Purification and partial characterization of vertebrate smooth muscle vinculin

Robert Russell Evans
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PURIFICATION AND PARTIAL CHARACTERIZATION OF VERTEBRATE SMOOTH MUSCLE VINCULIN

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Purification and partial characterization
of vertebrate smooth muscle vinculin

by

Robert Russell Evans

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GENERAL INTRODUCTION

Prior to 1979 only two studies mentioning proteins having a molecular weight of 130,000 and isolated from smooth muscle were reported in the literature. The first report (1) discussed the possible role of a protein with a molecular weight of 130,000 that was believed to somehow regulate the interaction between actin and myosin. This conclusion was based upon the observation that Ca$^{2+}$-sensitive actomyosin contained the 130,000-dalton protein but that Ca$^{2+}$-insensitive actomyosin did not. Later, however, it was shown that the primary Ca$^{2+}$ control mechanism in vertebrate smooth muscle involved a Ca$^{2+}$-sensitive myosin light chain kinase system (2). The nature of the protein isolated by Driska and Hartshorne (1) remains unknown. Shortly after publication of the study by Driska and Hartshorne (1), a second report (3) appeared which also pointed out the presence of a protein with a molecular weight of 130,000 in smooth muscle actomyosin preparations, but these workers made no attempt to elucidate the role of the protein.

Following these earlier studies, Geiger (4) reported the isolation from gizzard muscle of a protein having a molecular weight of 130,000 and its intracellular localization at the termini of microfilament bundles in cultured chicken cells. The protein was isolated from chicken gizzard smooth muscle by a modification of the procedure developed by Singh et al. (5) in our laboratory for preparation of alpha-actinin. Geiger (4) isolated the 130,000-dalton
protein from the water extract of washed myofibrils by precipitation with ammonium sulfate. The protein was then purified by DEAE-cellulose chromatography with subsequent rechromatography on the same column if contaminants were still obvious. From his study, Geiger (4) concluded: 1) the 130,000-dalton protein was soluble over a wide pH range (4-8.5) in aqueous buffers; 2) storage at 4°C resulted in breakdown of the protein, with appearance of two polypeptides having molecular weights of 110,000 and 85,000 along with other low molecular weight polypeptides; 3) the 130,000-dalton protein was globular as determined by gel filtration; and, 4) by peptide mapping and antibody specificity experiments, the 130,000-dalton protein was unrelated structurally or antigenically to other known muscle proteins. Immunofluorescent labeling of cultured embryonic chicken cells indicated that the 130,000-dalton protein was organized somehow in the intracellular plaques present at the termini of stress fibers. The 130,000-dalton protein was subsequently named vinculin (Latin - vinculum = link) by Geiger (6).

Shortly after the work reported by Geiger (4), Feramisco and Burridge (7) also reported the preparation of a 130,000-dalton protein that they isolated by high temperature (37°C), low ionic strength extraction of water washed myofibrils. They subjected the low ionic strength extract to sequential precipitation with magnesium chloride and ammonium sulfate. The pellet from the final ammonium sulfate precipitation was redissolved in Tris acetate buffer, and subjected to chromatography on a DEAE-cellulose column. The 130,000-dalton
protein, which they claimed was "highly purified" (7), was collected in the first fraction from the DEAE-cellulose column. Subsequent to this report, Burridge and Feramisco (8) reported that the 130,000-dalton protein was localized intracellularly at the ends of actin microfilament bundles (stress fibers) and in parallel association with fibronectin that is located extracellularly. They named the 130,000-dalton protein "focin", due to its focal distribution pattern inside the cell. The term "vinculin" will be used throughout this dissertation for the 130,000-dalton protein because Geiger's work (4) was reported first, and because this is the name now used for the protein in general.

In subsequent studies conducted on vinculin by Geiger (9), he reported vinculin had a sedimentation coefficient of 6.4 in 20 mM Tris acetate, pH 7.6. The sedimentation value was not altered by changes in pH, from 5 to 8, or by protein concentration. From this data, together with gel filtration data, a frictional ratio \(f/f_o\) of 1.3-1.35 was calculated, which indicated that vinculin has an ellipsoid shape and an axial ratio of approximately 6-8 to 1. The amino acid composition of vinculin indicated the molecule is rich in acidic amino acids and contains only 14 tyrosine residues (9). Immunofluorescent labeling studies also localized vinculin in several other cell contact regions in vivo including the a) zonula adherens of epithelial cells, b) dense plaques in smooth muscle cells, and c) intercalated disc of cardiac muscle cells. Vinculin-rich areas also were found in association with the acetylcholine receptor clusters in cultured
myotubes, although no explanation for the closeness of these
structures was given. An examination of the immunolocalization
patterns of vinculin in several cell types by electron microscopy (10)
demonstrated that vinculin was situated close to the membrane at sites
of possible actin/plasma membrane interaction. Tokuyasu et al. (11)
and Geiger et al. (12) reported that vinculin is situated closer to
the membrane in the fascia adherens of intercalated discs of chicken
cardiac muscle than alpha-actinin. From this and previous studies (4,
6, 10), it was concluded that vinculin may be involved in linkage of
microfilament bundles to the membrane, but that it is a "peripheral"
protein and not an "integral" membrane protein.

In an interesting study, Avnur and Geiger (13) reported a method
for isolation of ventral membranes from cultured chicken gizzard cells
that had adhered to the supporting media. By the use of a zinc
chloride incubation and shearing with a "stream" buffer, whole ventral
membranes or, dependent upon the shear force applied, enriched focal
contacts were obtained. Immunofluorescent localization studies of
alpha-actinin in these focal contacts showed the protein was arranged
in hexagonally organized dots (forming a "collar" type of
arrangement), which were tightly bound to the ventral membrane. In
contrast, immunofluorescent localization of vinculin showed that it
was organized in a linear array of dots over the entire focal contact
area. Although fibronectin also was found in association
(extracellularly) with the substrate, it was not associated with the
focal contacts. This result was in agreement with those of Birchmeier
in which it was shown that the fibronectin was localized in areas around the focal contacts (sometimes termed "feet"). Singer and Paradiso (15) and Singer (16) reported that vinculin was, in fact, coincident with extracellular fibronectin, but only in quiescent cells cultured under low serum conditions (0.3% fetal bovine serum). This lent support to a possible transmembrane vinculin-fibronectin association. However, in actively growing cells cultured in high serum conditions (5% fetal bovine serum), fibronectin was absent from the focal contact region. Apparently, the increased surface activity and the rapid flux of focal contacts of these more actively growing cells lead to the formation of fibronectin-negative contacts, which are probably the least stable type of typical adhesion site (16).

Koteliansky et al. (17) have demonstrated that human plasma fibronectin will bind to chicken gizzard vinculin as tested by an in vitro enzyme-linked immunoassay. Although they suggested the two proteins may interact directly to link microfilament bundles to the cell membrane, this seems unlikely.

Geiger (18) has related the localization of vinculin with isovariants observed by two-dimensional electrophoretic analysis. Vinculin was separated into four to five isoforms ranging in pH from 6.8 to 7.3, all with a molecular weight of 130,000. The three major variant isoforms were named alpha, beta, and gamma (alpha being most acidic). Two minor variants were designated alpha' and beta' because of their position relative to alpha and beta forms, respectively (18). All isoforms were closely related in primary sequence as shown by
tryptic peptide mapping experiments. In subsequent work, Geiger (19) compared vinculin isolated from different species and found that avian (chicken or turkey) vinculin was more basic (approximately 0.8 pH unit) than vinculin isolated from mammalian sources (human, mouse, pig, or rat). He also showed that the isovinculin forms from cultured chicken gizzards cells could be fractionated, with the beta-vinculin almost exclusively found in a detergent soluble fraction and the alpha-vinculin (along with the intermediate alpha'-vinculin form) enriched in the "organized" fraction. Interestingly, the gamma-vinculin form that is present in samples of intact tissue was absent from the cultured cells. He suggested this latter result may be due to the lack of intercellular contacts that are necessary for induction of the gamma-form (19).

A preliminary report by Evans et al. (20), followed by a more detailed study by Evans et al. (21), confirmed the presence of the three major isoelectric variants in vinculin isolated from turkey gizzard smooth muscle. We found, however, that additional purification of DEAE-cellulose-purified vinculin (7) by CM-cellulose chromatography was necessary to remove contaminants routinely present in the DEAE-cellulose purified vinculin. The additional chromatography step on CM-cellulose resulted in a highly homogeneous protein preparation that contained no additional proteins. The purified vinculin, in 100 mM NaCl, 20 mM Tris-acetate, pH 7.1, sedimented as a single peak ($s_{20,w}^0 = 5.9$) in the analytical ultracentrifuge. The ultraviolet absorption spectrum of vinculin
indicated a maximum at 278 nm and a measured $\frac{E_{278}^1}{\text{mol}^{-1} \cdot \text{cm}^{-1}}$ of 4.64. Vinculin contains approximately 50% alpha-helix as determined from its circular dichroism spectra. As part of this study, we also showed that vinculin is degraded by the Ca$^{2+}$-activated neutral protease isolated from muscle by the procedure of Dayton et al. (22, 23). Major proteolytic fragments appeared with molecular weights of 98,000, 85,000, and 26,000.

In a comparative study, Endo and Masaki (24) isolated vinculin from chicken skeletal, smooth, and nonmuscle cells and then characterized the vinculins with respect to tissue and differing stages of development. All vinculins were shown to be similar as determined by peptide mapping and cross-reactivity in double immunodiffusion and immunoblotting. On one- and two-dimensional isoelectric focusing gels, gizzard vinculin contained at least seven isoelectric variants having values from pH 6.26 to pH 6.58. Although all variant forms of vinculin were present in the tissues examined, relative amounts changed in relation to the samples monitored. For instance, their variant number "6" (pH 6.45, similar to the gamma-vinculin described by Geiger (19), was barely present in brain and 10- and 16-day old embryonic gizzard, but it increased in amount with later development of gizzard. It also varied in amount in relation to tissue origin (nonmuscle < smooth < skeletal).

It has been reported by Hunter (25) and Sefton et al. (26) that vinculin is a substrate for the protein kinase, p60$^{src}$, which is responsible for transformation of cells by Rous sarcoma virus. The
transforming protein, p60^{src}, modifies vinculin and several cellular proteins by phosphorylation of tyrosine residues. Because phosphotyrosine modifications are relatively rare, it has been suggested that vinculin is a primary substrate for the p60^{src} protein kinase and that this phosphorylation may be responsible for disruption of the microfilament bundles from the plasma membrane that occurs during the process of transformation (26). Besides disruption of microfilament bundles, transformed cells also undergo other morphological changes such as reduced adhesiveness and loss of cell surface fibronectin (27). When fibronectin is added back to transformed cells, they regain normal morphology and microfilament bundle organization (28, 29). The possible role of vinculin in anchoring actin-containing filament bundles to the plasma membrane gained considerable interest when the Rous sarcoma virus also was localized at adhesion plaques on the inner membrane surface of cells (29-31).

The overall processes of cellular infection and phosphorylation of vinculin and other targeted proteins that accompany cell transformation have been summarized by Hunter (25). A normal sequence of events by which this process may occur involves: 1) penetration of the plasma membrane by the Rous sarcoma virus; 2) reverse transcription of the viral RNA; 3) integration of the viral DNA into the cellular DNA; 4) transcription of the provirus; 5) translation of the src gene sequence; 6) association of the p60^{src} kinase with the plasma membrane at adhesion plaques; 7) p60^{src} kinase catalyzed
phosphorylation of vinculin with subsequent release of the microfilaments; and 8) loss of adhesion by the cell to the substrate. Even though direct evidence for this complete sequence has not yet been demonstrated, at least specific portions of it evidently occur based on results from several laboratories (26, 32-39). It also has been found that the viral p60^{src} is similar in protein kinase activity and primary structure to a normal cellular homolog called p60^{src} (31, 33, 40).

Ito et al. (35) have shown that vinculin is a poor substrate for the p60^{src} protein kinase *in vitro*. Phosphorylation, however, can be increased approximately 10-fold by the addition of anionic phospholipids (phosphatidylinositol or phosphatidylglycerol) to the *in vitro* assay. This effect may be the result of a conformational change in the vinculin molecule via binding of the phospholipids because vinculin is more susceptible to V8 protease cleavage following addition of phosphatidylglycerol, phosphatidylinositol, or phosphatidylserine (41). Although phosphorylation of vinculin's tyrosyl residues does not change in normal cells undergoing mitosis (42), vinculin phosphorylation is necessary for transformation in Rous sarcoma virus-infected cells (43). Recent results by Werth et al. (44) and Werth and Pastan (45) also show that vinculin is phosphorylated by protein kinase C. They suggest such phosphorylation may be involved in the normal cellular regulation of vinculin function.

As pointed out earlier, it has been suggested that vinculin is
involved in the linkage of actin bundles to cell membranes (4, 7-9) because of its localization at the termini of stress fibers in focal contacts of cells (4, 7-9, 19). To investigate the possible interaction between actin and vinculin, Jockusch and Isenberg (46) used low shear viscometry to measure the effect of vinculin on the formation of F-actin filaments. Low shear viscometry has been a useful technique for the characterization of actin filament networks (47-49). By this technique, one can measure a wide range of apparent viscosities (approximately three orders of magnitude). Upon addition of DEAE-cellulose-purified vinculin to actin, Jockusch and Isenberg (46) observed a decrease in the viscosity of F-actin. They suggested this may be because vinculin acted to bundle actin filaments. They subsequently suggested (50) that vinculin induces formation of actin bundles that are similar in structure to those formed by the addition of 50 mM MgCl$_2$. Jockusch and Isenberg (51) have also presented evidence showing that vinculin does not act to cap or bind to the ends of actin filaments. In contrast, Wilkins and Lin (52), who also observed the decrease in the low shear viscosity of actin filaments upon addition of DEAE-cellulose-purified vinculin, feel the reason is because vinculin acts as a capping protein. They, and Burridge and Feramisco (53) have reported that vinculin binds to the barbed end of actin filaments (1 vinculin to 1,500-2,000 actin monomers).

Evans and associates (21, 54) have recently demonstrated that the factor(s) responsible for the inhibitory activity in DEAE-cellulose-purified vinculin can be recovered in a peak when the protein is
subjected to further purification on a CM-cellulose column. That peak contains several contaminants, but not vinculin. The specific inhibitory factor(s) have not been identified, but may be one or a combination of proteins having molecular weights of 165,000, 67,000, 30,000, and/or 23,000. When added to F-actin, CM-cellulose-purified vinculin causes a 30% increase in the viscosity of F-actin networks at a concentration of 30 μg/ml due to increased cross-linking of actin filaments (21, 54). In contrast, the contaminant peak, which was isolated as the first peak eluting from the CM-cellulose column, decreased the viscosity of F-actin by approximately 95% at only 20 μg protein/ml. Structural examination showed that the marked decrease in viscosity caused by the contaminants resulted from the shortening and bundling of actin filaments in the absence of calcium. Using a technique termed gel overlay, Otto (55) and Wilkins et al. (56) recently demonstrated the binding of 125I-labeled vinculin to actin and other proteins having molecular weights of 220,000, 190,000, 170,000 and 100,000 (55) and 215,000, 205,000 and 185,000 (56). The identity of most of these proteins is unknown.

A protein with a molecular weight of 152,000 has been isolated from smooth muscle (57). It has both structural and immunological properties similar to those of vinculin as examined by peptide mapping and antibody cross-reactivity. The 152,000-dalton protein exhibits immunofluorescence localization patterns in smooth muscle cells that are indistinguishable from those of vinculin. Cell-free translation of messenger RNA from smooth muscle indicates that vinculin and the
152,000-dalton protein are separate gene products and probably not a precursor-product pair or due to post-translational modification (57). The 152,000-dalton protein decreases the low shear viscosity of actin filaments in a concentration-dependent manner, but only in the presence of Ca^{2+} (57). Siliciano and Craig (58) have also isolated a protein with a molecular weight of 150,000 from smooth muscle that has similar antigenic properties to vinculin. They termed their protein meta-vinculin (Greek - meta = among). From its solubility properties, it was proposed that meta-vinculin might be an integral membrane protein that anchors actin filaments to the plasma membrane (58).

Recent immunofluorescence studies (59-61) have shown that vinculin is localized near the sarcolemma of skeletal muscle in a two-dimensional orthogonal lattice. The staining has a pattern similar to that of the periodicity of sarcomeres, with vinculin encircling the inside of the muscle cell. They termed this pattern "costameres" (Latin - costa = ribs), with the vinculin localized on either side of the Z line and directly above the I band region of the peripheral sarcomeres (60, 61). They proposed that the vinculin present in these costameres interacts with the Z line structures via gamma-actin or spectrin, which also are localized in these regions of the muscle cell.

Avnur et al. (62) have provided some evidence showing that the association of vinculin with focal contacts is actin-independent in chick embryonic gizzard and heart cells. Removal of actin from isolated ventral membranes by addition of a protein called fragmin did
not alter the binding of vinculin to focal contacts when added to the actin-free membrane surfaces.

Burridge and Connell (63, 64) recently discovered the presence of a protein with a molecular weight of 215,000, which they have localized in fibroblasts by double-label immunofluorescence. The protein is present in focal contacts and ruffling membranes and is nearby, but distinct, from the pattern of vinculin localization. The protein possesses no cross-reactivity with vinculin as determined by immunoblots and immunoprecipitation. Burridge and Connell (63, 64) have named the 215,000-dalton protein "talin" (Latin - talus = ankle). In more recent studies, Burridge and Mangeat (65) have demonstrated an interaction of talin with vinculin but not between talin and actin, thus implicating talin as a potential anchoring protein for actin filaments in vivo.

It is clear from this review that little is yet known with certainty regarding vinculin's role or what other proteins really interact with vinculin. In this dissertation, I have prepared highly purified vinculin and have determined selected biochemical properties of the purified protein. I also have investigated the interaction of vinculin with F-actin under varying conditions of temperature, pH, and ionic strength by low shear viscometry and electron microscopy. The major portion of this dissertation is comprised of two chapters which represent two full length papers. The first has just been published (54). The second paper is in the process of being submitted. The first paper deals with the further purification of vinculin and
selected properties of the purified protein. The second paper describes the interaction of vinculin with actin in relation to temperature, pH, and ionic strength.
SECTION I: PROPERTIES OF SMOOTH MUSCLE VINCULIN
Properties of Smooth Muscle Vinculin†

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Running Title: Properties of Smooth Muscle Vinculin
Vinculin, isolated from turkey gizzard smooth muscle, was purified by chromatography on CM-cellulose after isolation from a DEAE-cellulose column. Two-dimensional gel electrophoretic analysis of crude muscle fractions demonstrated that: 1) much of the -130,000-dalton protein present in smooth muscle did not co-isoelectrically focus with the purified 130,000-dalton vinculin and 2) the purified vinculin consisted of three major, closely spaced isoelectric variants that were present only in small amounts in the original smooth muscle sample. Purified vinculin sedimented as a single peak with a sedimentation coefficient $s_{20,\text{w}}^0$ of 5.9. Circular dichroism spectra of purified vinculin indicated a considerable degree of secondary structure, with an alpha-helical content of approximately 50% as measured at 208 nm. The ultraviolet absorption spectrum of vinculin gave a measured $\frac{\epsilon}{c \lambda}$ of 4.64. Digestion of vinculin, much of which is located at the cytoplasmic surface of the cell membrane, with Ca$^{2+}$-activated neutral protease purified from skeletal muscle yielded major fragments with molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 98,000, 85,000, and 26,000. The factor(s) in DEAE-cellulose-purified vinculin responsible for decreasing the low shear viscosity of actin was removed and found in a crude fraction isolated by CM-cellulose chromatography. The purified vinculin had a small, but positive effect on the MgCl$_2$-induced polymerization of actin as measured by low shear viscometry.
INTRODUCTION

Vinculin is a 130,000-dalton cytoskeletal protein that primarily is located at the termini of microfilament bundles in focal contacts of cultured cells (1-5). Vinculin also has been detected in vivo near membrane/actin-filament attachment sites in the zonula adherens of intestinal epithelium, dense plaques of smooth muscle, the fascia adherens of cardiac intercalated discs (6, 7), and costameres of cardiac and skeletal muscle (8-10). Vinculin is situated closer to the cell membrane than is the protein alpha-actinin (6, 7, 11, 12). Primarily because of its specialized location where microfilaments abut the cytoplasmic face of plasma membranes, it has been suggested, albeit without direct evidence, that vinculin is involved in linkage of actin bundles to cell membranes (1, 2, 4, 5). This hypothesis gained interest and support when it was shown that vinculin is one of the intracellular targets of the src gene kinase (13-15), the transforming protein of the Rous sarcoma virus, much of which also is associated with adhesion plaques at the plasma membrane (16-18). In transformed cells, focal contacts are fewer and smaller, vinculin and alpha-actinin are altered in their organization, and actin-containing microfilament bundles are largely disrupted (17, 19, 20). Vinculin may play an important role in the events of transformation, although precise details of its involvement remain unclear (21, 22). The disruption of focal contacts and actin bundles seemingly could arise via modulation of vinculin function by phosphorylation or via cleavage
by a cellular protease (23).

It has been reported that vinculin binds directly to actin (24-30), but the nature of the interaction is not very clear. In several studies, it has been shown that vinculin suppresses the expected viscosity increase during actin polymerization (24-29, 31). Whether this is because: 1) vinculin forms para-crystalline-like bundles with F-actin (24, 25, 29), which has led to the suggestion that vinculin may organize the actin filaments into a configuration required in the formation of focal contact points (26, 32), and/or 2) vinculin reduces filament/filament interaction (29, 31), and/or 3) vinculin inhibits actin filament assembly, perhaps by binding to filament ends (5, 29, 31) is unclear.

In view of our overall interest in understanding proteins that may help anchor actin filaments to their attachment sites such as alpha-actinin (33-37), which evidently comprises the Z-filaments that bind actin filaments in the muscle Z-line (38, 39), we have examined selected properties of vinculin (40, 41). As part of our findings, we show that vinculin is a substrate for calcium-activated neutral protease isolated from muscle and demonstrate that vinculin preparations, depending upon chromatographic purification method, vary markedly in their ability to inhibit the low shear viscosity of F-actin solutions.
EXPERIMENTAL PROCEDURES

Purification of Vinculin

All steps were carried out at 0-4°C in a cold room or on ice unless stated otherwise. The pH adjustment of buffers was done at the temperature at which the buffer was to be used. Solutions were prepared by using double-distilled water that had been deionized before each distillation. Frozen (partially thawed just before use) turkey gizzards were used. The muscle was trimmed free of fat and connective tissue and ground in a prechilled meat grinder before being weighed. To reduce possible proteolysis, all solutions contained 0.1 mM phenylmethylsulfonyl fluoride (Sigma). The method used for the initial preparation of crude vinculin extract and "DEAE-cellulose-purified vinculin" was essentially that described for preparation of vinculin by Feramisco and Burridge (4) with the following slight modifications noted.

Step I: Preparation of crude vinculin extract

Ground muscle, routinely 800 g, was extracted twice with H₂O to prepare washed myofibrils. The myofibrils were resuspended in low ionic strength buffer for 1 h instead of 30 min at 37°C in order to enhance the extraction of vinculin from the myofibrils, and most of the desmin and actin in the resulting vinculin-containing supernatant was then precipitated with MgCl₂ (4). This supernatant was then fractionated by addition of 20.5 g of (NH₄)₂SO₄/100 ml of supernatant, yielding a pellet constituting the crude vinculin extract.
Step II: Preparation of DEAE-cellulose-purified vinculin

The ammonium sulfate pellet comprising the crude vinculin extract was dissolved in Buffer A (20 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, 20 mM Tris-acetate, pH 7.6) and then concentrated and dialyzed against Buffer A in an Amicon DC-2 hollow fiber apparatus (Amicon Corp., Lexington, MA). After clarification by centrifugation at 186,000 x g for 45 min, the protein was loaded on a DEAE-cellulose (Cellex D, BioRad) column (2.5 x 45 cm) equilibrated in Buffer A. Protein was eluted from the column by the use of a linear gradient of NaCl in Buffer A. Fractions were collected and examined by SDS- polyacrylamide gel electrophoresis and appropriate fractions were pooled to constitute DEAE-cellulose-purified vinculin.

Step III: CM-cellulose chromatography of DEAE-cellulose-purified vinculin

The pooled DEAE-cellulose-purified vinculin was concentrated and dialyzed against Buffer B (20 mM NaCl, 15 mM 2-mercaptoethanol, 0.1 mM EDTA, 20 mM sodium acetate, pH 5.0) by using an Amicon stirred cell connected to an external reservoir. Before dialysis, phenylmethylsulfonyl fluoride and leupeptin (California Medicinal Chemistry Corp., San Francisco, CA) were added, each to a final concentration of 0.1 mg/ml, to control proteolysis. The dialyzed protein was then applied to a Whatman CM52 cellulose column (1.6 x 40 cm) (The Anspec Company, Inc., Ann Arbor, MI) that had been poured and equilibrated in Buffer B. After loading, the column was washed in Buffer B at a flow rate of 12 ml/h until the absorbance at 280 nm attained a steady baseline
Vinculin was then eluted from the column with a gradient made with 250 ml each of Buffer B that contained 200 mM NaCl and Buffer B that contained 500 mM NaCl. The salt gradient was monitored by an LKB 5300B conductolyzer equipped with a 5313B flow cell (LKB Instruments, Inc., Rockville, MD). Column fractions were analyzed by SDS-polyacrylamide gel electrophoresis and pooled to comprise the purified vinculin used for further studies. The purified vinculin was passed through a Millex-GS 0.22 μm sterile filter (Millipore Corp., Bedford, MA) before further analysis.

**SDS-polyacrylamide Gel Electrophoresis**

SDS-polyacrylamide gel electrophoresis was performed essentially according to the procedure of Weber and Osborn (42). Insoluble pellets were dissolved by suspending the samples in 1% SDS, 10 mM sodium phosphate, pH 7.0, and immediately heating them in a boiling water bath for 10 min. Any remaining insoluble material was removed by centrifugation at 2,000 x g for 15 min. All protein samples were heated at 100°C for 10 min in tracking dye mixture (1.75% SDS, 10% 2-mercaptoethanol, 6.5% glycerol, 0.01% bromphenol blue, 20 mM sodium phosphate, pH 7.0). Electrophoresis was performed on cylindrical 7.5% polyacrylamide gels 5 mm in diameter by 80 mm long at 8 mA/tube until the bromphenol blue tracking dye was approximately 0.5 cm from the bottom of the gel. Gels were stained overnight in 0.1% Coomassie brilliant blue R (Sigma), 50% methanol, 7% acetic acid and destained electrophoretically in 7.5% acetic acid, 5% methanol. Molecular
weights were determined from plots of log $M_r$ versus relative mobility (42) obtained by comparison with proteins (filamin, myosin, alpha-actinin, desmin, actin, and tropomyosin) of known subunit molecular weights. Quantitative densitometry of SDS gels was used to calculate the percentage of vinculin in selected fractions during purification by the procedure of Huiatt et al. (43)

Two-dimensional Electrophoresis

Isoelectric focusing in the first dimension was done essentially as described by O'Farrell (44) in 4% polyacrylamide gels that were 2.5 mm in diameter by 120 mm long with the following modifications noted. Ampholytes used in the lysis buffer and isoelectric focusing gels were composed of 1.2% Ampholine, pH range 3.5 to 10 (LKB Instruments, Inc.) and 0.8% Ampholine, pH range 5 to 8, with the sample overlay solution composed of the above ampholytes in the same ratio to yield a 1% mixture. Following the specified prerun (44), the gels were run for 18 h at 400 volts followed by 1 h at 800 volts. Samples insoluble in nondenaturing solvents were extracted overnight with freshly prepared 8 M urea at 4°C and clarified at 150,000 x g for 1 h. The supernatants were equilibrated in sample buffer as described for soluble samples (44). Measurement of the pH gradient in the isoelectric focusing gels was done by placing 5-mm sections of duplicate gels in individual vials with 2 ml of degassed H$_2$O as described by O'Farrell (44). SDS-polyacrylamide gel electrophoresis in the second dimension was done on 9.5% polyacrylamide slab gels,
with no stacking gel, by the method of Allen et al. (45) with the buffer system of Laemmli (46). Gels were stained and destained as described previously for cylindrical SDS gels.

Turkey gizzard actin used as a standard in two-dimensional gels was extracted and purified from conventionally prepared acetone powder by the method of Spudich and Watt (47).

CD Spectra

Ultraviolet (195-250 nm) circular dichroic spectra of purified vinculin were obtained at 25°C by using a Cary model 60 spectropolarimeter and a Jasco ORD/UV-5 spectropolarimeter, both equipped with a circular dichroism attachment, in 1.0-mm and 1.0-cm path length cells, respectively. Both spectropolarimeters were purged with nitrogen before and during analysis. Vinculin samples were dialyzed extensively against 5 mM sodium phosphate, pH 7.1, and clarified before measurement by passage through a Millex-GS sterile filter. A mean residue weight of 110 was calculated from the amino acid composition of purified vinculin. An estimate of percentage alpha-helix of purified vinculin was made from the ellipticity at 208 nm by using Method I as described by Greenfield and Fasman (48) for estimation of the alpha-helical content of polypeptide chains.
Proteolytic Digestion of Vinculin by Ca\(^{2+}\)-activated Neutral Protease

Ca\(^{2+}\)-activated neutral protease, prepared by the method of Dayton et al. (49), was added to vinculin in the presence of 100 mM NaCl, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 100 mM Tris-acetate, pH 7.5, (these are final concentrations present after subsequent addition of CaCl\(_2\)) at 25°C. Digestion was initiated by addition of a stock solution of CaCl\(_2\) to bring the final Ca\(^{2+}\) concentration to 5 mM. Aliquots were removed at specified intervals and digestion was stopped by addition of EDTA to a final concentration of 10 mM. The samples were heated at 100°C for 10 min after addition of tracking dye and then analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis. Additional conditions are given in the legend for figure 8.

Other Analytical Methods

Protein concentrations were determined by the biuret method (50) as modified (51) or by the Folin-Lowry method (52) as modified (53). The UV absorption spectrum of vinculin was measured by using a Cary model 1605 spectrophotometer and a Beckman DB-GT spectrophotometer. Sedimentation velocity experiments were done on a Spinco Model E analytical ultracentrifuge equipped with an electronic speed control using Schlieren optics. All runs were done with double sector Kel-F centerpieces, and the plates were measured with a Nikon 6C profile projector. Amino acid analyses were done on a Durrum D-400 Amino Acid Analyzer (Durrum Instruments, Sunnyvale, CA). Protein hydrolysis was done in vacuo at 115°C in the presence of methane/sulfonic acid for
24, 48, and 72 h as described by Simpson et al. (54). Any cystine and cysteine were measured as cysteic acid (55).

Low Shear Viscometry

Low shear viscometry was done by the method of MacLean-Fletcher and Pollard (56), with measurement of the velocity of the falling ball done at an angle of 70° from vertical. Porcine skeletal actin was prepared by the method of Spudich and Watt (47), with further purification by gel filtration on Sephadex G-150 as previously described (57). Purified actin in 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl$_2$, 2 mM Tris-HCl, pH 8.0, was used immediately after preparation. CM-cellulose-purified vinculin and other isolated chromatographic fractions to be tested were extensively dialyzed against 1 mM sodium bicarbonate, pH 7.5, and used immediately for analysis. Polymerization of actin (0.5 mg/ml) in the presence of varying amounts of vinculin or isolated chromatographic fractions was done in 1 mM ATP, 1 mM EGTA, 2 mM MgCl$_2$, 10 mM imidazole-HCl, pH 7.5 (24). Samples were incubated at 25°C for 2 min before initiation of polymerization by addition of the MgCl$_2$. Following addition of MgCl$_2$, samples were immediately drawn into capillary tubes and incubated at 25°C. Velocity of the falling ball was measured after 10 min. Results are expressed as normalized viscosity (24) in order to minimize differences between actin preparations.
RESULTS

Purification of Vinculin

Preparation of vinculin from gizzard smooth muscle by two fairly similar methods (i.e. extraction of vinculin from myofibril fractions in low ionic strength solution followed eventually by DEAE-cellulose chromatography) has previously been described (1, 4). Both procedures yield preparations of similar purity in our hands, but we have used the method of Feramisco and Burridge (4) because it is somewhat shorter and results in a higher final yield (>2-fold) of DEAE-cellulose-purified vinculin. Electrophoretic analysis of selected fractions by using this procedure (4) are shown in Fig. 1. The original whole gizzard homogenate (Fig. 1a) shows the presence of the major smooth muscle myofibrillar/cytoskeletal proteins, including myosin (M_r ~ 200,000 heavy chains), desmin (M_r ~ 55,000), actin (M_r ~ 42,000), and beta- and alpha-tropomyosin (M_r ~ 38,000 and 35,500, respectively). In addition, lesser amounts of filamin (M_r ~ 250,000), M_r ~ 130,000 protein (includes vinculin), and alpha-actinin (M_r ~ 100,000) are present. Preparation of washed myofibrils (Fig. 1b) by short extractions of the whole muscle homogenate with water removes the majority of the tropomyosin subunits along with much of the endogenous cytoplasmic proteins. Extraction of the washed myofibrils with a low ionic strength, slightly alkaline buffer for 1 h at 37°C (composition of this extract is shown in Fig. 4a) followed by MgCl_2 precipitation and ammonium sulfate fractionation steps yields a crude
**Fig. 1.** SDS-polyacrylamide gel electrophoresis of selected fractions obtained during preparation of purified vinculin

a, original gizzard homogenate; b, washed myofibrils; c, crude vinculin extract (before DEAE-cellulose chromatography, end of Step I); d, DEAE-cellulose-purified vinculin (end of Step II); e, purified vinculin (after CM-cellulose column, end of Step III). A total of 30 μg of protein was loaded on gels a–c and 20 μg on gels d and e. Positions of myosin heavy chains (M, 200,000 daltons), 152-kDa protein (152K, 152,000 daltons), vinculin (V, 130,000 daltons), alpha-actinin (alpha-A, 100,000 daltons), desmin (D, 55,000 daltons), actin (A, 42,000 daltons), and tropomyosin (TM, beta-subunit of 38,000 daltons and alpha-subunit of 35,500 daltons) are shown at the left.
vinculin extract (corresponds to end of Step I under "Experimental Procedures") that, as shown in Fig. 1c, contains primarily alpha-actinin and lesser amounts of vinculin and other proteins such as filamin and a 152,000-dalton protein. Application of the crude vinculin extract to a DEAE-cellulose column and elution with a linear salt gradient results in the separation of three major fractions corresponding to vinculin, filamin (152-kDa protein is in latter portion of this peak), and alpha-actinin in order of increasing salt concentration in agreement with previous results (4). The vinculin peak (termed DEAE-cellulose-purified vinculin herein and corresponds to the end of Step II under "Experimental Procedures" shown in Fig. 1d) consists primarily of a 130,000-dalton protein, but routinely also contains other contaminants including those with molecular weights of approximately 165,000, 67,000, and 35,000, which are seen if sufficient protein is loaded on the polyacrylamide gel. Representative data from 20 separate preparations indicate that, at this stage, vinculin is only approximately 70-80% pure as determined by densitometry of one-dimensional gels. As a result, additional purification of the DEAE-cellulose-purified vinculin was deemed necessary before further characterization studies of vinculin.

The DEAE-cellulose-purified vinculin (Fig. 2, gel labeled DE) was applied to a CM52-cellulose column and eluted with a salt gradient (Fig. 2). We initially observed that lowering the pH of the DEAE-cellulose-purified vinculin solution from 7.6 to 5.0, which is well within the solubility range of vinculin (1), to retain the vinculin on
Fig. 2. Elution profile of the DEAE-cellulose-purified vinculin from a CM-cellulose column

A total of 86 mg of protein in 54 ml in Buffer B was loaded onto this column. Additional details are described under "Experimental Procedures". Insets show SDS-polyacrylamide gel electrophoresis of pooled fractions (fraction) as indicated. DE represents DEAE-cellulose-purified vinculin (i.e. the pooled peak of vinculin from a DEAE-cellulose column). Each gel was loaded with 15 µg of protein. Numbers to the right of the left set of gels indicate approximate migration distances of proteins having the indicated subunit molecular weights. 165K, for example, 165,000 daltons. Protein from pooled fractions 5 and 6 is the purified vinculin.
the column seemingly activated a protease(s) that partially degraded the 130,000-dalton vinculin (up to ~5% of the protein), generating fragments having $M_r = 98,000$ and $85,000$, as well as partially degrading the contaminating proteins in the extract. Addition of the protease inhibitors, phenylmethylsulfonyl fluoride and leupeptin, serves to effectively prevent most of the degradation of vinculin. Proteolysis of the contaminants also was decreased by addition of the inhibitors, but seemingly to a lesser extent. The first peak eluted from the CM52-cellulose column (Fig. 2) contained no vinculin, but several other components, including those with molecular weights of approximately 165,000, 67,000, 30,000, and 23,000 together with possible breakdown products of the contaminating proteins. None of the proteins in the first peak were found to react with vinculin antibodies in immunoblots (results not shown). The middle portion of the first peak (Fig. 2, fraction 2) was enriched with several proteins of unknown nature. The latter portion of the peak (Fig. 2, fraction 3) was comprised basically of proteins having $M_r = 165,000$, $67,000$, $35,000$, and 23,000. The area under the first peak relative to that under the second and only other peak eluted from the CM-cellulose column, which consists of vinculin, seems somewhat large in comparison to the amount of contaminants observed in SDS gels of the DEAE-cellulose-purified vinculin (Fig. 1d; Fig. 2, gel labeled DE). This is because the extinction coefficient at 280 nm of the protein contaminants in the first peak is much higher (~2-fold) than that of purified vinculin (value given later). Gels of the first part of the
vinculin peak (Fig. 2, fraction 4) occasionally showed diffuse light staining of components below vinculin. When this occurred, the vinculin in these fractions was not used for subsequent characterization studies. The latter portion of the second peak (Fig. 2, fractions 5 and 6) contained purified vinculin. At times, slight amounts of putative breakdown products of approximately 98,000 and 85,000 daltons from vinculin could be identified in the purified vinculin provided the gels were loaded with sufficient protein and not overly destained. These are likely to be breakdown products of vinculin because: 1) omission of phenylmethylsulfonyl fluoride and leupeptin prior to dialysis resulted in a slight increase in the amount of these polypeptides in the vinculin fractions, 2) digestion of vinculin with Ca$^{2+}$-activated neutral protease generates polypeptide fragments having these molecular weights (see also Fig. 8), 3) storage of purified vinculin for prolonged periods (>1 month) at 4°C resulted in an increase in the amount of these polypeptides (Fig. 8 and also see Ref. 1), and 4) vinculin and the 98,000- and 85,000-dalton components exhibit cross-reactivity as determined by immunoautoradiography with polyclonal antibodies prepared against the electrophoretically purified 130,000-dalton polypeptide (data not shown).

Preparations of vinculin, routinely consisting of over 97% 130,000-dalton protein, contained no detectable contamination other than the occasional 1-3% content of vinculin breakdown products as measured by one-dimensional SDS-gel electrophoresis (Fig. 1e; Fig. 2,
fractions 5 and 6) and two-dimensional gel electrophoresis (Fig. 3b). Typical yields of purified vinculin were 15-22 mg from 800 g ground gizzard. In calculating recovery of vinculin, quantitative densitometry of the vinculin fractions on one-dimensional SDS gels of fractions obtained before ammonium sulfate fractionation gave misleading results because of the presence of one or more additional proteins having a molecular weight of about 130,000. This problem was demonstrated clearly by two-dimensional gel electrophoresis experiments (Figs. 3 and 4). Examination of whole gizzard homogenate (Fig. 3a) shows that a protein (labeled 130A) is present that has a molecular weight similar to that of vinculin on one-dimensional SDS gels but that possesses a more acidic pI value than does vinculin. Immunoautoradiography of two-dimensional gels of whole gizzard homogenate demonstrates no labeling of this 130A protein by polyclonal vinculin antibodies (results not shown).

Two-dimensional Analysis of Purified Vinculin and Fractions Obtained at Intermediate Steps During Preparation

Although not easily distinguishable on normal one-dimensional SDS gels of whole muscle homogenates (Fig. 1a), vinculin and the 130A protein are clearly separated during isoelectric focusing (vinculin isoforms range in pI from about 6.25 to 6.50 and the 130A isoforms range in pI from about 5.70 to 5.85) and electrophoresis on the SDS slab gels in the second dimension (Fig. 3a). The purified vinculin consisted of six to seven isoforms, with three major, closely spaced
Fig. 3. Two-dimensional gels comparing vinculin in original muscle and after purification

The acidic end of the pH gradient in the isoelectric focusing dimension is to the left. Spots corresponding to the isoelectric variants of actin, desmin, tropomyosin (TM), 130A protein (130A), and vinculin are labeled. a, 8 M urea extract of whole gizzard muscle homogenate; b, final purified vinculin after CM-cellulose chromatography. Smooth muscle actin was added as an internal standard to the sample loaded on the isoelectric focusing gel in b. Purified vinculin (130K) and alpha-actinin (100K) standards were mixed together and loaded on slab gel b at the left before SDS electrophoresis in the second dimension. Very high molecular weight polypeptides such as the myosin heavy chains present in the sample in a do not enter the focusing gel and are not resolved.
ISOELECTRIC FOCUSING

130A  VINCULIN

DESMIN

ACTIN

130K

100K

VINCULIN

ACTIN β γ

SDS ELECTROPHORESIS

a

b
isoelectric variants present (pI from 6.30 to 6.45) and three or four lesser ones (Fig. 3b). To monitor the distribution of vinculin variants and to discern the steps at which 130A protein was removed, samples at selected stages of the purification procedure were monitored (Fig. 4). Low ionic strength extraction of washed myofibrils at 37°C generates a supernatant containing both 130A protein and vinculin (Fig. 4a). Examination of the pellet (muscle residue) remaining after this extraction (Fig. 4b) indicates that most of the 130A protein remains with the insoluble residue, whereas the vinculin has been released into the supernatant. Precipitation of the low ionic strength supernatant shown in Fig. 4a with MgCl\textsubscript{2} and analysis of the resulting supernatant (see Ref. 4 for sequential steps) shows that the MgCl\textsubscript{2} precipitation has effectively removed actin and desmin and has reduced the proportion of 130A protein in relation to vinculin (Fig. 4c). As expected from that result, the 130A protein is greatly enriched in the pellet resulting from the MgCl\textsubscript{2} precipitation, along with the actin and desmin (Fig. 4d). After precipitation of the MgCl\textsubscript{2} supernatant (Fig. 4c) with ammonium sulfate, all traces of the 130A protein have disappeared (crude vinculin extract shown in Fig. 4e). Thus, the only detectable 130,000-dalton components in the crude vinculin extract are the vinculin isoforms (pI = 6.25-6.50).

As shown in Fig. 4a, a very small amount of protein with a subunit M\textsubscript{x} ~ 152,000 (called 152-kDa protein herein) also was present in the original 1 h low ionic strength extract. This 152-kDa protein
Fig. 4. Two-dimensional gels of fractions obtained at intermediate steps during the preparation of vinculin

Only the upper two-thirds and right-hand portion of each gel, containing the spots corresponding to actin, desmin, alpha-actinin, vinculin, 130A protein (130A), and 152-kDa protein (152K) are shown. a, supernatant resulting from the 1 h, 37°C low ionic strength extract of washed myofibrils; b, pellet (muscle residue) remaining after the 1 h, 37°C low ionic strength extraction; c, supernatant obtained by precipitation of the low ionic strength extract shown in gel a with MgCl₂; d, pellet obtained by precipitation of the low ionic strength extract shown in gel a with MgCl₂; e, pellet obtained by ammonium sulfate precipitation of the MgCl₂ supernatant shown in gel c. Artifacts and/or smears in the areas of the actin and desmin variants are present because large loads were used in order to clearly identify other proteins present in lesser quantity.
was enriched along with the vinculin during preparation of the crude vinculin extract as shown in Fig. 4e. The 152-kDa protein also is easily observed in Fig. 1c. The 152-kDa protein, having a measured pI value of 5.89-5.95, probably is identical to the 152,000-dalton protein previously described (28) on the basis of its solubility, size, elution position from DEAE-cellulose columns (i.e. just after the filamin peak), and cross-reactivity with vinculin antibodies (see Ref. 28) as determined by immunoaautoradiography (results not shown).

Biochemical Characterization of Purified Vinculin

Analytical ultracentrifugation shows that the purified vinculin sediments as a single peak (Fig. 5; $s_{20,w}^0 = 5.9$) when examined in 100 mM NaCl, 20 mM Tris-acetate, pH 7.1. The ultraviolet circular dichroism spectrum of purified vinculin (Fig. 6), measured in 5 mM sodium phosphate, pH 7.1, is characteristic of proteins exhibiting considerable alpha-helical structure, with two negative extrema at 208 and 222 nm. The extremum at 208 nm gives a value of -18,500 deg·cm²·dmol⁻¹ and the extremum at 222 nm gives a value of -19,500 deg·cm²·dmol⁻¹. Calculation of the percentage alpha-helix from the mean residue ellipticity at 208 nm by using Method I of Greenfield and Fasman (48) results in a value of 50% alpha-helix. The pattern is similar to that obtained for poly-L-lysine containing about 50% alpha-helix and approximately equal amounts of beta-structure and random coil (48). The ultraviolet absorption spectrum of vinculin (Fig. 7), measured in 1 mM sodium bicarbonate, pH 7.0, indicated a maximum at
Fig. 5. Sedimentation velocity pattern of purified vinculin in 100 mM NaCl, 20 mM Tris-acetate, pH 7.1

Pooled vinculin (Fig. 2, fractions 5 and 6) from a CM-cellulose column was used at 6.82 mg/ml. Numbers represent minutes after reaching speed. Centrifugation was performed at 60,000 rpm at 20.0°C. Phase plate angle was 65°. Observed sedimentation coefficient was 5.3 S ($s_{20,w} = 5.9$).
Fig. 6. Circular dichroism spectrum of purified vinculin

The spectrum was measured in 5 mM sodium phosphate, pH 7.1, at a protein concentration of 0.51 mg/ml. Cell path length was 1.0 mm and temperature was 25°C.
Fig. 7. Ultraviolet absorption spectrum of purified vinculin

The spectrum was measured in 1 mM sodium bicarbonate, pH 7.0, at a protein concentration of 0.50 mg/ml. The spectrum shown is adjusted to that for a 1% (w/v) solution of protein (\(\varepsilon_{278}^{1} = 4.64\)).
278 nm, a measured $E_{278}$ of 4.64, and a ratio of absorbance at 273 nm to that at 260 nm of 1.60.

The amino acid analysis of the purified vinculin is shown in Table I and is compared with analyses obtained in other laboratories on DEAE-cellulose-purified vinculin (see "Discussion"). The relatively low value obtained for the extinction coefficient is in agreement with the low content of aromatic amino acids present in vinculin.

Digestion of Vinculin by Ca$^{2+}$-activated Neutral Protease

Digestion of vinculin with Ca$^{2+}$-activated neutral protease, which is a protease endogenous to muscle and many nonmuscle cells (49, 58, 59), generated major proteolytic fragments having approximate $M_r = 98,000, 85,000, and 26,000$ (Fig. 8). In this experiment, we used a sample of purified vinculin that had been subjected to prolonged storage (>1 month) at 4°C in order to compare breakdown products with polypeptides produced by proteolytic cleavage. Such samples (Fig. 8a) usually contained a small amount of polypeptides smaller than the 130,000-dalton vinculin polypeptide. Simply incubating this control vinculin for 30 min at 25°C in the presence of 5 mM Ca$^{2+}$, but no Ca$^{2+}$-activated neutral protease (Fig. 8b), or 10 mM EDTA, 5 mM Ca$^{2+}$, plus Ca$^{2+}$-activated neutral protease (Fig. 8c) results in a small increase in amount of these smaller polypeptides, especially those in the molecular weight range of 85,000-98,000. The major protein fragments of $M_r = 85,000$ and 98,000 resulting from Ca$^{2+}$-activated neutral
<table>
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<th>Amino acid</th>
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<th>Feramisco et al. (23)</th>
<th>Geiger (2)</th>
<th>Jockusch and Isenberg (25)</th>
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\(^a\)Values are expressed as mole percent.

\(^b\)Results are expressed as means plus or minus S.E. of analysis on five different preparations run in duplicate.

\(^c\)Recalculated from no. of residues per 130,000 M. W. polypeptide.

\(^d\)Values obtained by extrapolation to zero time hydrolysis.

\(^e\)Calculated from ultraviolet absorbance.

\(^f\)ND, not determined.

\(^g\)Determined as cysteic acid.
Fig. 8. Digestion of purified vinculin with Ca\(^{2+}\)-activated neutral protease

The Ca\(^{2+}\)-activated neutral protease to substrate ratio was 1/50 (w/w). Digestion at 25°C was initiated by addition of CaCl\(_2\) to a final concentration of 5 mM as described under "Experimental Procedures". Aliquots were removed at 2-, 5-, and 30-min intervals. a, control vinculin that had been stored at 4°C in order to show storage breakdown products; b, control vinculin incubated for 30 min at 25°C, but in the absence of added Ca\(^{2+}\)-activated neutral protease and Ca\(^{2+}\); c, control vinculin incubated for 30 min in the presence of Ca\(^{2+}\)-activated neutral protease, 5 mM CaCl\(_2\), and 10 mM EDTA; d, vinculin digested for 2 min; e, vinculin digested for 5 min; f, vinculin digested for 30 min. See "Experimental Procedures" for additional details. For the controls (a-c), 10 µg of protein were loaded on the gels and for the digests (d-f), 20 µg. 130k, for example, 130,000 daltons.
protease digestion (Fig. 8, d-f) comigrated with minor bands present in the stored sample of vinculin. Although Ca²⁺-activated neutral protease clearly digested vinculin during the first 2 min of incubation (Fig. 8d), digestion occurred at a slower rate during the remaining portion of the digestion (Fig. 8, e and f). This may result from the autodigestion of Ca²⁺-activated neutral protease (60).

**Vinculin/actin Interaction**

The interaction between purified vinculin and actin was measured by low shear viscometry with the falling ball viscometer (Fig. 9). Addition of DEAE-cellulose-purified vinculin to the actin decreased the viscosity in a concentration dependent manner (Fig. 9, •) with a decrease of approximately 85% occurring at a concentration of 30 µg DEAE-cellulose-purified vinculin/ml, which is similar to the results of others (24, 27-29). Addition of purified vinculin (i.e. CM-cellulose-purified vinculin) did not inhibit viscosity, but instead resulted in a small increase in a concentration-dependent manner (Fig. 9, △) with an increase of approximately 30% occurring at a concentration of 30 µg purified vinculin/ml. In view of the different effects of these two vinculin fractions on the low shear viscosity of actin, the protein in the pooled fractions from the first peak eluted during CM-cellulose purification (see Fig. 2) of the DEAE-cellulose-purified vinculin was tested (Fig. 9, ○). That fraction was noticeably more inhibitory than was the DEAE-cellulose-purified vinculin, reducing the viscosity of F-actin by approximately 95% at a
Actin, adjusted to a final concentration of 0.5 mg/ml in a total volume of 1 ml, was polymerized by the addition of MgCl₂ and incubated for 10 min at 25°C in the presence of selected protein samples being tested as described in "Experimental Procedures". (●), DEAE-cellulose-purified vinculin; (○), the pooled fraction of the first peak from the CM-cellulose column; (△), purified vinculin (i.e. CM-cellulose purified). Values have been normalized (24) to minimize variations among different actin preparations. Each data point represents the mean obtained from three different representative preparations of each sample tested in duplicate.
concentration of 20 μg/ml. The effects of the three protein fractions on actin viscosity shown in Fig. 9 were not calcium sensitive (results not shown). Also, to ensure that pH change alone during CM-cellulose chromatography was not a factor in these studies, the pH of the DEAE-cellulose-purified vinculin tested in the viscosity experiment also was adjusted to pH 5 and stored for a similar period of time before readjustment of pH and addition to actin, with no effect.
DISCUSSION

We found that vinculin isolated in low ionic strength extracts and purified by DEAE-cellulose chromatography by conventional procedures (1, 4) still contains contaminants that can alter results of characterization studies, especially those involving interaction of vinculin with actin. Others (15, 20) also have noticed the presence of impurities in DEAE-cellulose-purified vinculin (also see one-dimensional gel of vinculin in Fig. 1 of Ref. 3) prepared by conventional procedures (1, 4). Because so many actin-binding proteins have been identified (61, 62), some of which are quite active in altering actin behavior in even minor amounts, we believe it is essential to subject the vinculin to additional purification. Further chromatography on either CM-cellulose (studies herein) or a combination of hydroxyapatite and gel exclusion columns (results not shown) is simple and effective in removing impurities and results in vinculin having a consistent effect in its interaction with actin. The final yield of CM-cellulose-purified vinculin (1.8-2.8 mg/100 g ground gizzard) is understandably less than that reported for DEAE-cellulose-purified vinculin (12 mg/100 g) in Feramisco and Burridge (4), but is in the lower range of values reported for DEAE-cellulose-purified vinculin by others (3-5 mg/100 g in Ref. 2; 2-3 mg/100 g in Ref. 26). The yields of purified vinculin obtained in all studies seem low in comparison with that of alpha-actinin (10-fold higher) upon a cursory examination and comparison of the 100,000- and 130,000-
dalton bands on SDS gels of smooth muscle homogenates or myofibril fractions (e.g. see gels a and b in Fig. 1). The presence of considerable amounts of other proteins having similar subunit molecular weights to that of vinculin, such as that observed by two-dimensional gel electrophoresis and labeled 130A in this study, provides a reasonable explanation.

Our purified vinculin routinely exhibited six to seven isoforms by two-dimensional gel electrophoresis. Three of these were major, with the middle of the three being most abundant. The pattern of isovinculins shown herein is similar to those recently shown for gizzard vinculin (Fig. 1 in Ref. 3; Fig. 4 in Ref. 63). The three major variants seen in our study correspond, from acidic to basic, to the alpha, beta, and gamma isovinculins described in Geiger (3) and those labeled 3, 5, and 6 in Endo and Masaki (63). The range of observed pI values (6.30-6.45) of the three major isoforms obtained herein agrees well with values (6.34-6.46 for isovinculin numbers 3-6) in Endo and Masaki (63). In co-isoelectric focusing experiments (not shown) with our vinculin and a sample of vinculin (kindly provided by Darrel E. Goll, University of Arizona), which had been extracted by the low temperature, low ionic strength extraction method of Singh et al. (64) and Geiger (1) and then subjected to DEAE-cellulose, hydroxyapatite and gel exclusion chromatography, the patterns of isovinculins matched. Thus, the short, high temperature extraction method of Feramisco and Burridge (4) and the longer, low temperature extraction method of Geiger (1) result in essentially identical
It is somewhat difficult to compare amino acid composition data in detail because the results come from different laboratories and because of small technical differences in analyses; however, our vinculin composition data were most in agreement with those reported by Jockusch and Isenberg (25). There is a distinct difference in the amino acid composition of vinculin reported by Feramisco et al. (28), who found alanyl residues to be the most abundant residue rather than glutamyl residues as found by three other laboratories (Refs. 2 and 25; studies herein). Interestingly, vinculin contains a higher content of nonpolar residues than does alpha-actinin (65). This may be correlated with the observations that: 1) although both proteins are present in focal adhesion plaques of fibroblasts and in other membrane/actin-filament attachment sites in vivo (1, 2, 5-9), vinculin is situated closer to the membrane than is alpha-actinin (2, 6, 7, 12) and 2) vinculin is only a good substrate for the purified src gene kinase in vitro in the presence of anionic phospholipids (15).

The Ca\(^{2+}\)-activated neutral protease purified in this laboratory (49, 60, 66) recently has been shown to be localized at the cytoplasmic face of the cell membrane in myoblasts and at the same sites plus myofibrillar Z-lines in differentiated (mature) muscle cells. Dayton and Schollmeyer (67) have suggested the protease, which apparently is fairly ubiquitous (59), might play a role in the release of microfilaments from membranes via digestion of protein(s) involved in the attachment sites. Interestingly, Hynes (23) recently has
suggested that disruption of cell membrane/cytoskeletal linkages via cleavage of an important component by a proteolytic enzyme could be involved in events occurring at the cell membrane during cell transformation. Although alpha-actinin is a very poor substrate for Ca\(^{2+}\)-activated protease (66), our results show that vinculin is a substrate for the protease. Further studies, however, are needed to demonstrate any relevance of vinculin digestion to the release of microfilaments from membrane anchorage sites.

An unexpected finding in this study was that the removal of contaminants present in DEAE-cellulose-purified vinculin altered the effect of the protein on the low shear viscosity properties of actin\(^3\). Our results with DEAE-cellulose-purified vinculin are in excellent agreement with those of others (24-27) who have used DEAE-cellulose-purified vinculin made by similar procedures (4) in that the vinculin decreased the low shear viscosity of actin solutions in a concentration-dependent manner. In contrast, as shown herein, vinculin that had been subjected to an additional chromatographic separation resulted in a small but consistent increase in low shear viscosity of actin. We found (results not shown) that subjecting the DEAE-cellulose-purified vinculin to gel exclusion chromatography on Sepharose 6B, as done by Wilkins and Lin (29), did not adequately remove contaminants or the inhibitory action of vinculin unless the gel filtration step was used in conjunction with hydroxyapatite or CM-cellulose chromatography. The loss of inhibition that we observed after additional chromatography of vinculin was not due to simple
inactivation/denaturation of the vinculin itself. The factor(s) responsible for the inhibitory activity, which was present in every preparation of DEAE-cellulose-purified vinculin tested, was recovered in a peak from the CM-cellulose column that contained several contaminants, but no vinculin. The identity and nature of the inhibitory factor(s) is under investigation. We have not unraveled the seemingly complex effect of vinculin on actin viscosity, but our preliminary results suggest the small, but consistent, positive effect of purified vinculin on actin viscosity is because vinculin cross-links actin filaments\(^1\). We find, however, no evidence that our purified vinculin causes bundling of actin filaments\(^3\) (i.e. formation of paracrystalline-like structures with actin filaments in parallel alignment) as reported by others (24-26). The results of this portion of our study may explain in part the lack of agreement (cf. Refs. 24-26 with Ref. 27 and 29) concerning vinculin's possible role in capping actin filaments and inhibiting actin polymerization. It is not inconceivable that the small number of reported (27, 29) high-affinity binding sites for vinculin on actin filaments (~1 vinculin to 1,500-2,000 actin monomers) was due to labeled contaminants that bound to the actin. And, whether vinculin actually causes the formation of actin bundles previously observed in vitro (24-26) or simply binds secondarily to bundles formed by contaminants in vinculin preparations also remains to be established.
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1 Some of the data presented herein have been presented at the 1982 American Society of Biological Chemists meeting (40) and at the 1983 American Society for Cell Biology meeting (41).

2 The abbreviations used are: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N''-tetraacetic acid.

3 Darrel E. Goll, University of Arizona, and Joann J. Otto, Purdue University, personal communications, have found that highly purified vinculin does not decrease the high or low shear viscosity of F-actin solutions; and in agreement with us, Dr. Otto finds no evidence that purified vinculin bundles F-actin filaments. Highly purified vinculin isolated from human platelets also has been shown not to decrease the low shear viscosity of F-actin (68).
SECTION II: EFFECT OF TEMPERATURE, pH, AND IONIC STRENGTH ON THE INTERACTION OF VINCULIN WITH ACTIN
Effect of Temperature, pH, and Ionic Strength on the Interaction of Vinculin with Actin

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Running Title: Interaction of Vinculin with Actin
We have recently demonstrated (Evans, R. R., Robson, R. M., and Stromer, M. H. (1984) J. Biol. Chem. 259, 3916-3924) that the factor(s) in conventional DEAE-cellulose purified vinculin responsible for the marked decrease in the low shear viscosity of actin solutions ("standard conditions" of 2 mM MgCl$_2$, pH 7.5, 25°C) can be isolated and removed by CM-cellulose chromatography. To further characterize the effect of highly purified vinculin on F-actin, we have examined the effects of temperature, pH, and ionic strength (NaCl) on the formation of actin filaments in the presence of varying amounts of vinculin by 1) low shear viscometry and 2) electron microscopy. Under standard conditions, addition of vinculin (30 μg/ml) causes a 30% increase in the viscosity of F-actin. Structural studies show this increase is paralleled by an increase in cross-linking of actin filaments. When the temperature is decreased to 15°C, vinculin still causes a slight, but consistent increase in the viscosity of F-actin due to an increase in cross-linking of the actin filaments. When the temperature was increased to 37°C, however, addition of vinculin had no measurable effect on actin viscosity or appearance. When the pH was increased to 8.0 (other conditions standard), a 60% increase in the low shear viscosity of F-actin resulted from addition of vinculin (30 μg/ml) and this effect was paralleled by additional cross-linking of actin filaments. Surprisingly, when the pH was lowered to 7.0, vinculin (70 μg/ml) caused a 40% decrease in F-actin viscosity. This
was reflected by the formation of "irregular-appearing" actin filaments. Elevation of the ionic strength by addition of NaCl resulted in an increase in the viscosity of the F-actin controls, but addition of vinculin (70 µg/ml) decreased the viscosity of these controls by approximately 65%. Electron microscope observations indicated that vinculin had disrupted the branched, paracrystalline-like bundles present in the F-actin formed at the higher salt concentrations. When tropomyosin was premixed with actin in a 1 to 7 molar ratio and low shear viscosity was measured at near physiological-like conditions (37°C, 150 mM NaCl), addition of vinculin (50 µg/ml) caused a 90% increase in apparent viscosity.
INTRODUCTION

Primarily because of its localization where microfilament bundles terminate at focal contacts in cultured cells (1-5), vinculin has been implicated in the linkage of actin bundles to the cell membrane (1, 2, 4, 5). This putative role gained support when it was shown that vinculin is a substrate for the src gene kinase (6-8), which is localized in association with adhesion plaques at the plasma membrane (9-11), as well as for protein kinase C (12, 13), which also is associated with the plasma membrane (14, 15).

Although it has been indicated that vinculin binds directly to actin (16-22), the nature of the vinculin/actin interaction is not clear. Several studies using conventionally prepared vinculin (4) have shown that vinculin markedly lowers the expected viscosity increase that occurs during polymerization of actin (16-19, 21). We recently demonstrated (23, 24), however, that conventional preparations of vinculin contain impurities that greatly alter the effect of vinculin on actin viscosity.

In this study, we have used low shear viscometry and electron microscopy to carefully examine the effect of vinculin on F-actin under differing conditions of polymerization. Our results indicate that although vinculin interacts with F-actin in a concentration-dependent manner under several experimental conditions, it does so in a seemingly complex manner. These results also should provide a
useful foundation for others investigating the effects of highly purified vinculin on actin \textit{in vitro}. \footnote{1}
EXPERIMENTAL PROCEDURES

Protein Preparation

Highly purified vinculin was prepared from turkey gizzard smooth muscle as recently described (24). The purified vinculin was extensively dialyzed against 5 mM Tris acetate, pH 7.5 (storage buffer) before analysis. Actin, prepared from porcine skeletal muscle by the method of Spudich and Watt (25), was further purified by gel filtration on Sephadex G-150 as described by MacLean-Fletcher and Pollard (25). Purified actin in 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl₂, 2 mM Tris-HCl, pH 8.0, was used immediately after preparation. Tropomyosin was prepared from ethanol/ether powders of porcine skeletal muscle as previously described (27), with additional purification by hydroxyapatite chromatography (28). The purified tropomyosin was dialyzed against 0.1 M NaCl, 1 mM NaHCO₃, pH 8.0, and used immediately after preparation. All purified protein preparations were passed through a Millex-GS 0.22 μm sterile filter (Millipore Corp., Bedford, MA) to remove any particulate matter present and to prevent microbial growth. Protein concentrations were determined by the biuret method (29) as modified (30).

Low Shear Viscometry

Low shear viscometry was done by the method of MacLean-Fletcher and Pollard (31), with measurement of the velocity of the falling ball done at a fixed angle (70° from vertical). The term "standard
conditions" in this study refers to polymerization of actin (0.5 mg/ml) in the presence of varying amounts of vinculin in 1 mM ATP, 1 mM EGTA, 2 mM MgCl₂, 10 mM imidazole/HCl, pH 7.5 at 25°C. Experiments involving effect of temperature used the same ionic milieu. For experiments involving effect of pH, polymerization of actin was done in 10 mM imidazole/HCl, pH 7.0, and 10 mM Tris acetate, pH 8.0, respectively, in place of the 10 mM imidazole/HCl, pH 7.5 (i.e., otherwise standard conditions). In experiments involving effect of ionic strength, variations in the salt content of the polymerization buffer were made by adding 1.0 M NaCl to the reaction mixture to give the final desired concentration of NaCl. All samples were incubated under the selected experimental conditions for 3 min before initiation of polymerization by addition of MgCl₂. Following addition of MgCl₂, samples were immediately drawn into capillary tubes and incubated. Velocity of the falling ball was measured after 10 min for samples run at 37°C or 25°C and after 30 min for samples run at 15°C. Concentrated solutions of vinculin and tropomyosin were used to prevent any effect of storage buffers on polymerization of actin. Additional conditions for individual experiments are given in the figure legends.

Electron Microscopy

Negatively stained preparations of F-actin filaments were prepared by placing a drop of a duplicate sample of the actin control or actin/vinculin mixture (0.25 mg/ml of actin), polymerized under
conditions identical to those used for viscometry, on a 400-mesh, glow-discharged copper grid that had been covered with a carbon support film. After standing for approximately 15 sec, excess solution was removed. A drop of 1% uranyl acetate was applied and allowed to stand for 5 to 10 sec. Excess stain was removed and the grid was permitted to air dry. Preparations were immediately examined in a JEOL 100 CX-II electron microscope operated at 80 kV, with representative areas photographed.
RESULTS

Interaction of Purified Vinculin and Selected Samples from the Vinculin Purification Procedure with Actin Under "Standard Conditions"

It has been demonstrated previously (23, 24) that "conventionally" prepared vinculin (4) contains several contaminants that markedly affect the low shear viscosity of F-actin solutions. Upon removal of the contaminants by CM-cellulose chromatography, addition of highly purified vinculin to actin resulted in an increase in the low shear viscosity of F-actin in a concentration-dependent manner (Fig. 1) (24). This result was in contrast to that resulting from addition of an inhibitory fraction that was recovered as the first peak from the CM-cellulose column. The inhibitory fraction reduced the viscosity of F-actin by approximately 95% at a concentration of 20 µg/ml (Fig. 1). When duplicate, identically prepared samples are examined by the electron microscope, it is apparent that the increase in viscosity caused by addition of CM-cellulose-purified vinculin (Fig. 2b) is due to an increase in amount of cross-linking among F-actin filaments as compared to actin polymerized in the absence of vinculin (Fig. 2a). Polymerization of actin in the presence of the inhibitory fraction isolated from the CM-cellulose column (see Fig. 2 in Ref. 24) results in the formation of shortened (possibly severed) actin filaments and bundles of associated actin filaments (two examples are shown in Fig. 2c and d). One explanation for both the shortening and bundling of actin filaments
Fig. 1. Viscosity of actin polymerized in the presence of samples selected from the preparation of purified vinculin as measured in the falling ball viscometer at 25°C.

Actin, adjusted to a final concentration of 0.5 mg/ml in a total volume of 1 ml, was polymerized by the addition of MgCl₂ and incubated for 10 min at 25°C in the presence of selected protein samples. DEAE-cellulose purified vinculin (○); the pooled "inhibitory" fraction from the first peak from the CM-cellulose column (○); purified vinculin (i.e. CM-cellulose purified vinculin) (△).
Fig. 2. Electron micrographs of actin polymerized in the presence and absence of selected fractions from the preparation of vinculin

Actin (0.25 mg/ml) was polymerized by the addition MgCl₂ (2 mM final concentration) and incubated for 10 min, pH 7.5, at 25°C. a) actin control sample containing no vinculin; b) actin polymerized in the presence of CM-cellulose purified vinculin (70 µg/ml); c and d) actin polymerized in the presence of the pooled fraction of the first peak from the CM-cellulose column. Bar, 0.2 µm.
effected by the inhibitory fraction is that more than one actin-binding protein is present in this fraction. In results not shown, we observed that addition of highly purified vinculin to preformed paracrystalline bundles of actin filaments by the addition of 50 mM MgCl₂ indicated that the highly purified vinculin is still able to bind to F-actin bundles in a manner similar to that shown by Isenberg et al. (18).

Effect of Temperature on Actin/Vinculin Interaction

The effect of altering temperature, from 25°C (standard conditions) on the vinculin interaction as measured by viscometry is shown in Fig. 3. Addition of 30 μg/ml of vinculin resulted in a 30% increase over the actin control (apparent viscosity, 26.1 cp) polymerized in the absence of vinculin. A small, but consistent increase also was noted when actin was polymerized at 15°C (Fig. 3), with a maximum effect occurring at approximately 20 μg vinculin/ml. The apparent viscosity of the actin control at 15°C, with a value of 67.1 cp, was significantly above that of the controls at 25°C or 37°C. When actin was polymerized at 37°C (Fig. 3), increasing amounts of vinculin resulted in no measurable effect on the low shear viscosity of F-actin.

The effect of temperature on vinculin/actin networks also was followed by electron microscopy (Fig. 4). In the absence of vinculin, actin polymerized at 15°C, pH 7.5, appeared as an isotropic gel network in which filaments are of uniform diameter and evenly
Actin (0.5 mg/ml) was polymerized by the addition of MgCl$_2$ (2 mM final concentration) and incubated as described in "Experimental Procedures" at 15°C (■), 25°C (▲), and 37°C (○), pH 7.5.
APPARENT VISCOSITY (cP)

VINCULIN (µg/ml)

0 10 20 30 40 50 60 70 80

10 20 30 40 50 60 70 80
Actin (0.25 mg/ml) was polymerized by the addition of MgCl₂ (2 mM final concentration) and incubated for 30 min at 15°C and 10 min at 37°C, pH 7.5. a) actin control polymerized in the absence of vinculin at 15°C; b) actin polymerized in the presence of vinculin (30 µg/ml) at 15°C; c) actin control polymerized in the absence of vinculin at 37°C; d) actin polymerized in the presence of vinculin (30 µg/ml) at 37°C. Bar, 0.2 µm.
distributed (Fig. 4a). Addition of vinculin to actin (molar ratio of approximately 25:1, actin to vinculin) causes the formation of an F-actin network in which the filaments are slightly more cross-linked (Fig. 4b) in comparison to actin polymerized in the absence of vinculin (Fig. 4a). Polymerization of actin at 37°C, pH 7.5, in the absence of vinculin (Fig. 4c), leads to a gel network which is essentially indistinguishable from actin polymerized at 25°C, pH 7.5, without vinculin (cf. Fig. 2a). And no detectable change is obvious in the F-actin filaments formed at 37°C, pH 7.5, (Fig. 4d) upon addition of vinculin in comparison to the actin control (Fig. 4c).

Effect of pH on Actin/Vinculin Interaction

To determine the effect of pH on the interaction of vinculin with F-actin, actin was polymerized at 25°C in the presence of vinculin at pH values of 7.0, 7.5, and 8.0, values close to those present in vivo. Standard conditions, with exception of pH, were used. Under standard conditions (2 mM MgCl₂, 25°C, pH 7.5), the addition of 30 μg vinculin/ml resulted in an increase of 30% in the low shear viscosity over the actin control (apparent viscosity, 26.1 cp) (Fig. 5). Increasing the pH of the polymerization buffer to 8.0 (10 mM Tris acetate, pH 8.0) caused a 31% decrease in the control F-actin value compared to the control observed at 25°C, but addition of vinculin still increased the low shear viscosity (60% at 30 μg vinculin/ml). A marked change in vinculin's effect on actin viscosity became apparent, however, when the pH was lowered to 7.0. The viscosity of the F-actin
Fig. 5. Effect of pH on the apparent viscosity of actin polymerized in the presence of vinculin as measured by the falling ball viscometer.

Actin (0.5 mg/ml) was polymerized by the addition of MgCl₂ (2 mM final concentration) and incubated for 10 min, 25°C at pH 7.0 (●), 7.5 (▲), and 8.0 (○).
control increased considerably to an apparent viscosity of 48.1 cp. But vinculin caused a concentration dependent decrease (about 40% at 70 μg/ml) in viscosity.

As just shown (Fig. 5), adjustment of the pH to 7.0 (10 mM imidazole, pH 7.0, 25°C) causes an increase in viscosity of the actin control over the actin control polymerized at pH 7.5. The actin filaments (control) formed at pH 7.0 (Fig. 6a) are evenly distributed and of uniform diameter. Upon the addition of vinculin at pH 7.0 (molar ratio of approximately 10:1, actin to vinculin) (Fig. 6b), the F-actin filaments appear "irregular" and have lost the smooth appearance of the filaments in the control sample (Fig. 6a). Increasing the pH to 8.0 (10 mM Tris acetate, pH 8.0) results in an actin control that contains filaments less uniform in length and with an increase in lateral association or side to side aggregation of actin. The filaments are also poorly defined with a greater number of "broken" filaments present when compared to actin polymerized at pH 7.5, 25°C (Fig. 2a). The presence of vinculin during the polymerization of actin at pH 8.0, 25°C (molar ratio of approximately 25:1, actin to vinculin) (Fig. 6d) serves to cross-link formed filaments in a gel-like pattern.

Effect of Ionic Strength on Actin/Vinculin Interaction

Before conducting a study of the effect of changing the ionic strength (NaCl) on the polymerization of actin in the presence of vinculin, the effect of increasing ionic strength on actin alone was
Fig. 6. Electron micrographs of actin polymerized in the presence and absence of vinculin at pH 7.0 and 8.0

Actin (0.25 mg/ml) was polymerized by the addition of MgCl₂ (2 mM final concentration) and incubated for 10 min at 25°C. a) actin control polymerized in the absence of vinculin at pH 7.0; b) actin polymerized in the presence of vinculin (70 µg/ml) at pH 7.0; c) actin control polymerized in the absence of vinculin at pH 8.0; d) actin polymerized in the presence of vinculin (30 µg/ml) at pH 8.0. Bar, 0.2 µm.
examined at 25 mM, 50 mM, and 150 mM NaCl. The viscosity of the actin controls was increased (Fig. 7) by addition of NaCl to the polymerization buffer (otherwise standard conditions). At a concentration of 50 mM NaCl, the viscosity of F-actin was increased 250% above the standard value (apparent viscosity, 25.1 cp) obtained when no NaCl was present in the polymerization buffer. The viscosity of F-actin was increased to an even greater extent at a NaCl concentration of 150 mM, with a value approximately 350% over the actin control at 0 mM NaCl (Fig. 7).

The effect of ionic strength on the polymerization of actin at 25°C, pH 7.5, in the presence of vinculin is shown in Fig. 8. Addition of 30 µg/ml of vinculin resulted in an increase of 30% in the low shear viscosity of actin over the actin control (apparent viscosity, 26.1 cp) under standard conditions (2 mM MgCl₂, 25°C, pH 7.5). Elevation of the ionic strength to a final concentration of 25 mM or 50 mM NaCl resulted in essentially similar effects on the viscosity of F-actin in the presence of vinculin (Fig. 8), with vinculin at a concentration of 70 µg/ml causing 71% and 63% decreases in the viscosity of F-actin, respectively. The apparent viscosity of the actin controls polymerized in the absence of vinculin were essentially the same (84.8 cp and 85.4 cp for the 25 mM and 50 mM NaCl samples, respectively). Polymerization of actin in the presence of 150 mM NaCl (Fig. 8) resulted in an apparent viscosity of 118.1 cp for F-actin alone. The presence of 70 µg vinculin/ml caused a decrease in the viscosity, yielding a value 59% lower than the control value.
Fig. 7. Effect of increasing salt concentration (NaCl) on the apparent viscosity of actin polymerized in the absence of vinculin as measured by the falling ball viscometer.

Actin (0.5 mg/ml) was polymerized by the addition of MgCl$_2$ (2 mM final concentration) and incubated for 10 min at 25°C, pH 7.5.
Fig. 8. Effect of increasing salt concentration (NaCl) on the apparent viscosity of actin polymerized in the presence of vinculin as measured by the falling ball viscometer.

Actin (0.5 mg/ml) was polymerized by the addition of MgCl₂ (2 mM final concentration) and incubated for 10 min, 25°C with NaCl at 0 mM (△), 25 mM (●), 50 mM (○), and 150 mM (▼), pH 7.5.
Thus, addition of NaCl to the polymerization buffer markedly altered the effect of vinculin on actin's low shear viscosity.

Addition of NaCl to a final concentration of 50 mM at pH 7.5, 25°C results in the formation of some paracrystalline-like bundles (~4-5 filaments/bundle) of F-actin filaments (Fig. 9a). The majority of filaments are found to be closely associated in this type of lateral aggregate, but some F-actin filaments branch from these bundles and appear to interact with other bundles within the gel network. Upon addition of vinculin to actin (molar ratio approximately 10:1, actin to vinculin) polymerized in the presence of 50 mM NaCl, the tightly associated bundles are no longer present (Fig. 9b). Instead, the F-actin filaments are loosely associated with one another, and there is an increase in the number of single filament cross-link points in the gel (i.e. more single filaments are being cross-linked).

Polymerization of actin in the absence of vinculin at a concentration of 150 mM NaCl also resulted in filaments which are formed into tightly associated paracrystalline-like bundles (Fig. 9c). The filaments, at this concentration of NaCl, also may become more susceptible to physical damage as evidenced by the presence of some short filaments in the background. Upon addition of vinculin to actin polymerized in the presence of 150 mM NaCl (molar ratio approximately 10:1, actin to vinculin), the tightly associated bundles are no longer formed (Fig. 9d). Cross-linked filaments, which appear loosely associated with one another, are the predominant filaments present.
Fig. 9. Electron micrographs of actin polymerized in the presence and absence of vinculin at 50 mM and 150 mM NaCl

Actin (0.25 mg/ml) was polymerized by the addition of MgCl₂ (2 mM final concentration) and incubated for 10 min at 25°C, pH 7.5. a) actin control polymerized in the absence of vinculin, 50 mM NaCl; b) actin polymerized in the presence of vinculin (70 μg/ml), 50 mM NaCl; c) actin control polymerized in the absence of vinculin, 150 mM NaCl; d) actin polymerized in the presence of vinculin (70 μg/ml), 150 mM NaCl. Bar, 0.2 μm.
Effect of Tropomyosin on the Actin/Vinculin Interaction at Physiological-like Conditions

When actin was polymerized in the absence of tropomyosin at 150 mM NaCl, 37°C, pH 7.5 (Fig. 10), addition of vinculin gave no measurable effect on the viscosity of the F-actin. When tropomyosin was added, the apparent viscosity of the control actin sample (50.5 cp) was increased. Addition of 30 μg vinculin/ml increased the viscosity by approximately 90% (apparent viscosity, 96.6 cp, Fig. 10). Electron microscope studies show that F-actin filaments reconstituted with a 7:1 molar ratio of actin to tropomyosin and polymerized in the presence of 150 mM NaCl, 37°C, pH 7.5, are associated with one another in a random manner (Fig. 11a), which is comparable to the appearance of filaments polymerized in the absence of tropomyosin at 37°C, pH 7.5 (Fig. 4c). Addition of vinculin to actin (molar ratio of approximately 25:1, actin to vinculin) polymerized in the presence of tropomyosin (150 mM NaCl, 37°C, pH 7.5), results in the formation of a more highly cross-linked gel network (Fig. 11b), which seemingly overrides the effect of temperature on F-actin filaments (Fig. 4b) formed in the presence of vinculin.
Fig. 10. Effect of vinculin on the apparent viscosity of actin polymerized in the presence of tropomyosin as measured by the falling ball viscometer

Actin (0.25 mg/ml) was polymerized by the addition of MgCl$_2$ (2 mM final concentration) in the presence of tropomyosin (0.061 mg/ml) and incubated for 10 min at 37°C with 150 mM NaCl, pH 7.5 •, added vinculin; ○, actin polymerized at 37°C in the presence of 150 mM NaCl, pH 7.5 but no tropomyosin.
Fig. 11. Electron micrographs of actin polymerized in the presence of tropomyosin and in the presence and absence of vinculin

Actin (0.25 mg/ml) was polymerized in the presence of tropomyosin (0.061 mg/ml) by the addition of MgCl₂ (2 mM final concentration) and incubated at 150 mM NaCl, 37°C, pH 7.5. a) actin/tropomyosin mixture control polymerized in the absence of vinculin; b) actin/tropomyosin mixture polymerized in the presence of vinculin (30 μg/ml). Bar, 0.2 μm.
DISCUSSION

Previous studies have indicated that the interaction of vinculin with actin was inhibitory with respect to the low shear viscosity of F-actin solutions (16-21, 32). This inhibition by vinculin was supposedly due either to formation of paracrystalline-like F-actin bundles (16, 17, 21) or to inhibition of actin filament assembly by binding of vinculin to the ends of growing filaments (5, 21, 24, 32). We have recently shown (24), however, that the factor(s) present in DEAE-cellulose-purified vinculin (4) and responsible for the dramatic decrease in the low shear viscosity of F-actin solutions is due to contaminants present in the added vinculin. Removal of this inhibitory fraction from DEAE-cellulose-purified vinculin by CM-cellulose chromatography results in vinculin that routinely causes an increase in the low shear viscosity of F-actin when polymerized under what we have termed "standard conditions" (see Fig. 1). Our results were recently confirmed by Burridge and Mangeat (33). We show, herein, that the rather small, but consistent increase in viscosity caused by addition of highly purified vinculin under these conditions is apparently caused by an increase in cross-linking of actin filaments (Fig. 2b). Addition of the inhibitory fraction, removed from conventionally prepared vinculin by CM-cellulose chromatography, to actin under "standard conditions" results in the formation of shortened actin filaments, plus some bundles of F-actin filaments (Fig. 2c and d). Thus, it is possible that more than one actin
binding protein is present in the inhibitory fraction recovered from the CM-cellulose column (24).

In this study, we have examined the effect of several experimental variables on the effect of vinculin on actin filaments as measured by low shear viscometry and appearance of actin filaments by electron microscopy. We previously observed that the interaction of vertebrate smooth muscle vinculin with actin is not dependent upon the presence of Ca$^{2+}$ (24). This is in contrast to other actin-binding proteins such as villin, fimbrin and actinogelin, which have a Ca$^{2+}$-dependency with respect to their interaction with actin (34-36). One of the variables we tested was temperature. In contrast to polymerization at 25°C or 37°C, polymerization of actin at 15°C produces a marked increase in the apparent viscosity of the actin control. This is in accord with previous studies (37, 38) showing that at lower temperatures the rate of polymerization of actin is decreased, but the average length of filaments is increased due to the smaller number of "nuclei" present (39). Although vinculin causes a small increase in viscosity at 15°C or 25°C, it is difficult to determine if this increase is due solely to an increase in the number of cross-links which we observed in the structural studies or due also to an increase in the average length of filaments. Low shear viscometry is unable to detect small changes in filament length (40, 41). When the temperature was raised to 37°C (otherwise standard conditions), vinculin had no noticeable effect on either the low shear viscosity or appearance of actin filaments when viewed by the electron
microscope. Increasing the temperature increases the polymerization rate of actin (37, 38), thereby allowing actin to achieve a steady state equilibrium much faster than at lower temperatures. It is possible that at the higher temperature the actin filaments are in a greater state of flux with regard to breaking and reannealing of filaments, and that this continuous state of filament disruption prevents vinculin from effectively stabilizing F-actin filaments via cross-linking. It also, however, cannot be ruled out that vinculin simply is unable to bind to the actin filaments at 37°C. The lack of observed interaction between vinculin and actin at 37°C is rather similar to the interaction between alpha-actinin and actin which interact effectively at low temperatures (42-44), but interact much less at 37°C (43).

It was evident from our study that vinculin increased the low shear viscosity of actin polymerized under "standard conditions" at pH 7.5, or when the pH was increased to 8.0, and that this increase was reflected in a more cross-linked appearance of the actin filament network. A clear change, however, was observed when the pH was lowered only slightly to 7.0. First, a marked increase in the apparent viscosity of the actin control occurred in comparison to that of the actin control at pH 7.5 or 8.0. This may be related to the fact that increasing the hydrogen ion concentration increases the rate of polymerization of actin (37, 45) and the random side-by-side aggregation of actin filaments (45), while in turn causes an increase in the apparent viscosity of the actin. Second, in contrast to
results obtained at higher pH, addition of vinculin at pH 7.0 causes a concentration-dependent decrease in low shear viscosity. This was reflected in fewer cross-links in the actin filament network in the structural studies. Addition of some actin-binding proteins serves to shift the hydrogen ion-induced polymerization of actin to higher pH values (46). It is conceivable that vinculin is able to bind along the actin filament and thereby prevent or inhibit side-by-side aggregation of F-actin filaments at pH 7.0. Binding of vinculin to actin at this pH may counteract the effect of low pH in increasing actin's viscosity and thus result in a concomitant decrease in the low shear viscosity of F-actin.

Elevation of ionic strength by addition of NaCl causes a dramatic increase in the low shear viscosity of the F-actin controls (other conditions standard, which include 2 mM MgCl\(_2\)). This result is similar to that reported by others observed upon addition of KCl as the polymerizing agent to highly purified actin (46). Addition of monovalent salts has long been known to increase the rate of polymerization of actin supposedly through suppression or screening of negative electric charges on actin (57, 47). Although Na\(^+\) has been shown to be more effective in the polymerization of G-actin than K\(^+\) (47), we are unaware of any electron microscope studies dealing specifically with the effect of increasing Na\(^+\) on the polymerization of actin. We observed that Na\(^+\) not only enhanced polymerization, but also causes side-by-side aggregation of F-actin filaments. Addition of vinculin to actin polymerized in the presence of NaCl causes a
disruption of this lateral aggregation of F-actin filaments and a concomitant decrease in the low shear viscosity of the actin/vinculin solution. Compared to actin, the vinculin molecule (pI 6.3-6.45, 24) is much more basic. It is possible that vinculin prevents the side-by-side aggregation of filaments formed in the NaCl-containing solutions. Thus, although ionic strength affects actin cross-linking molecules, the effect of vinculin may not be due solely to the number of cross-links (46).

Addition of tropomyosin results in an increase in the low shear viscosity of F-actin under physiological-like conditions (37°C, 150 mM NaCl, 2 mM MgCl₂, pH 7.5). When examined by electron microscopy, there is an increase in the degree of cross-linking of the tropomyosin decorated actin filaments, but the major cause of the viscosity increase is probably due to increase in stability (rigidity) of the actin filaments (37, 48, 49). Addition of vinculin to the actin/tropomyosin mixture resulted in an even greater increase in low shear viscosity, reflecting cross-linking of the more stable actin filaments. Thus, binding of vinculin to tropomyosin-coated actin filaments is evidently enhanced compared to actin filaments alone.
REFERENCES


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1 Some of the data presented herein have been presented at the 1984 American Society for Cell Biology meeting.

2 The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid.
The initial interest in the 130,000-dalton protein (later named vinculin by others; 6) in our laboratory arose from an investigation into the possible purification of a 130,000-dalton protein we observed in gizzard smooth muscle extracts and which had not been previously identified in the literature. The specific objectives in this study were to develop a procedure by which vinculin could be reproducibly prepared, followed by the biochemical characterization of selected properties of this protein. Vinculin's possible role in vivo was examined by measuring its susceptibility to proteolytic degradation by an endogenous cellular protease and by examining its interaction with actin filaments. My preliminary experiments involved the isolation of the 130,000-dalton protein by ammonium sulfate precipitation of supernatants resulting from low ionic strength extraction of gizzard muscle, a procedure similar to the procedure used in extracting alpha-actinin. I also observed in preliminary studies that it was possible to isolate the 130,000-dalton protein from KCl extracts of washed myofibrils. During these early experiments, two procedures were developed and published by independent laboratories for the purification of the 130,000-dalton protein (4, 7). Utilizing the procedure of Feramisco and Burridge (7) for low ionic strength extraction of the 130,000-dalton protein at high temperature (37°C), vinculin could be prepared in a fairly short time, and in acceptable quantities. Examination of the DEAE-cellulose fraction containing
vinculin, however, indicated that other contaminating proteins were present. We felt that before beginning biochemical studies, it was necessary to further purify this fraction. I initially utilized gel filtration or hydroxyapatite chromatography to further purify DEAE-cellulose-purified vinculin, but neither procedure by themselves resulted in a highly purified form of vinculin. In contrast, I found that chromatography on a CM-cellulose column at pH 5.0 resulted in very good separation of vinculin from the contaminating proteins as judged by SDS-gel electrophoresis. Although vinculin did undergo a slight amount of degradation due to proteolysis during purification, the protein was judged to be about 97% pure, with the other 3% composed of vinculin breakdown products. Typical yields of purified vinculin were approximately 2.5 mg from 100 g of ground gizzard, substantially less than the DEAE-cellulose-purified vinculin of 12 mg from 100 g of muscle.

Two-dimensional gel electrophoresis of purified vinculin and of vinculin in whole muscle homogenates demonstrated the presence of six to seven isoforms, ranging in pI from 6.25 to 6.50, with three major, closely spaced variants as the predominate pattern. Another protein, termed 130A in this study, was also present in whole muscle. The 130A protein had a similar molecular weight to that of vinculin, but had different pI values (5.70 to 5.85) in comparison to those of vinculin. This explained why a constant amount of a protein, having a molecular weight of 130,000, was always left in the residues remaining after extraction of washed myofibrils at high temperature and low ionic
strength. Some of the 130A protein was extracted along with the vinculin, however, and was carried through most of the early stages in the preparation of crude vinculin extracts. It was not, however, precipitated by ammonium sulfate at the same step as vinculin. A second protein, previously reported by others (57, 58) as having structural and immunological properties similar to those of vinculin, but with a molecular weight of 152,000, was also isolated along with vinculin up to the first stage of chromatography. Vinculin antibodies I prepared did react with the 152,000-dalton protein as determined by immunoautoradiography.

Following my purification studies, experiments were done to determine specific biochemical properties of vinculin. Analytical ultracentrifugation of vinculin demonstrated that it sedimented as a single peak ($s_{20,w}^0 = 5.9$) in 100 mM NaCl, 20 mM Tris acetate, pH 7.1. The ultraviolet circular dichroism spectrum of vinculin, measured in 5 mM sodium phosphate, pH 7.1, showed two negative extrema at 208 and 222 nm, resulting in a value of 50% alpha-helix and approximately equal amounts of beta structure and random coil. Ultraviolet examination of vinculin, in 1 mM sodium bicarbonate, pH 7.0, indicated a maximum at 278 nm with an $E_{278}^\text{protein}$ of 4.54 and 278/260 ratio of 1.60. The amino acid analysis of vinculin indicated the protein contains a large amount of glutamyl residues and few aromatic residues, which agrees with the low extinction coefficient value obtained for vinculin.

My investigations into the possible role of vinculin in the cell
centered on its location in adhesion plaques and its possible interaction with actin. I found that digestion of vinculin by Ca^{2+}-activated neutral protease generated major fragments having molecular weights of 98,000, 85,000, and 26,000. The 98,000- and 85,000-dalton fragments were similar to those resulting from the storage of vinculin at 4°C for prolonged periods of time (>1 month) or to the small amount of these polypeptides generated during preparation of vinculin, as determined by immunoblots. The interest in these experiments stems from the observation that some of the Ca^{2+}-activated neutral protease is localized at the cytoplasmic face of the cell membrane in muscle cells (66). Those workers suggested the protease might play a role in release of microfilaments from the membrane via digestion of proteins involved in the attachment sites. Vinculin is one candidate for this possible mechanism.

The possible interaction of highly purified vinculin with F-actin was first studied by the use of low shear viscometry with the falling ball viscometer, under what I refer to in this dissertation as standard conditions (25°C, pH 7.5, 2 mM MgCl₂). I found that addition of the DEAE-cellulose-purified vinculin to actin decreased the viscosity of F-actin by approximately 85% at a concentration of 30 µg protein/ml, a result in agreement with those of others (46, 50-53, 57). In contrast, addition of CM-cellulose-purified vinculin to actin resulted in an increase of 30% at a concentration of 30 µg vinculin/ml. The inhibitory activity of DEAE-cellulose-purified vinculin on actin viscosity was present in the first peak isolated
from the CM-cellulose column. This inhibitory fraction caused a 95% decrease in the viscosity of F-actin solutions at a concentration of 20 μg protein/ml. These results, thus, clearly showed that the reported effect of vinculin on actin viscosity was due to contaminants present in DEAE-cellulose-purified vinculin. To help explain these effects better, identically prepared samples were negatively stained and examined with the electron microscope. The highly purified vinculin increased the viscosity of F-actin due to additional cross-linking of actin filaments. The inhibitory fraction from the CM-cellulose column decreased the viscosity of F-actin by both shortening and bundling of actin filaments.

To more completely characterize the interaction of vinculin with actin, several experimental conditions (temperature, pH, and ionic strength) were selected that might affect the binding of vinculin to actin filaments. When the temperature is decreased to 15°C, vinculin still causes a slight, but consistent increase in the viscosity of F-actin due to an increase in cross-linking of the actin filaments. When the temperature was increased to 37°C, however, addition of vinculin had no measurable effect on actin viscosity or appearance. When the pH was increased to 8.0, a 60% increase in the low shear viscosity of F-actin resulted from addition of vinculin (30 μg/ml) and this effect was paralleled by additional cross-linking of actin filaments. Surprisingly, when the pH was lowered to 7.0, vinculin (70 μg/ml) caused a 40% decrease in F-actin viscosity. This was reflected by the formation of "irregular-appearing" actin filaments. Elevation
of the ionic strength by addition of NaCl resulted in an increase in
the viscosity of the F-actin controls, but addition of vinculin (70
µg/ml) decreased the viscosity of these controls by approximately 65%.
Electron microscope observations indicated that vinculin had disrupted
the branched, paracrystalline-like bundles present in the F-actin
formed at the higher salt concentrations. When tropomyosin was
premixed with actin in a 1 to 7 molar ratio and low shear viscosity
was measured at near physiological-like conditions (37°C, 150 mM
NaCl), addition of vinculin (50 µg/ml) caused a 90% increase in
apparent viscosity. Although vinculin affects the viscosity of F-
actin in a seemingly complex manner, it has a definite effect on the
low shear viscosity of actin filaments, most notably at physiological-
like conditions.
REFERENCES CITED IN GENERAL

INTRODUCTION AND OVERALL SUMMARY

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