Vinculin Nucleates Actin Polymerization and Modifies Actin Filament Structure

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Vinculin links integrins to the actin cytoskeleton by binding F-actin. Little is known with respect to how this interaction occurs or affects actin dynamics. Here we assess the consequence of the vinculin tail (VT) on actin dynamics by examining its binding to monomeric and filamentous yeast actins. VT causes pyrene-labeled G-actin to polymerize in low ionic strength buffer (G-buffer), conditions that normally do not promote actin polymerization. Analysis by electron microscopy shows that, under these conditions, the filaments form small bundles at low VT concentrations, which gradually increase in size until saturation occurs at a ratio of 2 VT:1 actin. Addition of VT to pyrene-labeled mutant yeast G-actin (S265C) produced a fluorescence excimer band, which requires a relatively normal filament geometry. In higher ionic strength polymerization-promoting F-buffer, substoichiometric amounts of VT accelerate the polymerization of pyrene-labeled WT actin. However, the amplitude of the pyrene fluorescence caused by actin polymerization is quenched as the VT concentration increases without an effect on net actin polymerization as determined by centrifugation assays. Finally, addition of VT to preformed pyrene-labeled S265C F-actin causes a concentration-dependent decrease in the maximum amplitude of the pyrene fluorescence band demonstrating the ability of VT to remodel the conformation of the actin filament. These observations support the idea that vinculin can link adhesion plaques to the cytoskeleton by initiating the formation of bundled actin filaments or by remodeling existing filaments.

Cell migration is critical for embryonic development, adult homeostasis, inflammatory responses, and wound healing. To migrate, a cell must coordinate a number of different inputs into appropriate cellular responses. The cell must polarize in the direction of migration and extend lamellipodial and/or filopodial protrusions. Nascent adhesions that assemble within the branched actin network of the lamellipodium must link to the underlying actin cytoskeleton. This process allows for the maturation of adhesions to structures that anchor the protrusion. These adhesions also provide the traction forces necessary to pull the cell body forward and break older adhesions at the cell rear. Perturbation of any of these events affects a cell’s migratory ability. For example, nascent adhesions that do not form linkages to the actin cytoskeleton cannot effectively anchor the protrusion to the substratum. The result is an extension that folds back upon itself, forming a membrane ruffle that cannot provide the traction forces necessary for migration.

How adhesions establish links to the underlying actin cytoskeleton has been an area of intense investigation. Integrin-containing structures are active areas of actin polymerization suggesting that adhesion plaques can initiate actin filament formation (reviewed in Refs. 1–3). Focal complexes are small integrin clusters that are found exclusively at the tips of lamellipodia and filopodia. Formation of these structures is closely coupled with actin assembly in protruding regions of cells. Accumulating evidence indicates that adhesion complex components recruit the Arp2/3 complex, a potent nucleator of actin polymerization. Our work (4) and that of others (5–7) demonstrates that the Arp2/3 complex is recruited to focal complexes or transient adhesion structures reminiscent of focal complexes by binding vinculin. FAK has also been implicated in linking focal complexes to the actin cytoskeleton by virtue of its ability to recruit and activate the Arp2/3 complex (8). Furthermore, efficient focal complex assembly requires the actin-binding protein, cortactin, which could affect adhesion assembly by interacting with the Arp2/3 complex (9). Hence, many of the known mechanisms for initiating filament formation involve recruitment of the Arp2/3 complex, which initiates the formation of branched actin filaments (55). It is surprising then that the earliest detectable forms of actin-associated adhesions are interconnected by short actin bundles, not branched filaments (10). These observations suggest that our current understanding for how nascent adhesions initiate filament formation is incomplete.

The earliest detectable actin-associated adhesions are “dots or doublets of dots” and are highly enriched in integrins, paxillin, and vinculin (10), suggesting that one of these molecules has the capability to initiate actin filament formation from such a plaque. Vinculin has long been implicated in linking adhesion plaques to the actin cytoskeleton by virtue of the ability of its tail to bind (11) and bundle F-actin (12). The interaction of vinculin with actin has been extensively studied from the perspective of vinculin (11, 13–23). Studies of recombinant proteins identified two regions of the vinculin tail (VT)2 that bind F-actin independently (21, 17), but mapping these sites onto the VT crystal...
structure reveals that these peptides do not correspond to distinct sites (25). Upon binding actin, vinculin undergoes a conformational change that promotes dimerization suggesting that vinculin self-association may be important for its bundling activities (15).

Less is known with respect to the effect of vinculin on actin filament formation and structure. This lack of knowledge stems from the fact that many of the early studies showed vinculin to have no effect on actin dynamics (26–28). However, these experiments were performed using chicken gizzard vinculin, which exists almost exclusively in a conformation where the actin binding sites are inaccessible, or from preparations that contain contaminants that produce false negatives (29). More recently, recombinant VT proteins were shown to cross-link and bundle actin (23). However, the interaction of vinculin with G-actin and the effect of vinculin on actin filament dynamics have not been explored. In this study, we have assessed the interaction of vinculin with pyrene-labeled wild-type and mutant yeast actins. We show that the VT can promote the formation of an actin nucleus from which filaments arise and alter the assembly and structure of actin filaments. These findings provide novel insights into how adhesion plaques may be linked to the actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Actin Purification and Labeling**

The actins were purified from the lysates of frozen yeast cells by a combination of DNase I affinity chromatography, DE52 anion-exchange chromatography, and polymerization/denaturation cycling as described previously (30). Wild-type actin was obtained from yeast cakes purchased at a local market, whereas S265C actin was obtained from cells we had previously generated carrying the mutant actin (31). Covalent attachment of N-(1-pyrenyl)maleimide (Sigma) with actins was performed as described in Feng et al. (31). To conjugate actin at Cys-374 with Oregon Green 488 maleimide or N’-(3-maleimidopropionyl)biocytin, the maleimide derivatives were resuspended in DMSO and incubated with yeast F-actin at a molar ratio of 3:1 and 5:1, respectively, for 3 h at room temperature. The F-actin was separated from the reactants by centrifugation, and the resulting pellet was resuspended in G-buffer. Rabbit skeletal muscle actin was purified from acetone powder as previously described (32). Purified unmodified and modified actins were stored as the Ca\(^{2+}\)-bound form in calcium G-buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM CaCl\(_2\), 0.2 mM ATP, and 1 mM DTT) at 4 °C and used within 4 days of purification.

**VT Purification**

*Escherichia coli* carrying the plasmid pGEX-4T1, which encodes amino acids 881–1066 of chick vinculin, was obtained from David Critchley (University of Leicester). For expression of this vinculin fragment, known as the vinculin tail (VT), the cells were grown to early log phase and induced for 1 h at 37 °C in a Ti60 rotor at 40,000 rpm in a Beckman ultracentrifuge. The supernatant solution was filtered through four layers of gauze and passed over a GST-Sepharose column equilibrated with lysis buffer. The column was washed with HTND buffer (10 mM Tris-HCl, pH 8, 500 mM NaCl, and 1 mM DTT) followed by washes with LTND substituting 25 mM NaCl for 500 mM NaCl. The GST-VT was eluted from the column with LTND containing 10 mM reduced glutathione (Sigma), and the GST was cleaved from the GST-VT with 40 units of human thrombin (Calbiochem) for 1 h at room temperature. The VT peptide, 881–1066, was separated from the GST and thrombin by passing the solution over a DE52 DEAE column equilibrated with 10 mM Tris-HCl, pH 8.0, and 25 mM NaCl and collecting the flow-through fractions. Protein concentration was determined by measuring the A\(_{280}\) (ε = 18,470 M\(^{-1}\)cm\(^{-1}\)).

**Actin-VT Interaction (Pyrene Fluorescence Assays)**

Either pyrene-labeled or unlabeled yeast calcium G-actin in Ca-G-buffer was converted to the magnesium form 2 min prior to use by dilution of the concentrated stock actin at least 10-fold into Mg\(^{2+}\)-G-buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM MgCl\(_2\), 0.2 mM ATP, 0.1 mM EGTA, and 1 mM DTT). For assessing the actin-VT interaction in Mg\(^{2+}\)-G-buffer, the actin was diluted with G-buffer to a concentration of 1 μM, and the baseline fluorescence was determined. VT was added, and the resulting change in fluorescence was followed until a steady state was obtained. For all assays, the excitation wavelength was 365 nm. For WT actin, single wavelength kinetic assays were carried out with an emission wavelength of 386 nm. For studies involving S265C actin, emission spectra were recorded between 375 and 550 nm.

For VT-actin co-polymerization assays, VT was added to 1 μM G-actin, labeled or unlabeled in Mg\(^{2+}\) G-buffer. For pyrene assays, the increase in fluorescence was allowed to reach steady state after which 2 mM MgCl\(_2\) and 50 mM KCl were added to induce actin polymerization. The subsequent change in fluorescence was recorded as a function of time. All fluorescence assays were performed in a final volume of 120 μl in a microcuvette housed in a thermostatted sample compartment of either a Fluorolog-3 or FluoroMax-3 spectrometer (Jobin Yvon-Spex).

**Actin-VT Co-sedimentation Assays**

To quantitate the amount of actin filaments or bundles formed and the amount of actin-bound VT, the appropriate concentration of VT was combined with 1 μM Mg\(^{2+}\) G-actin in Mg\(^{2+}\) buffer as above. The sample, either before or after addition of MgCl\(_2\) and KCl to induce polymerization, was subjected to centrifugation in a TLA.1 rotor at 25 °C using a Beckman TL-100 centrifuge. To assess filament formation, centrifugation was for 15 min at 80,000 rpm. For assessment of bundle formation, centrifugation was at 20,000 rpm for 30 min. The supernatant fractions were carefully removed, and the pellets were resuspended in 30 μl of distilled water. The entire pellet and one-fourth of the supernatant fractions were
resolved by SDS-PAGE using 12% acrylamide gels. The protein bands were visualized by Coomassie Blue staining, and the density of the bands was quantitated using an Epson Perfection 2450 photo scanner and ImageJ software (National Institutes of Health) ensuring that the intensities of the bands were within the linear response range of the instrument. Band densities for actin and VT were normalized based on their relative molecular weights, and the resulting data were utilized to calculate VT/actin ratios in the pellet fractions.

**Electron Microscopy**

To visualize actin filaments and filament bundles, a 3-μl sample from an actin–VT solution was deposited on a carbon-coated Formvar grid (400 mesh) and negatively stained with 1% uranyl acetate. The sample was then observed using a JEOL 1230 transmission electron microscope housed in the University of Iowa Central Microscopy Facility.

**Data Analysis**

Two types of experiments were performed to examine the actin-VT interaction. In one, the increase in pyrene fluorescence following VT addition was followed as a function of time. In the second, VT-induced actin bundling was followed as a function of the amount of material pelleted in a co-sedimentation assay as described above. For the G-actin co-sedimentation study, we utilized two different types of data from the same experiment. One was the percentage of total actin pelleted (Fig. 3A), and the other the molar ratio of VT/actin in the pelleted material (Fig. 3B). We then generated Fig. 3C, a graph of VT-bound versus VT total using the Equation 1, where \( T = \text{total} \).

\[
[\text{VT}]_{\text{actin-bound}} = \frac{[\text{VT}]_{\text{precipitated}}}{[\text{actin}]_{\text{precipitated}}} \times \frac{[\text{actin}]_{\text{precipitated}}}{[\text{actin}]_T} \times [\text{actin}]_T
\]

(Eq. 1)

The data in Fig. 3C were then fitted to a quadric binding equation (Equation 2) in which the value of \( F \) was set at 1.

\[
[\text{VT}]_{\text{actin-bound}} = F \\
\times ([\text{actin}] + [\text{VT}] + K_d) - \sqrt{([\text{actin}] + [\text{VT}] + K_d)^2 - 4 \times [\text{actin}] \times [\text{VT}]_T} \]

(Eq. 2)

In this analysis, to obtain a good fit, we needed to assume that each actin monomer contains two independent VT binding sites. Thus, the total actin concentration shown is the concentration of sites rather than the actual actin concentration. \( K_d \) values were then obtained as previously described (33).

For the pyrene assays we used the same basic quadric equation to analyze the increase in fluorescence over time. However, we multiplied the right side of Equation 2 by the total change in fluorescence for each VT concentration used (represented by \( F \)). For the quenching-based assay in Fig. 9, we used the same quadratic equation but, instead of net fluorescence change, we used the value of 1 − \( F \).

**Total Internal Reflection Microscopy**

**Sample Cell Preparation**—The flow cell was essentially prepared as described by Kuhn and Pollard (34). Cells were coated with avidin (NeutrAvidin, Invitrogen) by allowing 15 μl of 50 nm avidin dissolved in Tris-buffered saline (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl) to enter the cell via capillary action. The loaded cell was incubated in a humid chamber for at least 30 min at room temperature, and the excess avidin was removed by washing the cell with Tris-buffered saline with 1% bovine serum albumin and then TIRF buffer (10 mM imidazole, pH 7, 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 100 mM DTT, 0.2 mM ATP, 15 μM glucose, 0.5% methylcellulose, 20 μg/ml catalase, 100 μg of glucose oxidase).

**Polymerization Experiments**—Calcium-actin mixtures containing 52% unlabeled actin, 33% Oregon Green 488 maleimide-actin, and 15% \( N^\prime\)-(3-maleimidopropionyl)biocytin-actin.
tin were converted to Mg-actin as described above. For monitoring actin polymerization, the Mg-actin mixture was diluted to a final concentration of 2 μM in TIRF buffer. In a separate tube, VT was diluted to 5.0 μM in a modified TIRF buffer containing 0.2 mM MgCl2. The two samples were combined resulting in a mixture containing 1.0 μM actin and 2.5 μM VT. A 10-μl aliquot was immediately loaded into the cell by capillary action, and the cell was mounted on a Leica AM TIRF MC imaging system. Images were acquired every 10 s and analyzed using the ImageJ software package.

RESULTS

Migration of many vertebrate cells requires both actin polymerization and adhesion to the substratum. The earliest

FIGURE 2. VT promotes actin filament bundling from G-actin. Electron micrographs of negatively stained pyrene-labeled G-actin alone (A) or pyrene-labeled G-actin (1 μM) incubated with VT (2.5 μM) (B). C, a portion of the image in B was magnified 4 ×. Bar = 0.2 μm. The dark arrows indicate tight bundles, and the white arrows indicate loose bundles.

FIGURE 3. VT co-sediments with yeast actin bundles. G-actin (1 μM) was incubated with increasing concentrations of VT in low ionic strength solution for 30 min at room temperature. The resulting mixtures were centrifuged at low speed (“Experimental Procedures”), and the contents of the pellet (P) and 25% of the supernatant (SN) were analyzed by SDS-PAGE and Coomassie Blue staining. This centrifugation speed is routinely employed to recover bundled actin material but not single filaments. A, the amount of pelleted actin material in the various fractions was quantified using densitometry and plotted as a function of VT concentration. B, the amount of VT that was recovered in the pellet was plotted as a function of the total VT concentration in the sample. The error bars in A and B represent the standard deviation from three independent experiments. C, a graph of VT-bound versus VT total that was generated and fitted to the quadratic binding equation as described under “Experimental Procedures.” The error on the Kd value in C represents the error on the fit.
detectable actin-associated adhesions are vinculin-rich and interconnected by short actin bundles (10). This observation suggests that vinculin, itself, may trigger bundle formation. We thus wished to assess the effect of vinculin on actin monomer and filament dynamics using the vinculin tail, VT, as a probe because it contains the actin-binding sites for the whole protein.

Interaction of VT with Mg$_{2}^{2+}$ G-actin—We first determined if VT would bind to the actin monomer. For this work, we converted G-actin to the expected Mg$_{2}^{2+}$ form, the predominant species found in the cell and labeled the active -SH of Cys-374 with pyrene-maleimide. If the two proteins interact, and this interaction alters the environment of the pyrene, its fluorescence properties will change providing an assay for the interaction. We thus combined VT with Mg$_{2}^{2+}$ G-actin in a low ionic strength solution that will prevent polymerization of pure actin (Mg$_{2}^{2+}$ G-buffer). Fig. 1A shows that this combination resulted in a biphasic time-dependent change in fluorescence consisting of a rapid first phase increase and a slower subsequent phase until a new steady state was reached after ~20 min. Fig. 1B shows that this VT-induced change was saturable with respect to the amount of VT added to a constant amount of actin.

The biphasic fluorescence increase observed in Fig. 1A suggested that, following an initial actin-VT interaction, some subsequent assembly step, possibly polymerization, might be occurring despite the fact that the proteins were combined in G-buffer. The polymerization-dependent increase in fluorescence of a pyrene at Cys-374 is, in fact, a commonly used assay for actin polymerization. To assess this possibility, aliquots of the VT-actin solutions at saturating VT concentrations were removed after the fluorescence increase had reached a steady state and visualized by electron microscopy after negative staining. Fig. 2 (B and C) shows the appearance of actin filament bundles that require VT to form (Fig. 2A). No evidence of bundle or filament formation was observed when we used actin

FIGURE 4. Binding of VT to pyrene-S265C G-actin causes excimer formation. A, G-actin (1 μM) labeled with pyrene at Cys-374 and S265C was incubated with G-buffer (black line), 1.0 μM VT (red line), or 5.0 μM VT (green line), and the fluorescence emission spectra were recorded. Addition of VT resulted in an increase in the fluorescence excimer peak (black arrow). B, pyrene-labeled G-actin (1 μM) was incubated with increasing concentrations of VT. The change in actin pyrene fluorescence at 485 nm (excimer peak) was plotted as a function of VT concentration. Error bars represent the standard deviation from three independent experiments.

FIGURE 5. VT accelerates actin polymerization and modifies pyrene actin fluorescence. A, mixtures of 1 μM of pyrene-labeled G-actin, including increasing amounts of VT as indicated, were polymerized by the addition of F-salts (indicated by the arrow). Polymerization kinetics was monitored by measuring an increase in fluorescence intensity. At all concentrations examined, VT accelerated the rate of actin polymerization. B, peak fluorescence values from the curves in panel A were graphed as a function of VT concentration. Error bars represent the standard deviation from three independent experiments.
with calcium rather than magnesium-bound to the high affinity divalent cation binding site (data not shown).

We next wished to determine the dependence of actin filament formation and bundling in G-buffer on VT concentration. Increasing amounts of VT were added to a fixed amount of actin, and the mixtures were then centrifuged at speeds sufficient to pellet actin bundles but not individual filaments. Aliquots of the pellet and supernatant fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The amount of VT that was recovered in the pellet was quantified by densitometry and plotted as a function of the total VT concentration in the sample. The open circles show the raw data, and the closed circles represent the same data set that has been fitted to the quadratic binding equation. The error bars represent the standard deviation from three independent experiments.

To determine if such were the case with our VT-G-actin interaction, we determined the molar ratio of VT/actin in the pellet at each VT concentration. Fig. 3B shows that, at the lowest VT concentration, the ratio was almost equimolar and that it increased to a ratio of 2:1 at saturation. We used the information obtained in Fig. 3 (A and B) to generate a graph of VT-bound versus VT total (Fig. 3C). This analysis reveals that maximal binding of VT to 1.0 μM actin occurs at a VT concentration of ~5–7.5 μM.

Although we observed VT-dependent actin filament formation in G-buffer, the ionic strength difference between it and F-buffer (G-buffer plus 2 mM MgCl₂ and 50 mM KCl) may have resulted in an aberrant filament structure. To examine this possibility, we utilized a mutant yeast actin S265C that we had previously characterized (31). This residue is at the tip of a hydrophobic loop between actin subdomains 3 and 4. Pyrene labeling of this actin results in the incorporation of 2 mol of probe per mol of actin, one at Cys-374 and one at Cys-265. Excitation of F-actin made with this doubly labeled protein results in the appearance of a new pyrene excimer band cen-
quenched as the VT concentration increases to saturating levels (Fig. 8). This result thus indicates that the filaments formed in G-buffer, described above, have very close to if not the same structure as those formed by pure actin alone in F-buffer and shows that the formation of this excimer depends on substantial binding of VT along the filament surface.

**Co-polymerization of VT and Actin in F-buffer**—We determined what effect VT would have on Mg-actin polymerization in F-buffer, which much more closely approximates the ionic strength conditions present in the cell. We thus examined the effect of preincubation of increasing VT concentrations on the polymerization kinetics of a fixed concentration of pyrene-labeled Mg-WT yeast actin. Following addition of VT to the actin in G-buffer, the increase in fluorescence was followed until it reached a steady state. MgCl₂ and KCl were then added as described under “Experimental Procedures” to induce polymerization, and the subsequent increase in pyrene fluorescence was followed as a function of time. Fig. 5A shows that, as VT increases, the lag time at the beginning of polymerization decreases with an apparent increase in the rate of elongation. However, the net change in pyrene fluorescence is more complicated (Fig. 5B). At lower VT concentrations, there is a concentration-dependent increase in net fluorescence. At higher concentrations, although net polymerization does not change based on co-sedimentation data (Fig. 6A), the extent of fluorescence increase becomes quenched as the VT concentration increases until saturable binding of VT to the actin is achieved. To determine if the VT-bundling effect was different in G- and F-buffer conditions, we examined the contents of a co-polymerization mixture under electron microscopy. Fig. 7A shows the absence of bundles and an abundance of filaments when VT is absent. Inclusion of 2.5 μM VT prior to salt-induced polymerization (Fig. 7B) resulted in the formation of bundles that were similar to those observed in G conditions in that they were tightly packed in some regions and more loosely packed in others. Finally, because VT-dependent and independent polymerization was occurring in this reaction, we examined the ratio of VT/actin in the pellet material as a function of increasing VT concentration. Fig. 6B shows that saturable binding of VT is reached at a 2:1 VT/actin ratio and a K_d of 0.34 ± 0.14 μM, similar to what we observed with the polymerization in G-buffer (K_d = 0.23 ± 0.12 μM). However, at lower VT concentrations, the ratio was substantially less in F-buffer than that found in the G-buffer case. This difference reflects the ability of the actin to polymerize independently of the action of VT in F-buffer conditions.

The quenching of Cys-374 pyrene fluorescence as VT reaches saturating levels (Fig. 5) suggests that the binding of VT to the filament might alter filament geometry. To explore this possibility further, we re-examined the VT-dependent effects on actin polymerization in F-buffer using pyrene-labeled S265C actin. Fig. 8A shows the emission spectra for the reactions of actin alone and in the presence of two VT concentrations. As the concentration increased, so did the extent of excimer fluorescence. Fig. 8B shows that as the amount of VT increased, the excimer increased in a saturable fashion reaching a plateau at about the same concentration seen in our other experiments. These results together indicate that not only can VT bind F-actin in a saturable fashion but, in so doing, it actually modifies the geometry of the filament resulting in an altered probe environment and the observed increase in excimer fluorescence.

**Vinculin Tail Binding to Pre-formed F-actin**—Co-polymerization data utilizing either pyrene-labeled WT or S265C actin suggested that the presence of VT on the actin filament can alter the geometry of the filament based on its ability to decrease the extent of the overall fluorescence change. We thus wished to determine if addition of VT to an actin sample following completion of polymerization produced the same type of probe effects we observed in the co-polymerization studies. Fig. 9 shows the results of the addition of increasing amounts of VT to a fixed concentration of F-actin. As before, the extent of polymerization-dependent increase in probe fluorescence is quenched as the VT concentration increases to saturating levels, and co-sedimentation data (not shown) yields a VT/actin binding ratio of 2:1 at saturation as seen before.
Vinculin Tail Binding to Skeletal Muscle Actin—To determine if the effects we observed were limited to yeast actin, we examined the effect of VT on rabbit skeletal muscle. For these studies we determined if VT could co-sediment with skeletal muscle under G-actin conditions or bind to preformed skeletal muscle filaments. We observed that, in response to VT addition, a substantial portion of VT and actin co-sedimented (Fig. 10). Incubation of VT with preformed filaments had a similar effect. Hence, the effects of VT that we observed with yeast actin can be recapitulated with other actin isoforms.

VT-induced Actin Polymerization in Real-time—We employed total internal reflection microscopy to visualize the assembly of actin induced by VT in real-time. Using this approach we observed that VT induced the formation of actin bundles in a very sporadic and spasmodic fashion that prevented further analyses of the kinetics of the reaction (Fig. 11 and supplemental videos). In some instances, the actin bundles formed and then joined with other bundled filaments (Fig. 11B). In good agreement with the findings presented with our electron microscopy analyses (Fig. 2) and sedimentation studies (Fig. 3), the structures formed under these conditions had the appearance of actin bundles in that they were much larger, thicker, and fluoresced brighter than filaments formed by the addition of F-salt in the absence of VT (Fig. 11C).

DISCUSSION
The vinculin-actin interaction has long been studied from the perspective of vinculin. However, other than its recognition as an actin-bundling protein, little is known with respect to the effect of vinculin on actin dynamics or filament structure. In this study we have examined the interaction of VT with monomeric and filamentous actin, and we have defined two novel effects of vinculin on actin. We show for the first time that VT binds to monomeric G-actin forming a nucleus from which genuine actin filament bundles assemble; filaments formed under these conditions had similar characteristics as those that are assembled under physiological conditions. VT also alters filament structure as was
evident by changes in a fluorescence excimer peak using an actin probe that reports on conformational changes. Hence, we have defined two novel effects of vinculin on actin: it can recruit G-actin to form a nucleus from which actin polymerization occurs, and it can modify the structure of actin filaments.

Vinculin-dependent de Novo Actin Polymerization—Two possible mechanisms may explain how VT might drive the assembly of actin into bundles in the absence of salt. One possibility is that the basic nature of the tail (pI = 9.6) initiates filament and bundle formation by masking the negative charge on actin, a property that is well appreciated for the polyamines spermine and spermidine (40, 56). If the polymerization was due simply to increased charge, then we would expect that, as concentration reached saturation. Thus, it is highly likely that the actin bundles arise from the fusion of multiple short VT and actin oligomers.

The amount of vinculin that can bind to actin filaments has been controversial for some time. Binding studies of recombinant vinculin fragments (21) identified two peptides that can bind F-actin independently. However, mapping these sites onto the VT crystal structure led to the idea that the previously identified two independent sites were, in fact, one overlapping site (17). In contrast, an atomic model for binding of the VT to actin filaments revealed that one VT (monomer) binds two unidentified sites on the actin filament (17). In this study, we have rigorously tested the interaction of VT with both G and F-actin at a wide range of molar concentrations using monomeric VT. We always observed that the stoichiometry of binding at saturation was two VT molecules per one actin (Figs. 3 and 6). The differences between our work and the computational studies (17) may be attributed to the fact that the atomic model was developed from a small fraction of single actin filaments that pelleted only in the presence of very low VT concentrations. In our studies we always found vinculin associated with actin bundles at a wide range of molar ratios of both G and F-actin. A third study using chemical cross-linking (15) reported that one VT binds to one F-actin monomer. The difference between this result and our finding could easily be reconciled if one of the VT molecules in the complex is not in close enough proximity to the actin molecule to be cross-linked or that the probability of cross-linking both VTs is small at the low concentrations of cross-linker employed.

VT Binding to Filaments—Vinculin is one of a number of proteins that can bind to the actin filament near Cys-374. Others include myosin (42, 43), tropomyosin (44–46), caldesmon (47), and α-actinin (48). When actin is labeled at Cys-374 with pyrene, this binding often, but not always results in an alteration of Cys-374 pyrene fluorescence. For example, no change is seen with tropomyosin binding. Such a fluorescence change may be due to an alteration in the local environment of the probe brought about by the mere presence of the binding pro-

**FIGURE 9.** VT modifies pre-formed filaments. Pyrene-labeled F-actin (1 μM) was incubated with the indicated VT concentrations. The fluorescence intensity at 386 nm for each mixture was plotted as a function of VT concentration.

**FIGURE 10.** VT effects are not limited to yeast actin. Co-sedimentation assays using rabbit skeletal muscle actin (1.0 μM) and VT (2.5 μM) were performed and analyzed as described in the legends of Figs. 3 and 6. Mg G-actin denotes mixtures of Mg²⁺-G-actin incubated with VT in G-buffer, and Mg F-actin denotes mixtures of Mg²⁺-G-actin that were polymerized into filaments by the addition of F-salts and then incubated with VT. P denotes the actin pellet, and SN denotes 25% of the supernatant.
tein without a concomitant change in filament geometry, or it could be the result of a significant change in overall filament conformation. It is not possible to distinguish between these two alternatives. However, the use of the labeled S265C protein does afford such an opportunity. The fluorescence excimer band observed with this actin is generated by the overlap of the two pyrene probes in the space between the strands of the filament far away from the binding sites of these proteins. Any change in the magnitude of this fluorescence excimer exerted by a binding protein should reflect a change in filament conformation, for example, a twisting or compression of the filament. Neither myosin nor tropomyosin can exert such a change (31). However, cofilin, which twists the filament leading to severing (49), and the yeast formin Bnr1, which nucleates filament formation, do exert such a change (50, 51). Our observation that vinculin can also alter excimer fluorescence of an actin filament suggests that the binding of VT to F-actin near an attachment plaque and the subsequent filament remodeling may be important components in attachment plaque assembly.

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Although vinculin may not be unique in its ability to modify actin filament structure, it is divergent from most other actin-binding proteins in its ability to nucleate filament formation and facilitate subsequent filament bundling when combined with G-actin in G-buffer. The myosin head will promote filament formation but not bundling (52–54). The yeast formin Bnr1, which normally nucleates and bundles actin filaments in F-buffer, will only nucleate but not bundle filaments starting with G-actin in G-buffer (51). The ability to bundle suggests that the interaction of actin with vinculin alters the vinculin conformation leading to a dimerization that allows filament cross-linking and subsequent bundling to occur. Such a model has previously been proposed based on the observation that homodimers of vinculin 884–1066 are detectable in the presence, but not in the absence, of F-actin (15).

**Biological Significance**—Focal adhesions are sites where bundled actin filaments (stress fibers) originate. Vinculin is highly enriched at these sites and is thought to be required for their linkage to the underlying actin cytoskeleton. How vinculin establishes these linkages is not well understood. Our observations that vinculin can nucleate polymerization and modify actin filament structure suggest that the linkage could be established by synthesizing bundled actin filaments, remodeling the existing ones, or some combination of the two. Bundled actin filaments could be established by the bundling of a number of single filaments or alternatively by the formation of an actin bundle concomitant with polymerization. At the wide range of

![FIGURE 11. VT induces G-actin polymerization in real time. Actin monomers (1 μM, 52% unlabeled, 33% Oregon Green-labeled, and 15% biotinylated) were combined with 2.5 μM VT in a modified TIRF buffer (50 mM KCl/0.2 mM MgCl₂/10 mM imidazole, pH 7.0/1 mM EGTA/100 mM DTT/200 μM ATP/15 mM glucose/0.5% methylcellulose/100 μg/ml glucose oxidase/20 μg/ml catalase) in a flow cell with a coverslip coated with avidin and blocked with 1% bovine serum albumin. Shown are frames representing the time course of polymerization for a VT-induced bundle that polymerizes in a non-uniform manner (A–E), VT-induced bundles that polymerize and then coalesce with one another (F–J), and for comparison, a single frame of actin filaments that are formed when the same actin monomer mixture is polymerized by the addition of F-salt (K). The times in A–J indicate the time after addition of VT; in K the time stamp indicates the amount of time that has lapsed since salt was added. The numbers indicate the ends of actin bundles.](image-url)
molar concentrations we examined, VT always forms bundled actin filaments supporting the idea that focal adhesion-bound stress fibers can be produced by the synthesis of a bundled structure. It is likely that, once these bundled structures are established by VT, other bundling proteins could further cross-link them to help establish an actin filament network capable of mediating tension and promoting adhesion plaque maturation. Such roles have been reported for \( \alpha \)-actinin, an actin cross-linking protein present in adhesion plaques. Taken together, these findings are beginning to provide us with a molecular understanding for the high degree of interdependency between stress fibers and focal adhesions.

Previously we (4) and others (5–7) showed that vinculin binds and recruits the Arp2/3 complex, a potent nucleator of actin polymerization to focal complexes. It is not readily evident why there is a need for vinculin itself to trigger filament formation and also recruit the Arp2/3 complex. The most straightforward possibility is that vinculin might promote the assembly of actin by forming a nucleus from which bundled actin structures arise (as described above) when physiological conditions dictate the need for a bundled actin filament, and vinculin plus the Arp2/3 complex might operate when a branched actin network, such as those that are present in lamellipodia, are needed. The other possibility is that vinculin itself may nucleate polymerization to link nascent adhesions to the actin cytoskeleton, and the vinculin-Arp2/3 interaction might be required to allow for further protrusion for these adhesion sites (reviewed in Ref. 1). More work is needed to better understand the physiological conditions under which the different mechanisms operate; this information will be invaluable toward generating a complete understanding for how nascent adhesions formed during cell migration are linked to the underlying actin cytoskeletal network.

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