due to a contaminant or contaminants.

The organization of the actin filament network that participates in a number of cellular activities including cell division, ciliary movement, cytokinesis, endocytosis, membrane reorganization, and secretion appears to be modulated by proteins that are subject themselves to higher order regulation (for review see references 1 and 2). One such protein, vinculin, with an M_r of 130,000, has been isolated from smooth muscle and HeLa cells (3, 4). By use of immunofluorescence methods, vinculin has been localized to adhesion plaques at the termini of actin stress fibers in cultured fibroblasts (3, 4) and in striated muscle (5), and to the junctional regions of cardiac muscle cells by use of immunoelectron microscopy (6–8). The biochemical studies on the effects of vinculin on actin assembly have been controversial. Jockusch and Isenberg (9) reported that vinculin binds to actin filaments and forms tightly packed paracrystalline bundles of 4–20 filaments that may be several hundred microns in length. They also (9) reported results of physical studies consistent with bundling, namely, a reduction in the viscosity of polymerized actin, but they demonstrated no influence on filament nucleation.

Wilkins and Lin (10) reported a calcium-independent reduction in the viscosity of F-actin by smooth muscle vinculin. Their results suggested that vinculin was bound tightly to the "+" ends of actin filaments, would increase the critical concentration of actin required for polymerization, and would inhibit both filament assembly nucleated by spectrin-actin complexes and filament-filament interactions. Wilkins and Lin (10) detected little paracrystal formation and suggested that bundling might be due to a low affinity binding of vinculin to actin. Using somewhat different experimental protocols, Burridge and Feramisco (11) reported results similar to those of Wilkins and Lin (10) for both smooth muscle vinculin and HeLa cell vinculin. The results with nonmuscle vinculin were reported to be similar to those with chicken gizzard vinculin except that calcium was necessary for activity.

Two studies have shown that platelets contain a 130,000

M_r vinculinlike protein. Langer et al. (12) used antibodies for vinculin, prepared against the chicken gizzard protein, to show the presence of a cross-reacting molecule in human platelets. Recently, Koteliansky et al. (13) purified a vinculin-like protein from platelets. This material cross reacts with antibodies against chicken gizzard vinculin, has physical properties similar to those reported for other vinculins (3, 4, 9, 10), and decreases the viscosity of F-actin solutions.

Recent biochemical work (14–17) shows that most of the preparations of vinculin used previously contain small amounts of other contaminating proteins. The report by Evans et al. (17) demonstrates little direct interaction of phosphocellulose-purified vinculin with actin. These studies suggest that other vinculin binding proteins, like those reported by Burridge (18, 19) and Otto (20), will be important in actin–vinculin linkages.

In this study, we have isolated a 130,000 M_r protein from human platelets with the characteristics of vinculin. Low shear viscometric studies showed an apparent effect of this DEAE-purified material on the viscosity of F-actin. This activity was lost upon further purification of the 130,000-mol-wt molecule by gel filtration. The results are similar to those reported for muscle vinculin in recent preliminary communications (14–17) and demonstrate that a nonmuscle vinculin does not interact directly with actin. We have also shown that antibodies against platelet vinculin cross-react with chicken gizzard vinculin, will stain adhesion plaques in WI38 cells, and show patterns of localization in spreading human platelets.

MATERIALS AND METHODS

Actin Purification: Actin was purified from rabbit skeletal muscle by the method of Spudich and Watt (21) then gel filtered on Sephadex G-150 following the procedure of MacLean-Fletcher and Pollard (22). We determined actin concentrations using an extinction coefficient at 290 nm of 0.65 for 1 mg/ml.

Platelet Extract Preparation: Platelet concentrates were used within 5 d after blood was drawn from donors. Erythrocytes and white blood cells were removed by centrifugation at 1,600 rpm, ~600 *g*, in a Sorvall HG-4L rotor (Dupont Instruments-Sorvall, Newton, CT) for 15 min. Platelets were pelleted at 4,200 rpm, ~4,500 *g*, in the same rotor for 30 min. The platelet pellets were gently resuspended in 25–30 vol of 125 mM NaCl, 5 mM KCl, 0.1 mM EDTA, 20 mM phosphate, pH 6.5, and 1 mg/ml of glucose, then collected by centrifugation at 3,700 rpm for 20 min in the HG-4L rotor. We repeated this washing procedure three times. Washed platelets were resuspended in ice-cold 1 mM ATP, 5 mM dithiothreitol, 20 mM EGTA, and 20 mM potassium phosphate, pH 7.0. Typically, 2 ml of buffer were used per unit, ~50 ml of platelet rich plasma. The resuspended platelets were then lysed by sonication with a sonifier (Branson Sonic Power Co., Danbury, CT). The total sonication time was ~9 min, consisting of 18 30-s bursts interspersed with 30-s rests to minimize heating. The crude platelet lysate was brought to a final concentration of 0.6 M KCl by the addition of solid KCl then stirred for 2 h at 4°C. A high salt supernatant was obtained by centrifugation at 100,000 *g* for 60 min then dialyzed against 50 mM KCl, 1 mM EGTA, 10 mM imidazole-HCl, pH 6.8. This dialysis step produces a substantial polymerization and gelation of actin and actin-associated proteins that eventually contract into a dense precipitate that we refer to as a contractile gel. This material was removed by centrifugation at 5,000 *g* for 10 min. The contractile gel consists of actin, myosin, and other actin-associated proteins, but little of the 130,000 M_r species. The gel-depleted extract was clarified further by centrifugation at 100,000 *g* for 60 min. A vinculin enriched fraction was obtained by ammonium sulfate fractionation of the 100,000 *g* supernatant. The pellet from a 0–35% ammonium sulfate cut was resuspended in, and dialyzed overnight against, buffer B from Feramisco and Burridge (23), 20 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, 20 mM Tris-acetate, pH 7.6, and clarified by centrifugation at 100,000 *g* for 60 min. We have used varying amounts of material but usually start with from 50 to 100 U of outdated platelets. This typically yields 75 to 150 ml of high salt extract at a protein concentration of 7 to 10 mg/ml. We have not attempted to determine the yield of vinculin from the following procedure with much precision because we have relatively little control over the quality of the starting material when working with large numbers of platelets. However, we can routinely recover 8–10 mg vinculin/100 U of the DEAE step and 6–8 mg from the gel filtration step. This is enough material for a number of experiments and can be combined with our procedures for isolation of gelsolin (24).

Isolation and Purification of Human Platelet Vinculin: Approximately 50 mg of the 35% ammonium sulfate fraction was loaded onto a 2.5 × 25 cm DEAE-Sephacel column equilibrated with buffer B. Alternatively, 200–250 mg of the contractile gel depleted extract was used after dialysis against buffer B. The column was washed with three column volumes of the equilibrating buffer and eluted with a 20–400 mM NaCl gradient, 1,000 ml total volume, approximately as described by Feramisco and Burridge (23). The first elution peak, which contained a highly enriched 130,000-mol-wt protein, was pooled, concentrated by ammonium sulfate precipitation (35%), and dialyzed against buffer B. For further purification, ~5 mg of the 130,000-mol-wt protein fraction was applied to a 2.5 × 100 cm Sepharose CL-4B column equilibrated and eluted with buffer B. The fractions containing the 130,000-mol-wt protein were pooled and reconcentrated by ammonium sulfate precipitation then resuspended in and dialyzed against buffer B.

Preparation of Antiserum Against Human Platelet Vinculin: BALB/c mice were immunized initially with 20 μg Sepharose CL-4B purified platelet vinculin these boosted three times at monthly intervals with 20 μg protein. We determined antibody titers 2 wk after each boost with an enzyme-linked immunoabsorption assay using horseradish peroxidase-conjugated goat anti-mouse antibodies from Cappel Laboratories, Inc. (Downington, PA) and 2,2′-azino-di-[3-ethyl-benathiazoline sulfonate (6) from Boehringer Mannheim as the enzyme substrate.

Vinculin Localization in Cells by Indirect Immunofluorescence: Cells of the human line WI38 grown on glass coverslips, were fixed in 3% formaldehyde-1% dimethylsulfoxide in PBS, lysed in acetone at −20°C, and processed for indirect immunofluorescence by using the mouse antivinculin serum as the first antibody and fluorescein isothiocyanate conjugated goat anti-mouse serum as the second. Human platelets were allowed to spread on glass coverslips for 20 min, then processed for immunofluorescence studies as described above. The fluorescein isothiocyanate-labeled goat anti-mouse antibodies were purchased from Miles Laboratories, Inc. (Elkhart, IN).

Cytoskeletal Localization of Vinculin: Confluent Marbin-Darby Canine Kidney (MDCK)[1] cell monolayer cultures were washed in PBS, then immediately lysed in a buffer containing 100 mM NaCl, 10 mM Tris, pH 7.4, with 0.5% Triton and either 0.1 mM EDTA or 0.1 mM $CaCl_2$. In some instances we dissociated cells from the culture plates with 0.1% trypsin before lysis.

Low Shear Viscometry: We did low shear viscometry using a falling-ball assay according to MacLean-Fletcher and Pollard (25). Approximately 0.25 mg/ml of gel-filtered actin in 25 mM KCl, 1 mM $MgCl_2$, 0.5 mM ATP, 20 mM PIPES, pH 6.8, with either 0.1 mM $CaCl_2$ or 0.275 mM EGTA was mixed with increasing amounts of the 130,000 protein, immediately drawn into a 100-μl capillary pipette, then allowed to polymerize for 3 h. A stainless-steel ball was pushed through the meniscus and timed over a 2-cm fall. Fall times were converted to apparent viscosity in centipoise using glycerol standards.

Actin Assembly: A fluorescent actin assembly assay (24) was used to test for nucleation activity associated with the 130,000-mol-wt protein during purification. We prepared fluorescent nitrobenzooxadiazole (NBD)-actin by the procedure of Detmers et al. (26). Rabbit skeletal muscle actin was derivatized at cysteine residue 374 with *N*-ethylmaleimide and then reacted with NBD chloride. Fluorescence measurements were done with an Aminco SPF-500 spectrofluorometer (SLM Instruments, Inc., Urbana, IL) temperature regulated at 25°C. The excitation wave length was 475 nm; the emission wavelength was 530 nm. NBD-F-actin has approximately a 2-to-2.2 fold higher quantum yield than NBD-G-actin and the fluorescence changes associated with polymerization are a direct measure of the conversion of monomer to polymer (26). The rate of fluorescence change during polymerization is a function of the number of growing filament ends, the rate(s) of monomer addition to these ends, and the concentration of monomeric actin. To monitor actin assembly, we mixed 1.1 ml of a solution of the 130,000-mol-wt protein in 1 mM $CaCl_2$, 10 mM Tris, pH 7.2, with 50 μl of a 1.8–2.2 mg/ml solution of NBD-G-actin. This was followed immediately by 50 μl of a 2 M KCl, 40 mM $MgCl_2$ solution to initiate assembly. The maximum slope of the reaction was used as a measure of NBD-actin polymerization.

Miscellaneous: We did immunoblots according to Towbin et al. (27) and ran SDS-polyacrylamide gels according to Laemmli (28). Protein concen-

[1] *Abbreviations used in this paper:* MDCK, Madin-Darby canine kidney; NBD, nitrobenzooxadiazole.

trations were determined by a modification of the Bradford method (29), with bovine serum albumin as a standard.

RESULTS

Purification of Human Platelet Vinculin

The procedure described by Feramisco and Burridge for chicken gizzard (23) was used to prepare a 130,000 M_r enriched protein fraction from human platelets. A protein profile from the DEAE-Sephacel purification step is shown in Fig. 1*A* with a corresponding SDS gel of various fractions (Fig. 1*B*). The first peak centered at fraction 40 is enriched in the 130,000-mol-wt protein. In this separation, fractions 35 to 44 were pooled and precipitated with ammonium sulfate, then dialyzed against buffer B. This material was assayed for effects on actin assembly or fractionated further by Sepharose Cl-4B chromatography. The protein profile for one Sepharose Cl-4B gel filtration experiment is given in Fig. 2*A*. The bulk of the protein elutes as a sharp peak resolved from higher molecular weight contaminants and aggregated material, which elutes in the early fractions. SDS gels of the pooled vinculin fractions are shown in the inset of Fig. 2 and in Fig. 5, lane *5*.

Gel-filtered Vinculin Does Not Affect the Viscosity of F-Actin

The effects of the 130,000-mol-wt protein, at two stages of purification, on the viscosity of F-actin were compared by using a falling-ball viscometer. The results are shown in Fig. 3. The filled symbols give the results for DEAE-purified vinculin in the presence of 0.1 mM Ca^{++} (triangles) or 0.275 mM EGTA (circles). The vinculin enriched fraction from DEAE Sephacel (filled symbols) chromatography reduced the viscosity of F-actin with an activity that was somewhat more effective at lower Ca^{++} concentrations. In EGTA this material reduced the low-shear viscosity of 0.25 mg/ml of F-actin by 90% at a concentration of ~20 μg/ml, with a half-maximal effect seen at ~8 μg/ml. The molar ratio of the major 130,000-mol-wt protein to actin at this latter concentration was ~1:100. The concentration of possible active factors must be 10- to 100-fold lower. In the presence of calcium a similar effect was seen; however, the concentration necessary for a half-maximal effect was approximately three times greater. Very different results were found for the vinculin purified further on Sepharose. The open symbols in Fig. 3 give the results for platelet vinculin purified by gel-filtration on Sepharose CL-4B after DEAE chromatography. There was no effect of this material on the viscosity of F-actin in either calcium or EGTA at vinculin concentrations as high as 60 μg/ml. The viscosity data argue against bundling and or crosslinking of filaments by purified platelet vinculin.

Platelet Vinculin Does Not Affect Actin Assembly

We used the NBD-actin assembly assay to look for effects

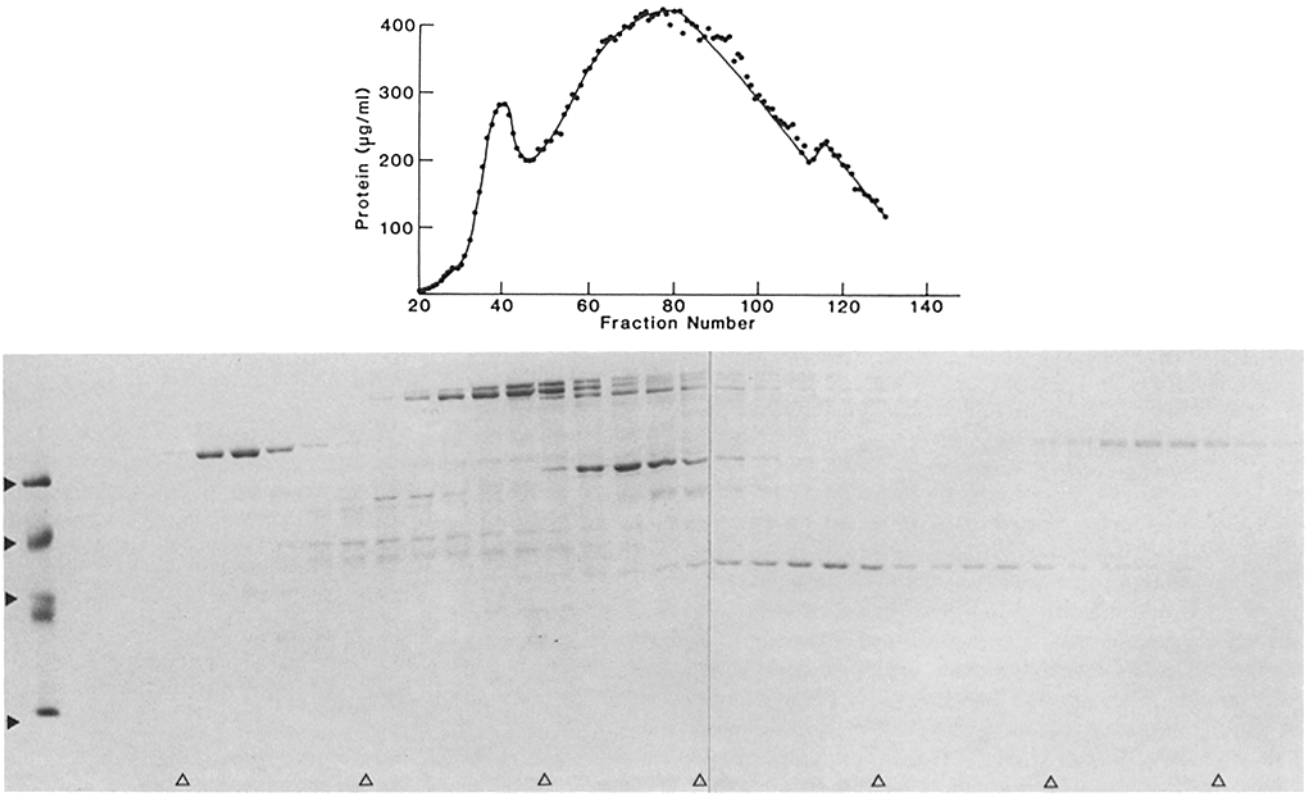

FIGURE 1 Fractionation of platelet extracts on DEAE-Sephacel. (*Top*) Protein profile. Approximately 250 mg of contractile gel-depleted extract, dialyzed against buffer B, was loaded onto a 2.5 × 25 cm column of DEAE-Sephacel and eluted with a 20 to 400 mM NaCl gradient in buffer B. 7.5-ml fractions were collected and analyzed for protein by the method of Bradford (29). Every third fraction from the column was analyzed on 7.5% SDS-polyacrylamide gels. The results are shown (*bottom*). Fractions 35–44 contained a prominent 130,000-mol-wt protein which was pooled and concentrated by ammonium sulfate precipitation as described in Materials and Methods. From the left, the open arrowheads in the lower panel indicate fractions 35, 50, 65, 80, 95, 110, and 125, respectively. The solid arrowheads indicate molecular weight markers: phosphorylase b, 94,000; bovine serum albumin, 68,000; ovalbumin, 45,000, and carbonic anhydrase, 30,000.

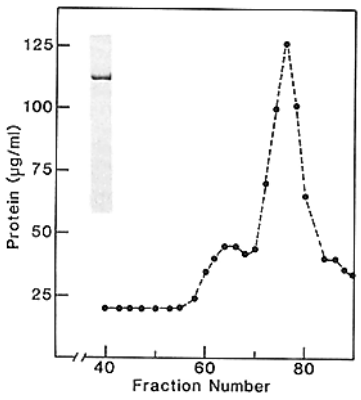

FIGURE 2 Fractionation of partially purified vinculin on Sepharose Cl-4B. The vinculin-containing fractions isolated by DEAE-Sephacel were pooled and concentrated by use of ammonium sulfate, then resuspended in and dialyzed against buffer B. 10 mg of this partially purified material was loaded onto a column (2.5 × 100 cm) of SepharoseCl-4Band eluted with buffer B. 3-ml fractions were collected and analyzed for protein by use of the method of Bradford (29). The protein profile is shown here. The purified vinculin in fractions 72–82 was pooled and concentrated by ammonium sulfate precipitation, then resuspended in and dialyzed against buffer B. The inset shows a 7.5% SDS gel run with 5 μg of the pooled protein.

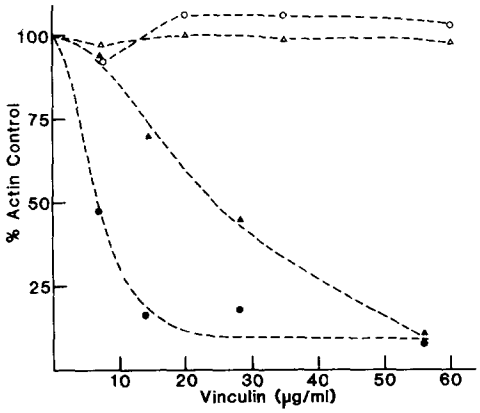

FIGURE 3 DEAE-purified vinculin affects the viscosity of actin. The effect of vinculin on actin viscosity was measured by use of the falling-ball method of MacLean-Fletcher and Pollard (24). Partially purified vinculin isolated by DEAE-Sephacel chromatography was assayed in the presence of 0.275 mM EGTA (filled circles) or in 0.1 mM calcium (filled triangles). Vinculin, purified through the additional Sepharose Cl-4B chromatography step, was assayed under the same conditions in EGTA (open circles) or calcium (open triangles). The data are expressed as the percent of the actin controls.

of gel filtered vinculin on both the rate of assembly and the final fluorescence values, which are a sensitive measure of the critical concentration of actin assembly. The results, shown in Fig. 4, indicate that DEAE-purified vinculin has no significant effect on either the rate or the critical concentration of assembly in either Ca^{++} (Fig. 4*A*) or EGTA (Fig. 4*B*) at vinculin/actin ratios of up to 1-to-15. We have found similar results for gel-filtered vinculin and have been unable to correlate the reduced viscosity seen in Fig. 3 with an end capping activity. In this assay, for example, 10 nM platelet gelsolin, whose capping activity we have established (25, 30), reduced the final fluorescence value 35 to 40% and increased the overall assembly rate about threefold.

Antibody Specificity

We produced antibodies in mice by use of the gel-filtered 130,000-mol-wt protein. Immunoblots were used to establish the specificity of the polyclonal antibodies. The left panel of Fig. 5 shows a Coomassie Blue-stained SDS polyacrylamide gel of various platelet fractions obtained during the purification of the 130,000-mol-wt protein (lanes *1–5*). The right panel of Fig. 5 shows companion gels, with corresponding lane numbers, immunoblotted with the mouse antiserum. In whole platelets, lane *1*, the 130,000-mol-wt protein is the major component that reacts with the antiserum, but we do detect a minor band at ~150,000 M_r, which may be metavinculin (31, 32). The 150,000-mol-wt species is always weakly stained and is seen most clearly in this gel in lanes *2* and *3* of the immunoblot. There are additional peptides, with molecular weights of 85,000–90,000 and one at 40,000, that appear to be degradation products since they vary from one batch of platelets to the next and increase at some of the early purification steps. Nearly all of the 130,000-mol-wt species (arrow) was solubilized by high-salt extraction, as judged by the intensity of staining (compare lanes *1* and *2* in the immunoblot), and there was an increase in the degradation

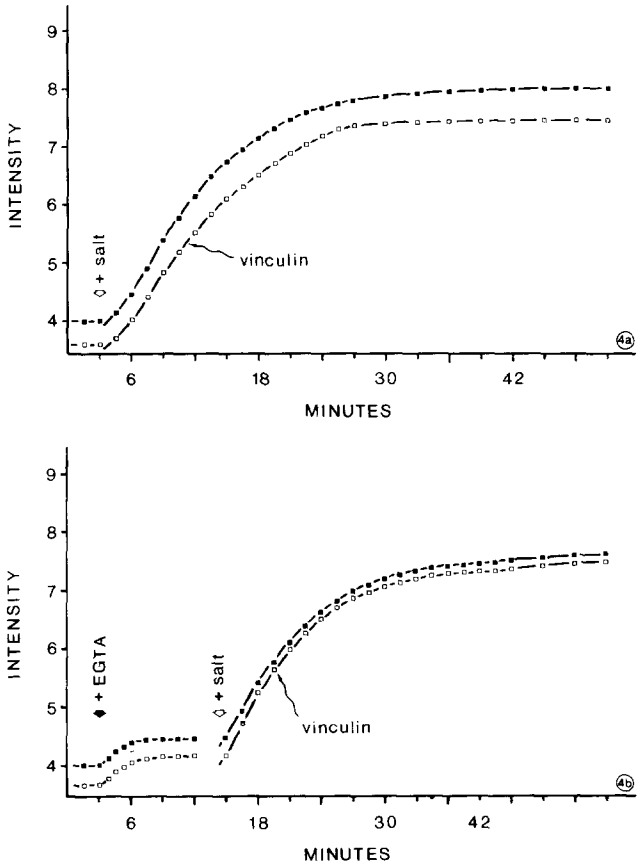

FIGURE 4 Partially purified platelet vinculin does not alter actin assembly. The assembly of actin was measured by use of NBD-actin at a final concentration of 2.5 μM. The base line of actin assembly in the presence of vinculin was offset in *a* and *b* to enhance clarity. (a) The effect of vinculin on actin assembly in the presence of 0.1 mM calcium. KCl and $MgCl_2$ were added at the open arrows to final concentrations of 100 and 2.5 mM, respectively, to initiate assembly. (*b*) Effect of vinculin on actin assembly in 1 mM EGTA. KCl and $MgCl_2$ were added at the arrow to initiate assembly, as indicated in a. The filled symbols are the control actin preparations; the open symbols are for a vinculin concentration of 25 μg/ml, an actin/vinculin molar ratio of about 15:1. Identical results were obtained with the protein purified through the additional Sepharose Cl-4B step.

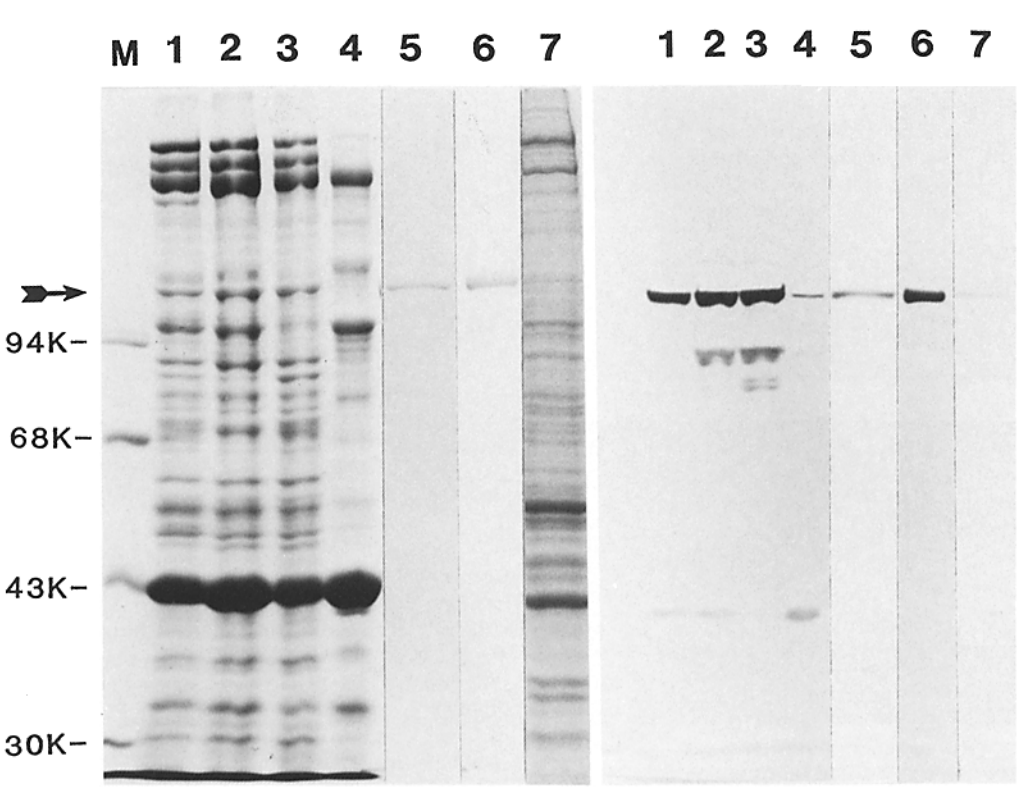

FIGURE 5 Immunoblot analysis using polyclonal antibodies against platelet vinculin. A mouse antiserum to human platelet vinculin was tested at a dilution of 1:1,000 against the following fractions, identified by lane numbers at the top of the Coomassie Blue-stained gel (*left*) and the corresponding immunoblot (*right*): (*1*) whole platelets immediately before sonication; (*2*) high salt extract after the 100,000 g centrifugation; (*3*) extract after dialysis and centrifugation to remove the contractile gel; (*4*) contractile gel; (*5*) vinculin after DEAE and Sepharose chromatography (see inset in Fig. 2 also); (*6*) chicken gizzard vinculin (kindly provided by Dr. Joann Otto, Purdue University, Lafayette, IN); and (*8*) whole WI38 cells. Lane *M* has the same molecular weight markers as those used in Fig. 1; the arrow indicates the 130,000-mol-wt position. K, thousands.

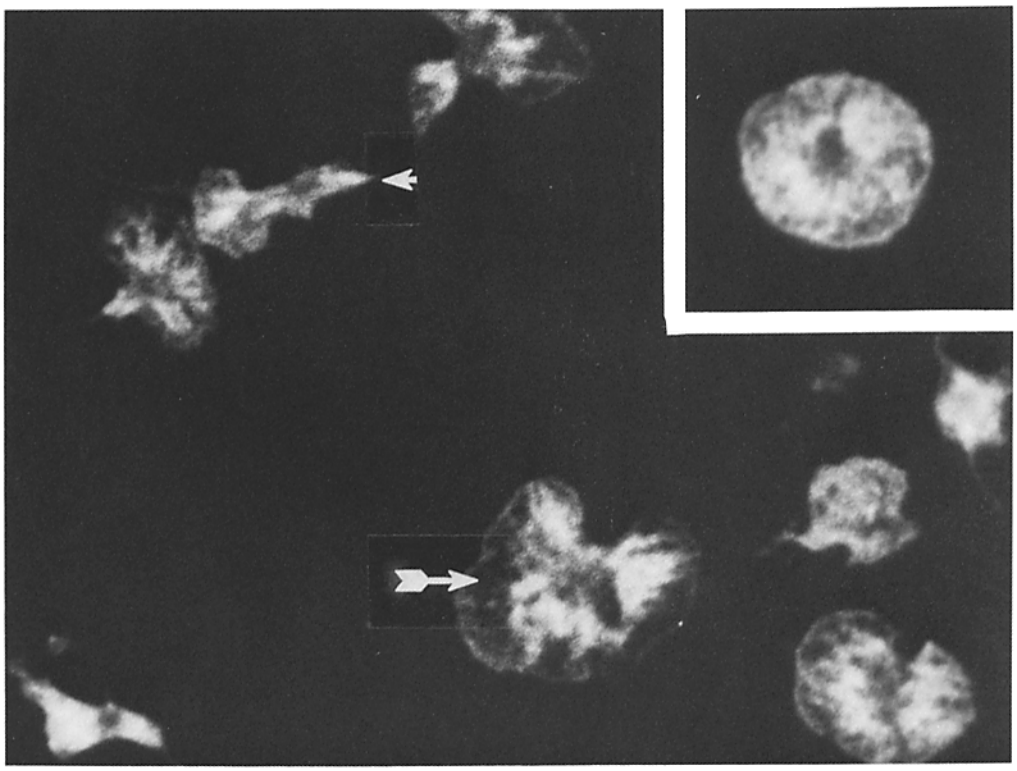

FIGURE 6 Vinculin localization in platelets. Variation in the immunofluorescent staining patterns of platelets seen using the mouse serum against platelet vinculin that was tested in Fig. 5. The inset shows a cell that has attached to the glass coverslip and has begun to spread. The arrow indicates a well-spread cell or several cells. The arrowhead indicates a well-spread cell that has a morphology that suggests movement toward the left. Vinculin is present in the leading edges and in larger aggregates in the central cytoplasm. (*Figure*) × 3,000; (*inset*), × 5,000.

products. The gelation step removes a substantial amount of actin, myosin, and alpha-actinin but little of the 130,000-mol-wt (compare the fourth lane in both panels). We have tried, unsuccessfully, to induce vinculin to associate with the contractile gel by varying the salt conditions during dialysis, by activating the platelets with ADP or Ca^{++} ionophores, and by using freshly drawn platelets. Lanes *5* and *6* compare the 130,000-mol-wt platelet protein with chicken gizzard vinculin and show that the mouse antibody also cross-reacts with the chicken protein. Lane *7* demonstrates cross-reaction of a 130,000-mol-wt species in the WI38 cells used for indirect immunofluorescence localization. We noted one potential artifact in doing gel electrophoresis and immunoblots with concentrated vinculin solutions. We occasionally observed the presence of high molecular cross-reacting material. This happened with samples that gave single bands on other runs

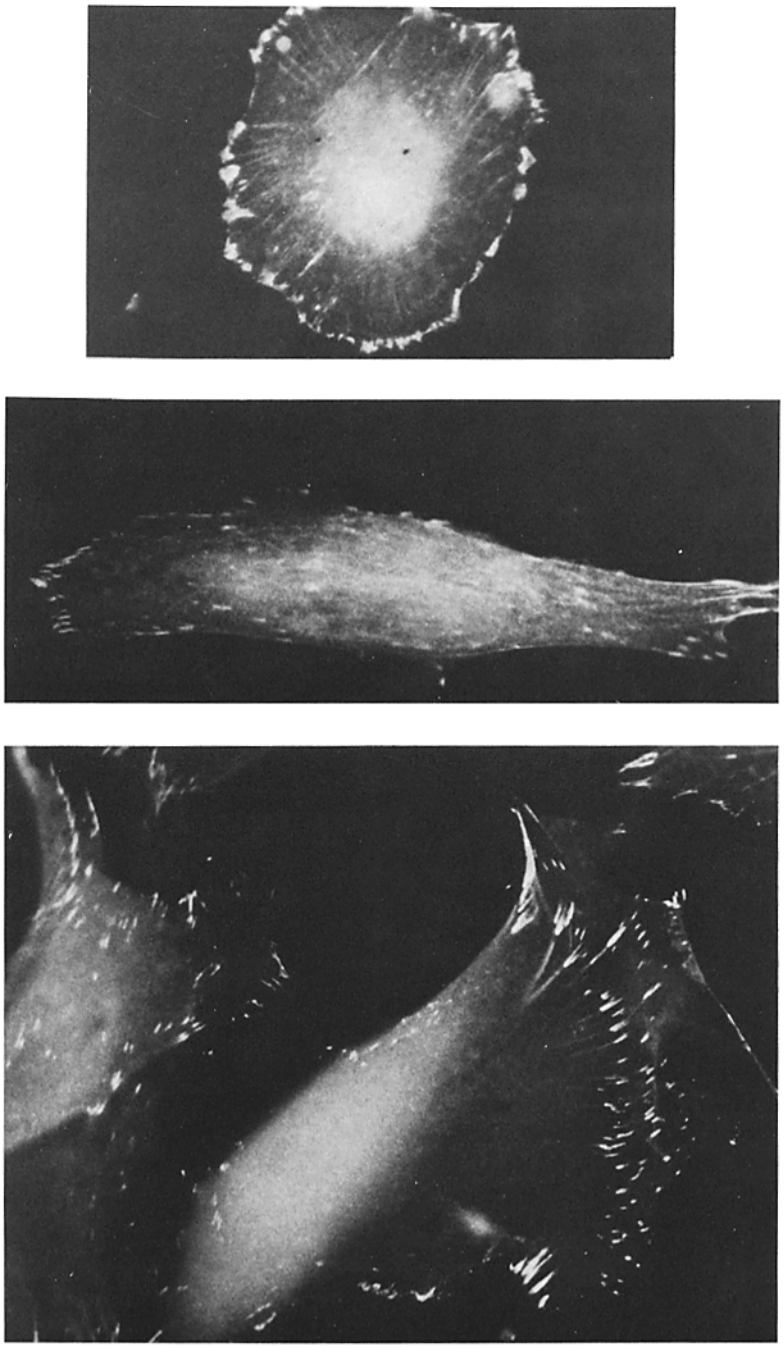

FIGURE 7 Vinculin localization in human WI38 cells. WI38 cells were processed for indirect immunofluorescence by use of the mouse serum against platelet vinculin tested in Fig. 5. The three panels show cells at various stages of spreading or moving. The cell in the middle, for example, seems to be moving to the left. All three panels show vinculin staining in discrete bright foci. Weaker staining along or under stress fibers is also apparent in some cells. × 1,700.

and could be eliminated by our using 2 rather than 1% SDS in the gel electrophoresis sample buffer and extending the heating time to 5 min.

Localization of the 130,000 M_r Protein in Human Platelets

We have made preliminary observations of vinculin localization in platelets. Fig. 6 shows the results of allowing platelets to attach and spread on a glass cover slip before processing for conventional indirect immunofluorescence. The staining pattern varies depending on the stage of platelet attachment and spreading. In cells that have attached and spread but that still have a circular profile (inset), there is a somewhat grainy or clumpy distribution of fluorescence in the cytoplasm and a clear demarcation of the cell boundary. In cells that we think are in the process of spreading (arrow), the fluorescence localization was more discrete and was focused primarily around the central core of the platelet. In some well-spread cells the fluorescence appeared fibrous and radially arranged (upper left of Fig. 6). Finally, some platelets looked like they were moving and had well-defined tails that were intensely stained (arrowhead).

Localization of the 130,000 M_r Protein in the Adhesion Plaques of WI38 Cells

In order to provide further evidence that the 130,000-mol-wt platelet protein was vinculin we used the antibodies to localize the cross-reacting protein detected in the immunoblots. This has been done for several cell types. WI38 cells

processed for indirect immunofluorescence showed, in Fig. 7, staining of foci similar in appearance to the adhesion plaques previously identified by others with antibodies against smooth muscle vinculin (3, 4). In a spreading cell (Fig. 7, top) staining was observed on these foci around the entire periphery. In motile cells (middle) antibody staining was observed in foci at the leading and trailing edges and scattered on the under surface. In cells at other stages of spreading (bottom) staining of discrete foci was also readily apparent. Some much weaker staining of stress fibers and the cell body was evident in some preparations. Elimination of the first or second step antibodies abolished staining completely.

Retention of Vinculin in MDCK Cell Cytoskeletons

Finally, we used the antibodies and immunoblots to study the distribution of the 130,000-mol-wt protein after cell lysis with Triton X-100. Confluent MDCK cell monolayer cultures were used to study the distribution of vinculin in attached cells lysed in the presence of Ca^{++} (Fig. 8, lanes *5* and *6*) or in the presence of EGTA (Figure 8, lanes 3 and 4). Alternatively, confluent MDCK cell monolayers were trypsinized to disrupt adhesion contacts with the substrate before lysis (Fig. 8, lanes *1* and *2*). The results show that the bulk of the vinculin was soluble in the lysis buffer but that some vinculin remained associated with the attached cytoskeleton and that the Ca^{++} levels made little change in the amount of material retained. Little if any vinculin was retained in the Triton X-100 residues from unattached cells. Geiger has reported similar results using anti-chicken gizzard vinculin and attached cells (33).

DISCUSSION

As part of an overall investigation to isolate and characterize human platelet proteins that modify actin assembly we have purified, as described in this report, a 130,000-mol-wt protein with the characteristics of vinculin, a protein previously isolated from chicken gizzards (23). The platelet protein has the same molecular weight and solubility properties as the chicken protein and can be purified using similar protocols (23). Antibodies against the 130,000-mol-wt platelet protein cross reacted with chicken gizzard vinculin, stained focal adhesion plaques in WI38 cells, and gave patchy fluorescence in human platelets. Our results extend, but are only in partial agreement with, the reports by Wilkins and Lin (10) and Jockusch and Isenberg (9) on smooth muscle vinculin, by Feramisco and Burridge (11, 23) on both smooth muscle and nonmuscle vinculins, and by Koteliansky et al. (13) on platelet vinculin. Like all of these authors we observed that DEAE-purified vinculin affects the viscosity of F-actin solutions. We were unable, however, to detect capping activity as described by Wilkins and Lin (10) and have no evidence that the reduced viscosity is due to shortened filaments. Gel filtration removes some minor components and eliminates the effect of this material on F-actin viscosity. This result agrees with the observations of others (14–16) and the report by Evans et al. (17) that DEAE-purified chicken gizzard vinculin has a contaminant that interacts with actin, and extends these observations to a nonmuscle vinculin. We have not identified the contaminating protein(s) that affect actin viscosity, but note that a minor component of the DEAE-purified vinculin is a high molecular weight peptide similar to that described recently by Otto et al. (20) and by Burridge and co-workers (18, 19). It is not clear if we have removed a co-factor that potentiates vinculin–actin interactions or removed a distinct actin-associated protein.

The cross-reactivity of the antibody against the 130,000-mol-wt protein with chicken gizzard vinculin and 130,000-mol-wt proteins from other cells plus the immunofluorescence localization in focal adhesions provide the strongest evidence that the platelet protein is vinculin. This is supported by the MDCK cell lysis experiments, which also indicate that disruption of the adhesions by trypsinization releases vinculin from the cytoskeleton. We have not established whether the vinculin retained in the Triton X-100 residue is a different isoform as recently described by Geiger (33). We have at-

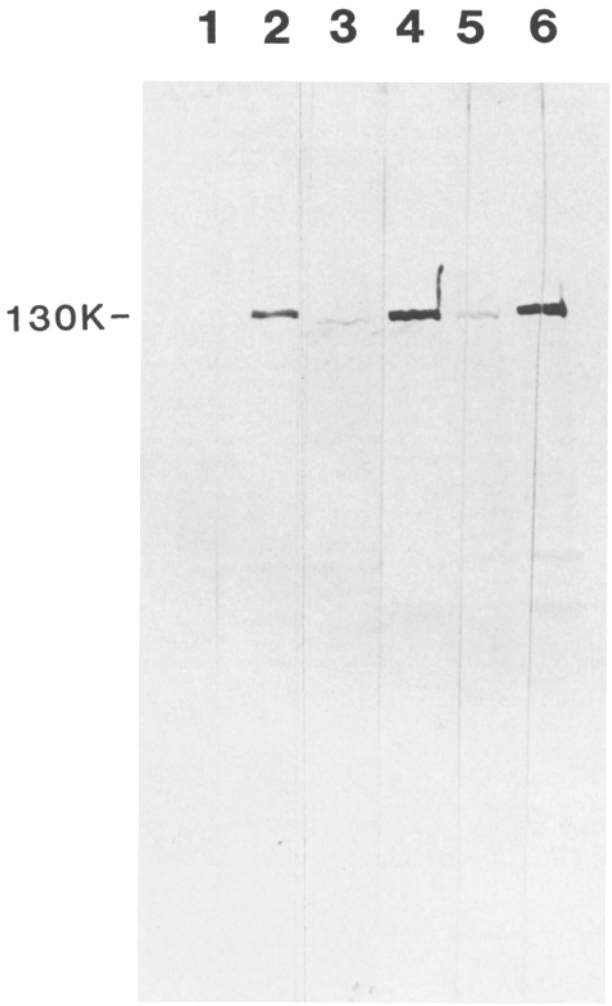

FIGURE 8 Vinculin is partially retained in the cytoskeleton of MDCK cells. Samples of Triton X-100 soluble and insoluble or cytoskeletal proteins from MDCK cell monolayers were analyzed by use of the mouse antiserum against platelet vinculin to immunoblot SDS gels. Lanes *1* and *2* are the insoluble and soluble fractions, respectively, from a trypsin-dissociated cell suspension. Lanes *3* and *4* are the insoluble and soluble fractions from attached cells lysed in Triton X-100 with 0.1 mM EDTA added, and lanes *5* and *6* are the corresponding fractions after lysis with Triton with 0.1 mM calcium added. Essentially all of the vinculin in the unattached control cells seems to be released by Triton lysis, whereas in the latter two extractions some vinculin is retained in the insoluble residue. K, thousands.

tempted to prepare platelet cytoskeletons and contractile gels that retain vinculin but have not been successful. Some vinculin, assayed by immunoblots, is retained in Triton X-100 residues (34) and in the contractile gels (Fig. 5), but the amounts do not exceed 2–3% of the total vinculin.

The staining of platelets with antivinculin does show some localization in spreading cells at the cell periphery and some indication of fibrous bundles. The evidence that platelets have well-developed adhesion plaques is, however, poor, and we do not observe discrete bright foci like those found in cultured cells. We are now trying to correlate the distribution of vinculin in platelets seen with immunofluorescence with close contacts visualized by interference-reflection microscopy. The initial results with freshly drawn platelets indicate that close contact regions are very dynamic and appear to be associated with vinculin.

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