Actin Activates a Cryptic Dimerization Potential of the Vinculin Tail Domain*

Robert P. Johnson‡ and Susan W. Craig‡§

From the Departments of ‡Biological Chemistry and §Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The tail domain of vinculin (Vt) is an actin binding module containing two regions that interact with F-actin. Although intact Vt purified from a bacterial expression system is a globular monomer, each actin binding region dimerizes when expressed individually, suggesting the presence of cryptic self-association sites whose exposure is regulated. We show that actin modulates Vt self-association by inducing or stabilizing a conformational change in Vt that allows dimerization. Chemical cross-linking studies implicate one of the actin binding regions in mediating dimerization in the presence of actin. Actin-induced Vt dimers may play a role in the filament cross-linking activity of this protein. The Vt dimers induced by actin are biochemically distinct from the Vt dimers and higher oligomers induced by acidic phospholipids such as phosphatidylinositol 4,5-bisphosphate, suggesting structural differences in Vt bound to these two ligands that may provide a mechanistic basis for inhibition of F-actin binding by phosphatidylinositol 4,5-bisphosphate. The ability of actin to regulate the dimerization state of an actin binding protein suggests that, rather than serving a passive structural role, actin filaments may directly participate in signal transduction and other cellular events that are known to depend on cytoskeletal integrity.

Adherens junctions are complex and dynamic cytoskeletal microenvironments formed by the interplay of actin, actin-binding proteins, and cellular signaling pathways (1, 2). Assembly of one type of adherens junction, the integrin-mediated focal adhesion, is regulated by members of the Rho family of small GTPases (3), through mechanisms that may lead to conformational changes in specific junctional components allowing their associations with one another to occur (4, 5). The availability of ligand binding sites in the adherens junction protein vinculin is regulated by an intramolecular interaction between the head (Vh) and tail (Vt) domains of the protein, which is thought to provide a mechanism for regulated recruitment of vinculin to junctions (4, 6). Members of the ERM protein family are other examples of junctional proteins in which masking of ligand binding sites in the native conformation is thought to control localization and function (7, 8). Whatever the specific mechanisms regulating recruitment of junctional components, once coassembled these proteins act in concert to create a dynamic, mechanical linkage to the extracellular substratum (9, 10).

Adherens junctions also provide a conduit for information transfer between the cell and its surroundings. A variety of protein kinases, phosphatases, and other cell signaling molecules localize at least transiently in adherens junctions (1, 11) and mediate adhesion-dependent control over such processes as cell division and apoptosis (2). Although the traditional view of the functional organization of an adherens junction is one of tenant signaling molecules residing dynamically within a framework of cytoskeletal elements (12), the extent to which components traditionally considered structural, such as actin, are in fact direct participants in junctional signaling events remains an unexplored question. In this report, we provide evidence that actin induces structural and functional changes in the Vt domain of vinculin.

Vt is an actin-binding module whose activity is regulated by the intramolecular interaction with the Vh domain (4). Several observations suggest that, if relieved of this regulatory intramolecular interaction in vivo, the Vt domain would mediate association of vinculin with F-actin. In vitro, free Vt domain binds F-actin and cross-links actin filaments into bundles and gels (4, 13), and a peptide ligand specific for the open conformation of vinculin can activate F-actin binding of intact vinculin when present at high molar excess (14). Expression of Vt in vinculin-deficient F9 cells results in a decrease in actin-dependent cell motility, which can be reversed by coexpression of Vh (15). In fibroblasts, Vt localizes to actin stress fibers and to focal adhesions when microinjected (13) or when expressed as a fusion with green fluorescent protein, in contrast to both native vinculin and green fluorescent protein-vinculin, which localize exclusively to focal adhesions. Conformational regulation of Vt binding to actin in intact vinculin suggests that this interaction is significant to vinculin function in vivo.

In studies seeking to identify the sequences in Vt responsible for actin binding, we found two regions of the tail, corresponding to amino acids 940–1012 and 1012–1066 of the chicken vinculin sequence (16), that individually appeared to constitute an actin binding motif when expressed as a fusion with glutathione S-transferase (17). This observation was confirmed by another group using similar tail constructs produced as fusions with the maltose-binding protein (18). The presence of two actin binding regions in Vt suggested that this domain cross-links F-actin by a mechanism involving simultaneous engagement of two actin filaments by a single Vt molecule. However, the existence of multiple actin binding regions does not rule out the possibility that Vt self-association is important for actin cross-linking. Electron microscopic evidence suggests that vinculin molecules in which the Vh-Vt interaction has been disrupted may be capable of oligomerization through their Vt domains (19, 20). Furthermore, maltose-binding protein fusions containing the actin binding regions of Vt are reported to self-associate (18).

* This work was supported by National Institutes of Health Grant GM41605. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 410-955-3666; Fax: 410-955-5759; E-mail: scraig@jhmi.edu.

This paper is available on line at http://www.jbc.org
We report here that purified Vт domain is a monomer but that F-actin induces its dimerization through a mechanism involving conformational changes in Vт that result in exposure of cryptic self-association sites in at least one of the actin binding regions. This insight allows us to begin to address the role actin itself plays in controlling the activity of an actin-binding protein. The finding that actin modulates the conformation and possibly the activity of an actin binding protein implies that rather than serving as a passive stage on which signaling events occur, actin filaments can instead play an active role in such cellular processes.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Vinculin 30-kDa tail fragment was produced by cleavage of purified chicken gizzard vinculin with V8 protease from *S. aureus* as described (6). Actin was purified from chicken skeletal muscle according to standard procedures (21, 22). PF was purchased from Avanti Polar Lipids (Alabaster, AL), and PIP2 was purchased from Sigma. Sephadex G75 and G150 superfine gel filtration media were from Sigma. The chemical cross-linkers DSS, DSP, EDC, and MBS were purchased from Pierce. 5,5'-Dithiobis(2-nitrobenzoic acid) (Ellman’s reagent) was from Fluka. A clone of rabbit antibody recognizing the Vт domain was raised by us using the proteolytically derived 30-kDa tail fragment of vinculin as an immunogen.

**Fusion Proteins—**Expression plasmids encoding His-tagged fusion proteins were constructed by polymerase chain reaction amplification of the appropriate region of the chicken vinculin cDNA (16) and subcloning of the amplified region into the pET15b vector (Novagen; Madison, WI); all fusion proteins were constructed with a translation stop codon following the final vinculin residue. Construction of His*-V985–1066 and His*-V961–970 has been described previously (24). Fusion proteins were produced in *Escherichia coli* BL21(DE3) cells and purified by Ni*²*-affinity chromatography essentially according to vendor instructions. Briefly, fusion protein expression was induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (Roche Molecular Biochemicals). C4 anti-actin monoclonal antibody was the generous gift of Dr. James Lessard (Children’s Hospital Medical Center; Cincinnati, OH). Polyclonal rabbit antibody recognizing the Vт domain was raised by us using the proteolytically derived 30-kDa tail fragment of vinculin as an immunogen.

**Gel Filtration—**Gel filtration analyses were performed at 4°C on 0.75 × 50 cm columns composed of either Sephadex G75 or G150 superfine resin. With either resin, the total included volume of AT rich sequences was 10 ml, and the void volume (based on the elution volume of blue dextran) was approximately 7 ml, yielding an included volume of approximately 13 ml. The column was washed with 2 ml Tris-HCl, 100 mM KCl, 2 mM MgCl₂, 0.2 mM CaCl₂, 0.02% NaN₃, pH 8.0, to maintain protein solubility. Although globular protein standards eluted essentially identically when chromatographed in either buffer, the apparent molecular weight of a test protein was always calculated based on the elution of globular standards under the same buffer conditions. Protein samples (50 μg) were applied in a 200-μl volume with 10% glycerol added to maintain a sharp sample boundary during loading. Fractions of ~330 μl were collected and analyzed for protein content using the Bio-Rad protein assay reagent. Eluting V985–1066 was instead detected by SDS-PAGE and silver staining because of interference of buffer components (100 mM guanidine HCl, 0.1% CHAPS) with the Bio-Rad assay.

**Chemical Cross-linking—**For chemical cross-linking experiments, fusion proteins stocks were dialyzed extensively against CL buffer (10 mM sodium phosphate, 100 mM NaCl, 0.02% NaN₃, pH 7.5). G-actin was dialyzed against buffer P (2 mM NaHCO₃, 0.2 mM CaCl₂, 0.02% NaN₃, pH 8.0). Actin-depletion experiments were performed in CL buffer or (for experiments involving F-actin) in buffer P supplemented with 100 mM NaCl and 2 mM MgCl₂ from a concentrated stock to induce actin polymerization.

**Immunoblotting—**Following SDS-PAGE, proteins were transferred electrophoretically to nitrocellulose filters (25). Filters were blocked in phosphate-buffered saline containing 5% nonfat dry milk. Primary antibody was either a rabbit polyclonal antibody to the vinculin tail domain, which was used at a 1:3000 dilution of immune serum, or 1 μg/ml C4 monoclonal anti-actin antibody. Secondary antibody was either goat anti-mouse IgG polyclonal antibody conjugated to horseradish peroxidase for detection of C4 anti-actin antibody or peroxidase-conjugated goat anti-mouse IgG polyclonal antibody conjugated to horseradish peroxidase for detection of C4 anti-actin antibody or peroxidase-conjugated goat anti-mouse IgG polyclonal antibody.
Two Actin-binding Subdomains of Monomeric Vt, Form Homodimers—We examined the oligomeric state of the tail domain of vinculin, isolated either by expression in a bacterial fusion system or by proteolysis of intact vinculin (6, 29). Vt and regions of Vt were expressed in bacteria as His-tagged fusions because proteolytic removal of the His tags proved very efficient, with little loss of the fused Vt portion. Vt fusion proteins will be referred to by the corresponding chicken vinculin residue numbers (16), since the His tags were removed from fusions before use in most of the experiments described. The fusion protein V884–1066 comprises most of the tail domain (16) and retains helical secondary structure (24) and F-actin binding activity (14). On gel filtration columns, V884–1066 migrates as a globular, monomeric protein (Fig. 1c and Table I). The concentration of V884–1066 eluting in this monomer peak was determined to be approximately 1 μM, comparable with the $K_d$ for Vt binding to F-actin and in the range of concentrations at which Vt bundles and gels F-actin (4). Depending on the gel filtration medium used, V884–1066 eluted with a $K_{av}$ corresponding to that of a globular protein of $\sim 18–21$ kDa, essentially identical to the predicted monomeric size for this protein (20,942 Da) based on its primary structure. The 30-kDa tail fragment, derived from cleavage of native vinculin with S. aureus V8 protease and corresponding to vinculin residues 858–1066, eluted at the position of a globular protein of $\sim 34.2$ kDa. This value, although higher than the predicted molecular mass of this fragment ($\sim 23.3$ kDa), is significantly lower than the expected size of a dimer of this species ($\sim 47$ kDa), suggesting that the additional proline-rich segment composed of residues 858–884 produces a more extended, asymmetric shape to the 30-kDa fragment. It is noteworthy that, as its name implies, the 30-kDa tail fragment also migrates aberrantly on SDS-PAGE gels in comparison with its predicted mass.

Two regions of the tail domain, V907–984 and V985–1066, corresponding roughly to the amino- and carboxy-terminal halves of Vt, were also produced as fusion proteins in bacteria. These sequences were chosen because they overlap with the regions (V940–1012 and V1012–1066) to which we had previously mapped F-actin binding sites (17) and because attempts to express and purify His-tagged fusions of V940–1012 and V1012–1066 were unsuccessful due either to very poor expression or to extensive proteolysis of the proteins in bacteria (data not shown). While the amino-terminal V907–984 construct was well behaved, the carboxy-terminal region (V985–1066) proved difficult to work with because of insolubility in the bacteria expression system, but it could be purified in a denatured state and refolded (see “Experimental Procedures”). As expected, both of the fusions retain the ability to cosediment with F-actin (Fig. 1b). The cosedimentation assays using His/V985–1066 were performed under somewhat stringent buffer conditions, including 100 mM guanidine HCl and 0.1% CHAPS, to maintain protein solubility. Although both the His-tagged and the thrombin-cleaved V985–1066 aggregate over time in more physiological buffers (see “Experimental Procedures”), no significant sedimentation of His/V985–1066 is observed in the absence of F-actin under the assay conditions used for this construct in Fig. 1. Binding of V907–984 to actin filaments is observed under standard actin cosedimentation assay conditions, where V907–984 does not pellet significantly on its own. Surprisingly, both of these subdomains of monomeric Vt eluted from gel filtration columns as dimers (Fig. 1c). Gel filtration was performed in buffer conditions corresponding to those used in the actin cosedimentation assays above for each protein. V907–984 eluted approximately at the position of a dimer (21.5 kDa), based on its calculated monomeric size (9006 Da). Refolded V985–1066 eluted from gel filtration columns with a major peak at an apparent molecular mass of $\sim 24.5$ kDa and a smaller peak at $\sim 14.7$ kDa. In SDS-polyacrylamide gels, the material in the second V985–1066 peak was identical in migration to that in the first peak (data not shown), indicating a difference in oligomerization state rather than mass. Since the expected size of a V985–1066 monomer is 9757 Da, these data suggest that the V985–1066 region folds into an asymmetric monomer that self-associates almost quantitatively into a dimer. The His-tagged V985–1066 used in the actin binding study in Fig. 1b eluted as a single peak of a size comparable with the dimer of thrombin-cleaved V985–1066 (data not shown). Thus, both V907–984 and V985–1066 fusion proteins exist as dimers under conditions where both are functional with regard to F-actin binding.

Because the V985–1066 constructs aggregated over time in the absence of $\geq 75$ mM guanidine HCl, we focused on the V907–984 region. We excluded the trivial possibility that dimerization of V907–984 was the result of aberrant disulfide bond formation occurring during expression in bacteria or subsequent storage by direct quantification of cysteine sulphydryl content using the Ellman reagent. V907–984 displays the number of free sulphydryl groups anticipated from its amino acid sequence (Table I). The sulphydryl content per V907–984 molecule was stable over prolonged storage ($\geq 2$ months) in buffer lacking reducing agents (data not shown), suggesting that the cysteine residues in this region are relatively insensitive to oxidation.

Chemical Cross-linking Confirms Distinct Oligomeric States of Vt and Its Amino-terminal Half—We employed chemical cross-linking to generate independent evidence for the dimeric state of V907–984. The homobifunctional compound DSS quantitatively cross-links V907–985 into a species migrating in SDS-PAGE at the expected position of a cross-linked dimer (Fig. 2a). The dimeric state of the V985–1066 region could not be confirmed by chemical cross-linking, since this fusion protein does not react with DSS or a variety of other cross-linkers to yield a dimer (data not shown). In contrast to the V907–984 region, the full-length Vt construct V884–1066 is not cross-linked significantly by DSS to form a dimer-sized species, although small amounts of dimer-sized products of V884–1066 can be observed with longer reaction times or higher concentrations of cross-linking reagent (Fig. 2a) or at higher concentrations of V884–1066 (Fig. 3). Although little dimer-sized product of V884–1066 is detectable following cross-linking, this protein reacts with DSS to form a species migrating slightly faster in SDS-PAGE gels than the unreacted monomer (Fig. 2a).

We hypothesized that the faster migrating V884–1066 species observed after DSS cross-linking represented an intramolecularly cross-linked monomer of V884–1066. To test this hypothesis, DSS cross-linking reactions were performed over a 30-fold range of V884–1066 concentrations (Fig. 3). The rela-
Cryptic Dimerization Sites in Vinculin Tail Domain

The elution position of globular monomeric standards is indicated at the total volume, 20.2 ml) as described under “Experimental Procedures.”

0.75 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), cytochrome C (12.5 kDa), and aprotinin (6.5 kDa).

Homodimers of V907–984 can also be detected by cross-linking with either MBS or EDC, although the reactivity with EDC is low and requires relatively high concentrations of cross-linker and prolonged incubation (Fig. 2b). A smaller amino-terminal region of the vinculin tail, V916–970, also migrates as a dimer on gel filtration columns and is efficiently cross-linked to a dimer in the presence of DSS and MBS (data not shown). This region probably contains the same dimerization motif as the overlapping V907–984 region. Neither 30–100 μM MBS or 1–10 mM EDC yield significant amounts of dimers of full-length V884–1066, although reaction with MBS does yield an intramolecularly cross-linked monomer species similar to that generated by DSS (data not shown). Taken together, the gel filtration and chemical cross-linking analyses indicate that V907–984 (and probably V985–1066) contains a site that mediates homodimerization of the isolated subdomain but not of intact Vt under the assay conditions. Intact Vt may have a weak dimerization potential that permits a low level of dimer formation at relatively high Vt concentrations. These data suggest the presence of self-association sites in Vt that are either masked or of low affinity in the native structure of this domain.

**Actin Induces Dimerization of Vinculin Tail Domain**—The coincidence of cryptic dimerization sites in Vt with the F-actin binding sites suggested the possibility that F-actin binding induces dimerization of the monomeric Vt domain via exposure of these self-association sites. Because oligomers of V985–1066 could not be detected by chemical cross-linking, we focused on the possibility that accessibility of a dimerization site in the V907–984 region is actin-dependent. If actin binding leads to the self-association of Vt through a mechanism involving exposure of the cryptic dimerization site in V907–984, such Vt dimers should be detectable under the same chemical cross-linking conditions at which dimers of isolated V907–984 are observed.

Although a relatively low concentration of the cross-linker DSS (30 μM) is effective for cross-linking the V907–984 dimer (Fig. 2a), little or no detectable dimer species of intact Vt is produced under these conditions in the absence of F-actin (Fig. 2a and Fig. 4, lane 2). Following incubation with actin filaments, however, cross-linking with 30 μM DSS reveals a significant increase in the level of Vt dimers (Fig. 4, lane 3). This was true for both the V884–1066 fusion protein and the proteolytically derived 30-kDa fragment (data not shown). We used immunoblotting to detect the actin-induced dimer of V884–1066, because its expected size (~42 kDa) is essentially identical to the size of monomeric actin. The homodimer of Vt cosediments with actin filaments (Fig. 4, lanes 8 and 9), indicating that this dimeric species is functionally active. This finding is consistent with the proposition that actin binding induces Vt dimerization.

DSS cross-linking of Vt in the presence of F-actin produces another novel species migrating at ~64 kDa, a mass corresponding to the expected size of an actin-Vt heterodimer (Fig. 4,
Cryptic Dimerization Sites in Vinculin Tail Domain

Table 1

Summary of gel filtration analysis of Vt fusion constructs

| Protein    | Mass (calc) | Mass (app) | Apparent oligomeric state | -SH/protein |
|------------|-------------|------------|---------------------------|-------------|
| V884–1066  | 20,942      | 17,930 ± 170 | Monomer | 3.06 ± 0.07 (3) |
| V907–984   | 9006        | 21,530 ± 240 | Dimer | 2.16 ± 0.15 (2) |
| V985–1066  | 9757        | 24,464 ± 331 | Dimer | ND |
| 30-kDa tail | 23,321      | 14,733 ± 593 | Monomer | ND |

*Calculated molecular mass based on amino acid sequence.
*Apparent molecular mass based on comparison with globular protein standards.
*Quantification of cysteine sulfhydryl content using DTNB (average ± error of two determinations); for each protein, the expected number of cysteines per monomer based on the predicted amino acid sequence is indicated in parentheses.
*ND, not determined.

Fig. 2. Distinct oligomeric states of Vt and its V907–984 subregion discernible by chemical cross-linking. a, Vt fusion proteins (5 μM V884–1066 or 13 μM V907–984) were incubated with varying concentrations of the chemical cross-linker DSS for the indicated times. Reactions were analyzed for cross-linking by SDS-PAGE and Coomassie Blue staining. Even at the highest DSS concentration and the longest reaction time, V884–1066 is not significantly cross-linked into dimers (d), although a faster-migrating monomer species (m) is apparent even at short times and lower cross-linker concentration. Cross-linking of isolated V907–984 region into a dimeric species is readily detectable at low cross-linker levels and short times and is essentially quantitative at the highest DSS concentration. b, differential reactivity of V907–984 region; dimers of V907–984 are readily detected by chemical cross-linking with low concentrations of either DSS or MBS, whereas even 10–100-fold higher concentrations of EDC yield little dimer species.

lane 3). Formation of this product depends on the presence of actin (Fig. 4, lane 2), consistent with its being the result of a cross-link between one Vt molecule and one molecule of actin. Immunoblotting confirms that the 64-kDa species represents a 1:1 complex of V884–1066 and actin, since this species also reacts with antibodies to actin (Fig. 4, lane 3, lower panel). The amount of cross-linkable Vt homodimer formed in the presence of actin depends on the concentration of F-actin in a biphasic manner over a range of Vt concentrations, suggesting a dependence on the ratio of Vt to F-actin (Fig. 5). These data indicate that actin-induced dimerization is not simply the result of spurious cross-linking of “crowded” Vt monomers that have become effectively concentrated on the surface of the actin filaments through binding. In such a scenario, at any specific Vt concentration in this range, the amount of cross-linked dimer should be maximal at the lowest F-actin concentration and should decrease with increasing actin concentration, as the amount of actin surface area available per bound Vt molecule increases.

Intramolecular Flexibility within Vt Is Important for Actin Binding—In the absence of actin, the principle cross-linked Vt species produced by DSS is an intramolecularly cross-linked monomer that migrates slightly faster on SDS gels than does the uncleaved monomer. In the presence of actin, a new species corresponding to Vt homodimers appears, suggesting that actin binding is associated with, or induces, a conformational change in Vt that leads to exposure of the cryptic dimerization sites lying within the actin binding regions. In several experiments, the presence of actin appeared to reduce the amount of intramolecularly cross-linked Vt species produced by DSS (e.g. Fig. 5a). This observation suggested that the conformation that Vt adopts in the absence of F-actin may be distinct from, and possibly incompatible with, its conformation when bound to actin filaments. Reproducible demonstration of this result was difficult under our assay conditions, however, because at concentrations of DSS that are fairly selective for detecting actin-induced Vt dimers, the efficiency of intramolecular Vt cross-linking is low, and the appearance of this species is not reliable. Moreover, disappearance of the intramolecularly cross-linked Vt product in the presence of actin would not necessarily indicate a conformational change resulting from actin binding. Therefore, we sought another strategy to test the possibility that, when bound to actin, Vt adopts a different structure from that detected by intramolecular DSS cross-linking in the absence of actin.

We utilized the cross-linking reagent DSP, which is essentially identical to DSS in its reactivity and solubility properties but has a disulfide bridge in its spacer arm to allow for reversible cross-linking by thiol-mediated cleavage. Intact Vt (V884–1066) was first reacted with DSP under conditions at which the tail construct is almost quantitatively converted to the intramolecularly cross-linked species, and the ability of this species to bind actin was tested in a cosedimentation assay before and after cleavage of the cross-link with DTT (Fig. 6). In the absence of DTT, most untreated Vt binds F-actin, while DSP-cross-linked V884–1066 is significantly reduced in its ability to bind actin filaments. Reversal of the intramolecular cross-link...
with DTT, however, leads to significant recovery of actin binding activity (Fig. 6). Intramolecular cross-linking of V884–1066 monomer with the irreversible DSS also led to loss of F-actin binding in cosedimentation assays (data not shown), but it was unclear whether abolition of F-actin binding in the DSS-treated monomer was due to loss of F-actin binding itself or to chemical modification of the targeted lysine residues. In contrast, the result using DSP-cross-linked Vt demonstrates that inhibition of actin binding is due to intramolecular cross-linking and not to side chain modification; DTT cleaves the spacer arm of DSP but does not reverse modification of the targeted lysine residues. Because an intramolecular cross-link in Vt would be expected to hinder the mobility of different subdomains of this protein relative to one another, this result suggests that conformational flexibility within the tail domain is important both for high affinity actin binding and for actin-induced dimerization.

Actin and Phosphoinositides Induce Biochemically Distinct Oligomers of Vt—

FIG. 3. Stable dimerization of V907–984 but not of V884–1066. Varying concentrations of intact V884–1066 and of isolated V907–984 region were cross-linked with 100 μM DSS for 20 min. Aliquots from each reaction containing 250 ng of protein were subjected to SDS-PAGE and visualized by silver staining. Detectable species include monomer (m) and dimer (d) of both proteins as well as intramolecularly cross-linked monomer (mi) of V884–1066. Dimers of V884–1066 monomer are detectable at high protein concentration but are dilution-sensitive, whereas intramolecular cross-linking of V884–1066 is not concentration-dependent. Cross-linking of the free V907–984 region into dimers is both quantitative and insensitive to dilution over this range, indicating that this polypeptide forms a stable dimer species.

FIG. 4. Vt dimerization induced by actin. Samples containing V884–1066 or F-actin alone or a mixture of the two proteins were cross-linked with 30 μM DSS for 40 min. Duplicates of some samples were spun in an Airfuge to separate free V884–1066 in the supernatant (S) fraction from actin filaments and bound V884–1066 in the pellet (P) fraction (lanes 6–11). Reaction products were analyzed by SDS-PAGE followed by Western transfer and immunoblotting with antibodies specific for either the vinculin tail domain (upper panel) or actin (lower panel). Homodimers of V884–1066 (~42 kDa) are detectable in the presence, but not in the absence, of F-actin. A heterodimeric species (~64 kDa) containing one actin monomer and one V884–1066 monomer is also apparent following cross-linking.

FIG. 5. The extent of actin-induced Vt dimerization depends on the ratio of Vt to actin. a, V884–1066 (2.5 μM) was cross-linked with DSS in the presence of various concentrations of F-actin. Reaction products were detected by immunoblotting following SDS-PAGE and Western transfer. b, V884–1066 (0.5–2.5 μM) was cross-linked with DSS in the presence of various concentrations (0–10 μM) of F-actin, and an aliquot of each sample containing 300 ng V884–1066 was subjected to SDS-PAGE and Western transfer. Following immunoblotting, the amount of V884–1066 homodimer formed in each sample was quantified by scanning and densitometry.
mediating association with acidic phospholipids such as PIP2 (24, 30). The binding sites for actin and for phospholipid may overlap, since Steimle et al. (13) have reported that PIP2 inhibits binding of Vt to actin filaments. Like F-actin, phospholipids may thus modulate Vt oligomerization. Recently, Rudiger and colleagues (30) observed that the acidic phospholipids PIP2 and phosphatidylserine induce formation of Vt oligomers. We reproduced these observations in order to compare lipid-induced Vt oligomers with the actin-induced dimer we have described.

We observed dimers and trimers of V884–1066 following DSS cross-linking in the presence of pure PIP2 micelles or of small unilamellar vesicles of pure PI (Fig. 7a). The level of phospholipid-induced Vt dimerization depended on the concentration of lipid in a biphasic manner, with the maximal amount of dimer and trimer forming at a lipid:protein molar ratio of approximately 3–10:1 in the case of PIP2 micelles and approximately 10–30:1 in the case of PI vesicles (Fig. 7b), suggesting a 3-fold difference in efficacy between these lipids. Essentially all of the PIP2 molecules should be accessible at the surface of a PIP2 micelle, whereas only about half of the PI in a small unilamellar vesicle would be exposed. If the data in Fig. 7b are corrected to account for this expected difference in surface exposure, the difference in efficacy between PIP2 and PI is only about 2-fold. Similar results were obtained when EDC was used as the cross-linking reagent (data not shown). With either cross-linker, the optimal ratio of PIP2 we observed is comparable with that found by Rudiger and co-workers (30) using a combination of EDC and N-hydroxysuccinimide for cross-linking. We did not observe significant amounts of lipid-induced Vt tetramer or pentamer species following cross-linking with DSS or EDC, although trimers were apparent in many but not all experiments (data not shown). In contrast, F-actin consistently induces only dimers of V884–1066.

The addition of 0.2% Triton X-100 substantially reduces the amount of cross-linkable Vt species formed in the presence of acidic phospholipids, whereas the amount of actin-induced Vt dimer is largely unaffected by the addition of detergent (Fig. 8a). The effect of Triton X-100 is not likely to be due to disruption of PIP2 binding to V884–1066, since under the same conditions PIP2 inhibits Vt binding to F-actin (Fig. 8b), as reported recently (13). Very low concentrations of PIP2 (10–25 μM) in 0.2% Triton X-100 are sufficient to inhibit actin binding by Vt completely, whereas even at PIP2 concentrations 100-fold higher than the concentration of V884–1066, the presence of Triton X-100 largely eliminated PIP2-induced oligomers of Vt (data not shown). These data suggest that the inhibitory effect of Triton X-100 is mediated by disruption of phospholipid aggregates and dispersion of the phosphoinositide molecules into micelles with Triton rather than by inhibition of Vt binding to the phospholipid.

The actin-induced Vt dimer differs from the phospholipid-induced dimers in its reactivity with different cross-linking reagents. Actin-induced dimers of V884–1066 can be detected readily by DSS or MBS cross-linking but only poorly by EDC.
treatment (Fig. 8c), a pattern of reactivity comparable with that of dimers of the isolated V907–984 region (Fig. 2b). EDC cross-linking does, however, produce a Vt-actin heterodimer of \(-64\) kDa, which is also produced by DSS treatment but formed only poorly by MBS cross-linking (Fig. 8c). The Vt homodimer induced by actin is thus specifically unreactive to EDC cross-linking. In contrast, both PIP2 and PI induce Vt dimers that can be cross-linked effectively by DSS, MBS, or EDC (Fig. 8c). These results suggest that at least two biochemically distinct species of Vt dimers are possible.

### DISCUSSION

**Conformational Changes Underlie Vt Dimerization and F-actin Binding**—Evidence for self-association of comparable actin-binding Vt subregions has been reported previously (18). In this study, we have extended this observation and elucidated further its significance by demonstrating that Vt is a monomeric protein with cryptic oligomerization potential that is activated by binding to actin. Gel filtration and chemical cross-linking studies show that both the amino-terminal (V907–984) and the carboxyl-terminal (V985–1066) halves of the vinculin tail domain form stable homodimers, whereas little or no self-association of intact Vt can be detected in the same assays. The presence of cryptic dimerization sites suggested that self-association of Vt is regulated through exposure of these sites in response to specific events. In addition to self-association sites, these regions of Vt also contain determinants mediating interaction with F-actin (17, 18), implicating actin in regulated exposure of the self-association sites. Chemical cross-linking assays demonstrate directly that F-actin binding is one type of event that can induce dimerization of Vt. The ability of this actin-induced dimer to react with different cross-linking reagents is similar to the range of reactivity of the homodimer formed by isolated V907–984, implicating this region of the tail in mediating dimerization of the intact domain in the presence of actin. Dimerization of the carboxyl-terminal region may also play a role in formation of the actin-induced dimer, but its contribution cannot be inferred from the present data.

Subdomain motion within Vt is critical both for F-actin binding and for exposure of cryptic dimerization sites. Chemical cross-linking of the Vt monomer with DSS or DSP in the absence of F-actin leads to formation of an intramolecular cross-link that results in faster migration through SDS-PAGE gels, suggesting that this species retains a more compact structure under denaturing conditions. This interpretation is supported by the observation that DSS-cross-linked monomer is significantly more resistant to papain digestion than untreated monomer (data not shown). F-actin binding reduces the efficiency of intramolecular cross-linking of Vt, while the prior introduction of an internal cross-link greatly reduces Vt affinity for F-actin. The use of the reversible cross-linker DSP confirms that cross-linking per se, not chemical modification of protein side chains, is responsible for inhibition of actin binding. These data suggest that DSS and DSP target residues on two secondary structural elements that must move away from one another for Vt to adopt a conformation competent to bind tightly to F-actin and to dimerize. Identification of the sites of intramolecular cross-linking in Vt should provide insight into the structural basis of this conformational change.

Vt Oligomerization May Be Induced by More than One Mechanism—Another type of event that can induce Vt oligomerization is binding to aggregates of acidic phospholipids such as PIP2 (30). This finding is of interest, because PIP2 is thought to modulate recruitment of vinculin to adherens junctions by associating with phospholipid binding sites on the tail domain (24, 30, 31) and thereby disrupting the intramolecular Vt-Vt interaction to allow binding to F-actin and talin (32, 33). We compared PIP2 with PI, which binds well to Vt (31) but has not been implicated in regulation of vinculin structure under physiological conditions (14, 32). PI induces at least dimerization and trimerization of Vt in a manner qualitatively and quantitatively similar to PIP2. Thus, it is unclear how lipid-induced oligomerization of Vt relates to the specific activation of intact vinculin by polyphosphoinositides observed in vitro (14, 32).

Two pieces of evidence indicate that the oligomers of Vt induced by acidic phospholipids are biochemically distinct from Vt dimers induced by F-actin. In the presence of actin, only...
Dimers of Vt were evident; in contrast, the major oligomeric species that we find in the presence of either PI or PIP$_2$ are dimers and trimers, while Hüttemaier et al. (30) also detected tetramers and pentamers induced by phosphatidylyserine or PIP$_2$. The Vt oligomers induced by actin and by phospholipids also differ in reactivity with different cross-linking reagents. Although the data on this point are preliminary in nature, it is tempting to speculate that these biochemical differences reflect an underlying structural difference resulting from distinct conformational changes in the Vt monomer induced by these ligands. This possibility is particularly appealing in light of recent evidence showing that PIP$_2$ inhibits the interaction of Vt with F-actin (14).

Dispersal of acidic phospholipids into micelles of the nonionic detergent Triton X-100 significantly reduces their ability to induce formation of Vt oligomers, despite the fact that when presented under such conditions (0.3–8 PIP$_2$ molecules/micelle) PIP$_2$ is capable of binding Vt and inhibiting its interaction with F-actin (14). It is noteworthy that Hüttemaier et al. (30) have reported that the addition of phosphatidylcholine, a neutral lipid that does not bind Vt, to even 10% (w/w), completely inhibits formation of Vt oligomers in the presence of PIP$_2$. These results suggest that lipid-induced Vt oligomerization is the result of binding to an aggregate of pure acidic phospholipid rather than of binding to small clusters or individual molecules of acidic phospholipid. Highly concentrated foci of PIP$_2$ may exist in vivo and play a role in regulating vinculin conformation (34). However, it should be noted that the sensitivity of phospholipid-induced oligomerization to surface dilution, as well as the broad range of oligomers sometimes observed (30), are also consistent with the possibility that such oligomers represent cross-linking events resulting from forced clustering of Vt monomers binding to the same limited surface area of a phospholipid aggregate.

A Model for Ligand-induced Vt Dimerization—Examination of the sequence of Vt suggests that dimerization is mediated by the V907–984 and V985–1066 regions through homophilic coiled-coil interactions that form subsequent to an activating conformational change. The MacStripe program, which is based on the Coils algorithm of Lupas (26, 35), detected significant coiled-coil propensity in residues 944–971 and residues 1014–1045 when the Vt sequence was analyzed using the MTK matrix (35) and a window size of 14–28 residues (Fig. 9a). Analysis using the MTK matrix (26) also yielded a high coiled-coil score (−0.8) for residues 1014–1045, but a much lower score (−0.1) for residues 944–971. These data suggest that the V985–1066 region and possibly the V907–984 region contain coiled-coil-forming motifs.

The high α-helical content of V907–984 and V985–1066 is consistent with the possibility of coiled-coil forming segments in these regions. Secondary structure prediction using the PHD program (28) suggests that Vt is composed of five or six α-helices, connected by short loop regions, with essentially no β-strand content (Fig. 9a). V907–984 and V985–1066 are both predicted to be composed largely of two long α-helices. Based on the amino acid sequence, these helices would be highly amphipathic in nature (24, 36), consistent with the predicted coiled-coil forming propensity. Circular dichroism analysis demonstrates directly a highly α-helical secondary structure content for both V884–1066 and V916–970, a phospholipid-binding fragment contained within V907–984 (24) that retains the dimerization site.

Together with the results reported above, these observations suggest a model for ligand-induced conformational change and dimerization of Vt (Fig. 9c). Based on our hydrodynamic data, Vt must adopt an overall compact structure, presumably consisting of at least two subdomains in which the hydrophobic faces of the amphipathic helices are sequestered in the interior. A conformational change associated with binding of F-actin or acidic phospholipid would result in movement of at least two subdomains relative to one another, exposing the hydrophobic faces of some of the helices. Specific, exposed hydrophobic regions could form coiled-coil interactions with corresponding regions on another activated Vt monomer, leading to formation of Vt dimers and potentially of higher order oligomers. The “opening” of the tail domain could also alter the nature of the interaction with the specific activating ligand. In the case of phospholipid binding, exposure of the hydrophobic surface of the V916–970 region probably leads to insertion of this face into the acyl region of the lipid bilayer, as we have previously proposed (24), while in the case of actin binding, such newly exposed surfaces may mediate high affinity interactions through van der Waals contacts with the filament surface.

Up to this point, we have referred to the ability of F-actin and of acidic phospholipids to “induce” conformational changes in Vt, but it should be clarified that a model in which these ligands act by perturbing an equilibrium between different conformational states of Vt is also plausible. In this view, a low level of activated Vt would be present normally as a result of confor-
nationally breathing of the monomer. F-actin or acidic phospholipids would bind preferentially to Vt molecules, which are in an activated, less compact conformation, thus stabilizing them and blocking transition back to the compact state. Such a model might better explain the low levels of V884–1066 dimers observed following chemical cross-linking in the absence of actin or phospholipid as well as the inhibitory effect of intramolecular cross-linking of Vt on its binding to F-actin. The latter result implies that F-actin binds poorly to the compact conformation of Vt, and it is difficult to reconcile with the notion that opening of Vt occurs subsequent to actin binding.

If our model of actin- and phospholipid-induced opening of Vt is correct, an interesting implication is that an allosteric mechanism may underlie intramolecular regulation of Vt by Vh in native vinculin. Binding of Vh to Vt diminishes the affinity of Vt for these ligands under physiological conditions (4, 24, 31) but does not induce Vt oligomerization, since native vinculin exists as a monomer in solution (6, 37). It is possible that Vh only binds to Vt monomers that are in a compact conformation incompatible with Vt oligomerization and high affinity actin binding, thus providing a mechanistic basis for inhibition of actin binding by Vh to Vt (4, 6). As a corollary, Vt oligomerization or the associated change in Vt conformation may play a role in stabilizing an activated form of vinculin. The balloon-on-a-string structure observed in electron micrographs of intact vinculin, in which Vh is highly extended (19, 38), may represent an activated form of vinculin where the Vh–Vt interaction is disrupted as a result of unfolding of Vt.

Relation of Actin-induced Dimerization to Vh Function—Although earlier studies mapping two F-actin binding regions in Vh (17) suggested a mechanism of actin cross-linking involving simultaneous engagement of two actin filaments by the same Vh monomer, the present results suggest the possibility of a different mechanism. In this view, Vh monomer is not itself competent to cross-link F-actin, but exposure of cryptic self-association sites associated with actin binding allows for dimerization of actin-bound monomer to form a cross-linking species. Thus, actin filaments might regulate their own assembly into bundles and gels by inducing certain actin-binding proteins to form cross-linking-competent species. If this hypothesis is correct, it is intriguing that each monomer of Vh has two actin binding regions, since logically only one actin binding site would be necessary to form a cross-linking species out of a protein that dimerizes. It is possible that the presence of multiple actin binding sites is mechanistically important; simultaneous engagement of both sites by F-actin may provoke or stabilize the conformational change that exposes masked dimerization sites. Further elucidation of the connection between actin cross-linking activity and actin-induced dimerization will be important.

The vinculin tail domain is the only F-actin-binding protein for which direct evidence of actin-induced oligomerization has been reported, although self-association induced by actin binding has been proposed to explain the cross-linking activity of scruin (39). Scruin is a monomeric protein with two actin binding domains. Three-dimensional reconstruction of scruin-decorated F-actin reveals that the two actin binding domains of a single molecule of scruin engage the same actin filament, while filament cross-linking is effected by self-association of scruin monomers bound to two different filaments (40). This suggests that binding of scruin to F-actin leads to its self-association. The oligomeric state of other monomeric actin cross-linking proteins, such as villin, fimbrin, and the Dictyostelium 30-kDa protein (41, 42), may likewise be modulated by F-actin in ways that affect their cross-linking activities.

The potential for actin to regulate the activity of its own cross-linking proteins is intriguing. With the large number of actin-binding proteins that also cross-link F-actin (43), it is unclear under what circumstances it would be advantageous to the cell for actin to potentiate its own cross-linking. One role might be in the control of actin bundle formation, which can be quite complex in vivo, involving several stages during which small, disorganized bundles coalesce and reorganize into larger and more ordered assemblies through the sequential action of multiple actin cross-linking proteins (44–46). Actin-binding proteins that undergo actin-induced self-association might be well suited for participating in critical stages of such processes.

The ability of F-actin to modulate the conformation and oligomeric state of its binding proteins suggests an active role for actin filaments in cell signaling. Rather than simply providing a framework for recruitment of signaling proteins, actin may also regulate the activities of some of the many signal transducers that localize to adherens junctions or to other specialized compartments of the actin cytoskeleton (1, 47). Such a role would help to explain the importance of cytoskeletal integrity to adhesion-dependent signaling events. Actin-binding proteins traditionally thought to play largely structural roles, such as vinculin, may acquire novel functionality when associated with actin filaments as a result of actin-induced changes in conformation or oligomeric state. An important goal of future studies will be to elucidate what role actin-induced dimers of vinculin may play in the assembly and function of cell adhesion sites.

Acknowledgments—We thank Dr. Paul Steimle for the His1/V884–1066 fusion construct and Dr. Jim Lessard for the C4 monoclonal anti-actin.

REFERENCES
1. Yamada, K. M., and Miyamoto, S. (1995) Curr. Opin. Cell Biol. 7, 681–689
2. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) Annu. Rev. Cell Dev. Biol. 11, 549–599
3. Ridley, A. J., and Hall, A. (1992) Cell 70, 389–399
4. Johnson, R. P., and Craig, S. W. (1995) Nature 373, 261–264
5. Gilmore, A. P., and Burridge, K. (1996) Structure 4, 647–651
6. Johnson, R. P., and Craig, S. W. (1994) J. Biol. Chem. 269, 12611–12619
7. Gary, R., and Bretscher, A. (1995) Mol. Biol. Cell 6, 1061–1075
8. Magendanz, M., Henry, M. D., Lander, A., and Solomon, F. (1995) J. Biol. Chem. 270, 25324–25327
9. Danowski, B. A., Imanaka Yoshida, K., Sanger, J. M., and Sanger, J. W. (1992) J. Cell Biol. 118, 1411–1429
10. Wang, N., Butler, J. P., and Inger, D. E. (1993) Science 260, 1124–1127
11. Miyamoto, S., Akiyama, S. K., and Yamada, K. M. (1995) Science 267, 885–885
12. Jossen, K., M., Bubek, P., Moschner, M., and Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. (1995) Annu. Rev. Cell Dev. Biol. 11, 379–416
13. Menkel, A. R., Kroemker, M., Bubek, P., Bubek, P., Ronsiek, M., Nikolai, G., and Jockusch, B. M. (1994) J. Cell Biol. 126, 1231–1240
14. Steimle, P. A., Hoffert, J. D., Asey, N. B., and Craig, S. W. (1999) J. Biol. Chem. 274, 18414–18420
15. Xu, W., Coll, J. L., and Adamson, E. D. (1998) J. Cell Sci. 111, 1535–1544
16. Coutu, M. D., and Craig, S. W. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8535–8539
17. Johnson, R. P., and Craig, S. W. (1995) Mol. Biol. Cell 6, 341 (abstr.)
18. Hotz, H., Bubek, P., Bubek, P., and Jockusch, B. M. (1997) Eur. J. Biochem. 247, 1136–1142
19. Milan, L. M. (1995) J. Mol. Biol. 184, 543–545
20. Guarna, M., Burzio, D., and Small, J. V. (1987) J. Muscle Res. Cell Motil. 18414–18420
21. Spudich, J., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
22. MacLean-Fletcher, S., and Pollard, T. D. (1980) Biochem. Biophys. Res. Commun. 96, 18–27
23. Ellman, G. L. (1958) Arch. Biochem. Biophys. 74, 443–450
24. Johnson, R. P., Niggli, V., Durrer, P., and Craig, S. W. (1993) Biochemistry 32, 1021–10222
25. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
26. Lupas, A., Van Dyke, M., and Stock, J. (1991) Science 252, 1162–1164
27. Rost, B., and Sander, C. (1997) J. Mol. Biol. 232, 584–599
28. Rost, B. (1996) Methods Enzymol. 266, 525–539
29. Groesch, M. E., and Otto, J. J. (1990) Cell Motil. Cytoskeleton 15, 41–50
30. Hotz, H., Bubek, P., Bubek, P., and Jockusch, B. M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. (1995) Annu. Rev. Cell Dev. Biol. 11, 549–599
31. Johnson, R. P., and Craig, S. W. (1995) Biochemistry 34, 1061–1075
32. Gilmore, A. P., and Burridge, K. (1996) Nature 381, 531–535
Cryptic Dimerization Sites in Vinculin Tail Domain

33. Weekes, J., Barry, S. T., and Critchley, D. R. (1996) *Biochem. J.* **314**, 827–832
34. Fukami, K., Furuhashi, K., Inagaki, M., Endo, T., Hatano, S., and Takenawa, T. (1992) *Nature* **359**, 150–152
35. Lupas, A. (1996) *Methods Enzymol.* **266**, 513–525
36. Tempel, M., Goldmann, W. H., Isenberg, G., and Sackmann, E. (1995) *Biophys. J.* **69**, 228–241
37. Evans, R. R., Robson, R. M., and Stromer, M. H. (1984) *J. Biol. Chem.* **259**, 3916–3924
38. Winkler, J., Lunsdorf, H., and Jockusch, B. M. (1996) *J. Struct. Biol.* **116**, 270–277
39. Sanders, M. C., Way, M., Sakai, J., and Matsudaira, P. (1996) *J. Biol. Chem.* **271**, 2651–2657
40. Schmid, M. F., Agris, J. M., Jakana, J., Matsudaira, P., and Chiu, W. (1994) *J. Cell Biol.* **124**, 341–350
41. Matsudaira, P. (1991) *Trends Biochem. Sci.* **16**, 87–92
42. Otto, J. J. (1994) *Curr. Opin. Cell Biol.* **6**, 105–109
43. Pollard, T. D., and Cooper, J. A. (1986) *Annu. Rev. Biochem.* **55**, 987–1035
44. Tilney, L. G., Tilney, M. S., and Guild, G. M. (1995) *J. Cell Biol.* **130**, 629–638
45. Tilney, L. G., Connely, P. S., Vranich, K. A., Shaw, M. K., and Guild, G. M. (1998) *J. Cell Biol.* **143**, 121–133
46. Furukawa, R., and Frischfield, M. (1997) *Int. Rev. Cytol.* **175**, 29–90
47. Clark, E. A., and Brugge, J. S. (1995) *Science* **268**, 233–239