Two Distinct Head-Tail Interfaces Cooperate to Suppress Activation of Vinculin by Talin*

Received for publication, December 30, 2004, and in revised form, February 16, 2005
Published, JBC Papers in Press, February 22, 2005, DOI 10.1074/jbc.M414704200

Daniel M. Cohen‡§, Hui Chen†, Robert P. Johnson†, Begum Choudhury‡, and Susan W. Craig‡**

From the ‡Department of Biological Chemistry, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205 and the §Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, Illinois 60611

Vinculin (V)1 is a 116-kDa cytoskeletal protein associated with focal adhesions and adherens junctions. Several functions, mostly related to cell adhesion and motility processes, have been ascribed to vinculin. Disruption of vinculin expression in mice results in an embryonic lethal phenotype, with severe cardiac and brain abnormalities (1). Moreover, heterozygotes show increased susceptibility to stress-induced cardiomyopathies (2), consistent with the paralysis and defects in muscle architecture associated with the knockout of vinculin in Caenorhabditis elegans (3). Interestingly, vinculin-null cell lines show defects in mechanical stiffness (4), Rac-mediated lamellipodial protrusion (5), cell shape, and spreading on fibronectin (1, 6, 7), as well as misregulation of apoptotic cues (8).

Whereas mechanistic insights into the molecular basis of these phenotypes are limited with a few notable exceptions (7, 8), the aforementioned effects are generally consistent with the idea that vinculin functions as a mechanical linker between the plasma membrane and actin cytoskeleton through cross-linking several adhesion proteins. This model derives from in vitro studies that identified interactions between vinculin and numerous focal adhesions proteins including talin (9), α-actinin (10, 11), paxillin (12), VASP (13), vinexin/ponsin family members (14, 15), and F-actin (16, 17). However, the binding of intact vinculin to these potential ligands is highly restricted by conformational regulation of vinculin structure. An autoinhibitory interaction between the vinculin head and tail domains directly masks binding sites for talin (18), F-actin (17, 19), and vinexin2 and has been correlated with changes in the vinculin affinity for α-actinin (11), VASP (19), and Arp2/3 (7). This has led to a proposed model of vinculin function in which cell adhesion transduces a signal that disrupts the autoinhibitory interaction and permits engagement of the vinculin cytoskeletal targets within the focal adhesion (17).

Recent crystallographic studies on the autoinhibited conformation of vinculin have revealed that the principal binding site for head-tail interaction is comprised of a large hydrophobic interface between the D1 domain of vinculin (residues 1–258) and the top of the five helix bundle structure comprising the vinculin tail (20–22). Importantly, this interface is disrupted upon binding of a talin-derived peptide (VBS3) to the D1 domain. Binding of VBS3 to D1 results in a dramatic conformational change, termed helical bundle conversion, which cannot be competed by the addition of excess tail domain (20). This observation led to a proposed mechanism for vinculin activation in which binding of talin serves as the driving force behind conformational change (20, 23). In fact, vinculin is activated by a talin-related peptide, pVR (24), or VBS3 (25), but only at concentrations of 500–1000-fold molar excess to vinculin. In light of the fact that the D1-Vt interface is nearly identical in the bimolecular complex (20) and in the structure of full-length vinculin (21, 22), the unexpectedly high concentration of pVR or VBS3 required to activated full-length vinculin (24, 25) has

---

* This work was supported by National Institutes of Health Grant GM41605 (to S. W. C.). 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.

** The on-line version of this article (available at http://www.jbc.org) contains Supplementary Materials.

† Recipient of a Howard Hughes Medical Institute predoctoral fellowship.

‡ Recipient of an American Heart Association postdoctoral fellowship.

†† To whom correspondence should be addressed. Tel.: 410-955-3866; Fax: 410-955-5759; E-mail: scrain@jhmi.edu.

1 The abbreviations used are: V, vinculin; Vt, vinculin tail domain residues 884–1066; Vh, vinculin head domain residues 1–851; D1, vinculin residues 1–258; D4, vinculin residues 710–836; F-actin, filamentous actin; VBS3, talin residues 1945–1970; YFP, yellow fluorescent protein; GFP, green fluorescent protein; PIP2, phosphatidylinositol 4,5-bisphosphate; NTA, nitritotriacetic acid; WT, wild type; Bicine, N,N-bis(2-hydroxyethyl)glycine; DMEM, Dulbecco’s modified Eagle’s medium; HEK, human embryonic kidney cells.

2 Chen, H., Cohen, D. M., Choudhury, B., Kioka, N., and Craig, S. W. (2005) J. Cell. Biol., in press.
Bipartite Autoinhibition Blocks Vinculin Activation by Talin

been interpreted by us as evidence that additional intramolecular components functionally lower the $K_d$ of the head-tail interaction below $10^{-9}$ (22). The crystal structure of intact vinculin led us to speculate that the high affinity autoinhibited state derived either from the intramolecular nature of the D1-Vt complex, or from cooperativity between multiple interdomain interaction sites, but we previously could not assess the relative contribution of these two factors.

Here we present results of biochemical and mutagenesis studies that support a model in which the autoinhibited state of vinculin is achieved by two separate interfaces; one of these comprises the D1-Vt interface seen in the structures of chicken and human vinculin (22, 21), and the second comprises the D4-Vt interface observed in the chicken vinculin structure (Fig. 1A). We find that D1 binds weakly to Vt in solution, with a $K_d$ near 10 $\mu$M, whereas Vt-Vt (1–851) binds with a $K_d$ of $0.1$ $\mu$M. We then show that the increased affinity of the autoinhibitory complex is attributable to the D4-Vt interface. This analysis redefines the autoinhibitory Vt domain as D1-D4, rather than D1 (20) or D1-D3 (22), and enables us to provide a detailed model for autoinhibition that accounts for the functional insufficiency of talin for activating native vinculin (22).

EXPERIMENTAL PROCEDURES

Expression Constructs—Vt-(884–1066) was cloned into the pET15b expression vector as previously described (24). pET15b/YFP-(1–258) and pET15b/V-(1–851) were constructed as follows. Using in-frame primers containing 5’-Nde and 3’-XhoI sites, appropriate regions of the chicken vinculin cDNA sequence (26) were amplified using the polymerase chain reaction (PCR) with Pfu Turbo polymerase (Stratagene, La Jolla, CA), followed by addition of 3’-adenosine overhangs with Taq polymerase (Invitrogen, San Diego, CA). The PCR products were ligated into the TOPO II plasmid (Invitrogen) and then subcloned into pET15b His-tagged expression vector (Novagen, Madison, WI) using NdeI and XhoI digestion of the multiple cloning site. To generate fluorescently tagged expression vectors, we cloned the yellow fluorescent protein (YFP) cDNA using a similar strategy as outlined above. Briefly, the YFP cDNA was amplified by PCR using the pYEPF vector (Clontech, Palo Alto, CA) as template and in-frame primers containing 5’- and 3’-Nde sites. Nde-flanked YFP was subcloned into pET15b/V-(1–851) or pET15b/V-(1–258) to yield pET15b/YFP-V-(1–851) or pET15b/YFP-V-(1–258), respectively. pEGFP/Vvinculin was constructed by ligating the EcorI-flanked vinculin cDNA from p1005 (26) into the pEGFP/C1 vector (Clontech). pEGFP and pET15b/YFP vinculin plasmids contain a 12-residue spacer between the EGFP and vinculin. pET30a/talin rod-(397–2541) was a generous gift from Drs. Baodong Xing and Stephen Lam. pET30a/talin rod-(397–2541) was a generous gift from Drs. Baodong Xing and Stephen Lam. pET30a/talin rod-(397–2541) was a generous gift from Drs. Baodong Xing and Stephen Lam. pET30a/talin rod-(397–2541) was a generous gift from Drs. Baodong Xing and Stephen Lam.

Site-directed Mutagenesis—The clustered charge-to-alanine mutations in pET15b/V-(1–851) were introduced by QuikChange PCR (Stratagene) per the manufacturer’s instructions. QuikChange PCR (Stratagene) was also introduced to utilize the T12, T8/19, and N773;E775A mutations into pEGFP/vinculin. The entire vinculin cDNA coding region in these plasmids was then verified by sequencing.

Screen for Head Binding Mutants in Vt—Binding of proteolytic Vh to Vt was recovered quantitatively on His-bind resin. His-tagged Vt, YFP/Vt, or D1 at 0.2 $\mu$M was incubated with His-tagged Vt, as indicated in the figure legends. Complexes were bound to NTA beads, and the fluorometry remaining in the supernatant was assayed by fluorometry.

RESULTS

Screen for Head Binding Mutants of Vt—Prior to determination of three-dimensional structural models, analysis of the vinculin primary sequence suggested a role for electrostatic interactions in the head-tail interaction. First, the pls of the
head and tail domains, 5.5 and 9.6, are complementary in nature, and second, a high proportion of lysine and arginine residues in vinculin tail are conserved evolutionarily. Based on these observations, we screened for head binding mutants of Vt using a charge-to-alanine mutagenesis scheme outlined in Fig. 1B. Twenty charge-to-alanine mutations were designed in windows of five to seven amino acids along Vt and introduced into pET15b/V-(884–1066).

The ability of the tail mutants to bind Vh-(1–851) was screened in pull-down experiments measuring loss of Vh in response to His-tagged tail domains immobilized on nickel-charged agarose beads (Fig. 2A). The large majority of mutations had little or no effect on head binding, suggesting that the mutations were not globally disruptive to tail structure and that specific side chain interactions played a more important role than overall charge character of Vt. The mutagenesis was highly successful in defining a subset of mutants affecting head binding, namely T8 (K944;R945A), T12 (D974;K975;R976;R978A), T14 (K996;K1002A), and T19 (K1047;R1049;D1051A).

When the structure of Vt was determined (32), we saw that these mutations implicate two structurally distinct head binding regions on Vt (Fig. 2B); namely a basic patch at the base of the helical bundle, defined by T12 and T19, and a cluster on the faces of helices 3 and 4 comprised of T8 and K996 of T14 (Fig. 2B). Interestingly, we also found that the effect of combining mutations from these two candidate head binding sites, such as in the T8/19 double mutant, potentiated the head binding defect, consistent with biochemically distinct contributions of the two interaction surfaces (Fig. 2A). These mutations appear to define specific residues involved in the head-tail binding site, as they have only minimal effects on the ability of Vt to cosediment with F-actin filaments (Fig. 2C).
Bipartite Autoinhibition Blocks Vinculin Activation by Talin

Quantitative Dissection of the Head-Tail Interaction Surface—The clustering of strong head-tail interaction mutations at the base of Vt is unexpected given the recently proposed structural model for the autoinhibitory head-tail interaction, in which the top of the Vt bundle exclusively contacts the D1 domain in vinculin head, and this interaction is proposed to account for the high affinity, autoinhibited state of vinculin (20). To investigate the significance of the head binding mutants of Vt, we decided to compare the D1 domain (residues 1–258) with Vt (residues 1–851) for Vt binding. A, binding isotherm for the YFP-Vt complex showing a high affinity, 93-nM interaction. YFP-Vt, 0.2 μm, was incubated with increasing concentrations of Vt, in phosphate-buffered saline + 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 0.1% β-mercaptoethanol, and 10 mM imidazole, pH 7.8, for 1 h at room temperature. Vt was captured using NTA-agarose beads with inversion for 30 min at room temperature. Free YFP-Vt in the supernatant was assayed by fluorometry and binding curves determined by the depletion of supernatant fluorescence. Error bars represent the S.E. from three independent experiments. B, YFP-D1, 0.2 μm, was incubated with Vt as described above. Vt concentrations were varied from 0–15 nM. Note that the affinity of D1 for Vt is ~120-fold weaker than is observed with full-length Vt (1–851).

Using the fluorescence supernatant depletion assay presented in Fig. 3, several of the head-tail interaction mutants were assayed quantitatively for loss-of-binding activity for YFP-Vt. Binding defects are expressed in terms of Kd values as well as fold increases relative to wild-type Vt. Kd values were derived from non-linear fits using the Scatchard equation, assuming that the binding curves reach saturation points equivalent to wild-type Vt.

| Vt sample | Kd (μM) | Fold increase |
|-----------|---------|---------------|
| WT        | 0.09    | 1             |
| T8        | 0.55    | 6             |
| T14       | 0.77    | 8.3           |
| R976A     | 0.75    | 8.1           |
| R978A     | 0.67    | 7.2           |
| T12       | >10     | >100          |
| T19       | 3.9     | 42            |
| T8/19     | 9.2     | 98            |

The 120-fold difference in Kd observed between the D1-Vt (11.5 μM) and Vt (93 nM) complexes is best reconciled by the existence of an auxiliary head-tail interaction site. Therefore, we tested whether the T12-T19 surface on Vt could account quantitatively for the additional binding energy manifest in the full-length Vt-Vt complex. Using the fluorescence supernatant depletion assay, we measured the binding affinities of the tail mutants for Vt. As shown in Table I, the T19 and T12 mutations increase the Kd for head binding by factors of 42- and 100-fold, respectively. In the case of the most severe mutant, T12, the overall loss of affinity closely predicts the difference in Kd values observed in Fig. 3B between D1 and full-length Vt. Single mutations in the T12 cluster, R976A and R978A have intermediate effects on binding to Vt (Table I). In contrast, the T8 and T14 mutations, which cluster near the structurally defined D1-Vt interaction site, exert relatively mild effects of 6–8-fold increases in the observed Kd for Vt binding. This result is consistent with the overall hydrophobic character of this interface as described in the co-crystal of D1 and Vt (20) and establishes function for the contacts noted between Lys844, Arg845, (T8), and D1 in that structure.

Definition of the Role of the D4 Domain in Head-Tail Interaction—The mutagenic studies predicted the existence of an auxiliary tail binding site in Vt that would make specific contacts with the T12/T19 surface on Vt. Upon solution of the crystal structure of intact vinculin (22), we were able to apply structural insights to the nature of the T12/T19 recognition domain in Vt. In this structure of intact vinculin, the base of Vt makes a small, but well ordered contact with the D4 domain of Vt (residues 710–836). As seen in Fig. 4A, this interaction surface is characterized predominantly by hydrogen bonding and salt bridge interactions. Three of the four residues in T12 (Asp974, Lys976, and Arg978) make direct side-chain contacts with the D4 domain, and Lys975 appears to be structurally important for the presentation of the other T12 side chains. In T19, Lys1043 and Arg1049 are peripherally associated with this interface, consistent with the smaller magnitude of their binding defect relative to T12.

Two side chains in the D4 domain of Vt, asparagine 773 and glutamate 775, appeared to participate in the principal interactions with Vt (Fig. 4A). Therefore, to directly test the role of D4 as a putative second Vt binding site, we designed a N773E775A double mutant in which both side chains were mutated to alanine, and measured its affinity for Vt in the fluorescence
assay. As shown in Fig. 4B, the N773;E775A mutation interferes strongly with head-tail interaction, increasing the $K_d$ to 6.3 µM, a 68-fold change relative to wild-type $V_h$. The N773;E775A mutant demonstrates conclusively the requirement for the D4-T12 interface to mediate high affinity interaction of the head-tail domains. Given that the D4 mutant behaves in a nearly identical fashion to the isolated D1 domain, it appears that the D4-$V_h$ interface is sufficient to account for the difference in binding affinity of full-length $V_h$ and D1 for $V_h$.

Interestingly, attempts to directly measure the $K_d$ of a bimolecular D4-$V_h$ complex using isolated D4 were unsuccessful. Several GST fusion constructs spanning vinculin residues 259–850 failed to mediate a direct interaction with $V_h$ in the absence of D1 domain. The interaction could be measured, however, as a ternary complex; GST/V259–850 was competent to bind to $V_h$ in the presence of D1 domain (Supplementary Fig. 1). This interaction appeared to be $V_h$-dependent, as D1 did not separately interact with GST/V (259–850). It is conceivable that this second binding site is structurally unstable in the absence of D1, or that thermodynamic coupling to the D1-$V_h$ interaction is required to offset costly entropic components to binding. This may account for why it has not been detected by conventional mapping assays defining the head-tail interaction (34).

FIG. 4. Structural and biochemical characterization of the D4-$V_h$ interface. A, structural view of the D4-$V_h$ interface as seen in the crystal structure of chicken vinculin (PDB file 1ST6). D4 domain backbone trace is shown in green and $V_h$ backbone is traced in blue. Charged or polar interactions predominate between the side chains represented as sticks colored in CPK format. Note especially the salt bridges between Asp$^{773}$–Arg$^{976}$ and Glu$^{775}$–Arg$^{98}$. B, binding isotherm for the YFP-$V_h$ (N773;E775A)–$V_h$ complex. The YFP-supernatant depletion assay, described in the legend to Fig. 3, was used to quantitate binding. Data are plotted as percent maximum binding in order to allow direct comparison with the D1-$V_h$ binding curve (also shown in Fig. 3B). Error bars represent the S.E. from three independent experiments.

FIG. 5. The N773;E775A mutation attenuates autoinhibition of the talin binding site in $V_h$ by $V_h$. YFP-$V_h$ and talin rod-(397–2541) were preincubated for 1 h at room temperature. $V_h$ was then added to a final concentration of 12 or 24 µM in a final reaction volume of 30 µl. Competition proceeded for an additional hour at room temperature, after which 10 µl of 50% glycerol was added to each tube. Complexes were resolved on 7.5% native polyacrylamide gel run at 120 V at 4 °C. YFP-fluorescence was visualized using a CCD camera/Alpha-Innotech system. Note that $V_h$ efficiently displaces wild-type $V_h$ from talin rod, but $V_h$ is a much weaker competitor for $V_h$ (N773;E775) and fails to form a stable, tightly migrating head-tail complex.

The D4-$V_h$ Interface Is Essential for Regulation of the Talin Binding Site in $V_h$—The functional consequences of tail binding do not apply equally to the D1 domain and full-length $V_h$, according to published findings. Whereas it has been reported that talin or a talin-related peptide, pVR, bind in a competitive fashion with $V_h$ to $V_h$ (18, 24), more recent studies on the D1-VBS3 complex show that $V_h$ fails to displace VBS3 from D1 (25, 20). We therefore tested whether these differences could be accounted for by the D4-dependent high affinity bimolecular interaction with $V_h$ in full-length $V_h$. Using a native gel assay, we tested the ability of $V_h$ to compete talin rod from YFP-tagged $V_h$, either wild-type or bearing the N773;E775A mutation.

In the case of wild-type YFP-$V_h$, 75% of the fluorescent signal comigrated with talin rod in the absence of $V_h$ (Fig. 5, lane 2); however, at 2-fold molar excess $V_h$ to talin rod, the $V_h$–$V_h$ complex predominates and less than 33% of the fluorescence remains in the complex with talin rod (Fig. 5, lane 3). Competition is maximal at 4-fold molar excess (lane 4 versus 5). This is consistent with roughly equivalent $K_m$ values for $V_h$ (400 nM) and talin-related peptide (300 nM) in solution-phase assays, as previously reported (24). Although the N773;E775A mutation did not perturb binding to talin rod (lane 7), it did attenuate the competition activity of $V_h$ versus talin rod. At 2-fold molar excess $V_h$ to talin rod, 56% of the YFP fluorescence of the N773;E775A mutant head remained associated with talin rod in a slow migrating complex (lane 8). Moreover, $V_h$ and $V_h$ (N773;E775A) did not form a complex that was stable enough to be maintained during migration through the gel (note the accumulation of free $V_h$ (N773;E775A) as $V_h$ is titrated to higher concentrations, lanes 8–10). Thus in the context of the high affinity bimolecular interaction, $V_h$ is sufficient to block talin binding to the D1 domain. These results provide an additional line of evidence that the tightly autoinhibited state of native vinculin is conferred by the full-length head, residues 1–851, as defined structurally by limited proteolysis (35).

Disruption of the Auxiliary Binding Site Partially Opens Full-length Vinculin—The results of the competition experiments could explain the mechanism by which head-tail interaction can effectively suppress talin binding in the context of full-length vinculin (22). However, interpretation of our experiments with reconstituted head-tail complex was limited by assumptions of native-like structure in recombinant vinculin fragments, and by the lack of intramolecular constraints present in the intact molecule. Therefore, it was important to ad-
Bipartite Autoinhibition Blocks Vinculin Activation by Talin

**Fig. 6. Mutation of the D4-Vt interface induces selective opening of the talin binding site in full-length vinculin.** Lysates of HEK293 cells expressing either wild-type, T12, or N773;E775A GFP-vinculin were prepared by swelling the cells (3 × 10⁶ cells/ml) in hypotonic buffer (20 mM Tris, 2 mM MgCl₂, 0.2 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM ATP, 0.02% azide, 2× PICS) for 20 min on ice. Cells were then lysed with a Dounce homogenizer and insoluble material removed by centrifugation (16,000 × g, 10 min, 4°C). Lysates were normalized to equivalent GFP-vinculin levels based on GFP fluorescence. A, 50 µl of lysate were supplemented with imidazole to 10 mM final and His-tagged talin rod to concentrations of 0.1, 0.3, 1.0 µM. Binding proceeded 30 min at room temperature. Vinculin-talin complexes were recovered on NTA-agarose, washed twice in phosphate-buffered saline, and analyzed by SDS-PAGE and Western blotting using monoclonal anti-vinculin (hVin1) or anti-talin (8d4) antibodies. Note that although talin, up to 1.0 µM, fails to bind any detectable wild-type EGFP or endogenous vinculin (lanes 2–4, the N773;E775A (lanes 6–8) and T12 (lanes 10–12) mutants co-fractionate with talin in a dose-dependent manner, indicating enhanced availability of the talin binding site in Vh, Lanes 1, 5, and 9 represent no talin controls. B, Lanes 13–15 show that equivalent levels of GFP-vinculin were present for WT, N773;E775A, and T12. Expression of the GFP-vinculin constructs did not cause any changes in the level of endogenous vinculin. C, N773;E775A and T12 mutant are unaffected for autoinhibition of the actin binding site. 1.5 µM F-actin was polymerized in cell lysates by the addition of KCl to 100 mM and MgCl₂ to 2 mM final concentrations. After 1.5 h of polymerization, F-actin was pelleted in an airfuge (28 psi, 30 min). Pellets were resolved by SDS-PAGE followed by Western blotting. Under these conditions, some vinculin sediments in the no actin control (lanes 16, 18, 20). The addition of F-actin (lanes 17, 19, 21) produced no increase in the amount of vinculin that could be sedimented, indicating that the actin binding site in vinculin is fully masked. D, as a positive control for actin binding in the HEK293 lysates, ectopic His-tagged Vt was added to a final concentration of 0.1 µM. The endogenous HEK293 actin is only present in the monomer pool, and Vt does not sediment on its own (lanes 22 and 25). However, at least 90% of Vt cosediments with F-actin under the conditions of this assay (lane 25 versus 24), showing that Vt efficiently competes with other components of the lysate in the cosedimentation assay and the absence of specific binding of vinculin to F-actin in C is a function of autoinhibition.

The function of the proposed second head-tail binding site in the context of full-length vinculin. One prediction of our competition studies was that disruption of the auxiliary binding site would leave vinculin susceptible to activation by talin, given that the residual D1-Vt complex would have an expected Kᵦ of 0.1 µM or higher (assuming intramolecular binding provided a maximum of 100x binding enhancement). We therefore introduced the D4 and T12 mutations into GFP-tagged vinculin, and assessed vinculin activation in HEK293 lysates using a pull-down assay with His-tagged talin rod (talin-(397–2541)). As shown in Fig. 6A, wild-type vinculin does not bind appreciably to talin (lanes 1–4), demonstrating the functional insufficiency of talin to activate vinculin. In contrast, the T12 (lanes 9–12) and N773;E775A (lanes 5–8) mutants display a dose-dependent increase in binding to talin, from 0–1 µM talin rod. The increased binding to talin rod reflects only a partial opening of vinculin; however, as the actin binding site remained fully masked in these mutants. As shown in Fig. 6C, vinculin cosedimenting with F-actin (lanes 17, 19, 21) is equivalent to baseline level of vinculin sedimentation (lanes 16, 18, 20) in this assay. Since the affinity of the vinculin-actin interaction is ~1 µM (17), it is consistent that the D1-Vt autoinhibitory interface is sufficient to inhibit actin binding in intact vinculin, whereas the 10–300 nM affinity of the talin-vinculin interaction (9, 25, 36) requires the bipartite high affinity head-tail complex to achieve full autoinhibition. Thus, native vinculin is normally in a high affinity autoinhibited state in which both talin and F-actin binding is prevented, but the presence of the T12 and N773;E775A mutations create a low affinity autoinhibited state, which is selectively able to interact with talin but not F-actin. These results confirm the importance of the D4-T12 interface in intact vinculin and demonstrate a functional correlation between strength of the head-tail interaction and ligand binding.

Activated Vinculin Induces Assembly of Hypertrophied Focal Adhesions—Next, we asked whether the increased association of talin with the head-tail interaction mutants had functional significance for organization of the F-actin cytoskeleton. Talin has been proposed to be an important mediator for transmembrane linkages between integrins and the actin cytoskeleton within the focal adhesion (37, 38). One potential consequence for increased talin-vinculin association might therefore be alterations in the structure of focal adhesions. To investigate this possibility, we transfected vinculin-null cells with GFP-tagged vinculin cDNA containing the head-tail interaction mutations. Activation of vinculin correlated with a loss of diffuse, cytosolic vinculin staining, and enrichment in focal adhesions. In cells expressing N773;E775A, T12, or T8/19, focal adhesions were more numerous, with particular abundance in central regions of the cell (Fig. 7). Most strikingly, however, we observed a dramatic elongation of focal adhesions in cells expressing high levels of activated vinculin mutants, T12, T8/19, compared with wild-type controls. This fibillar adhesion phenotype was limited to the most severe head-tail interaction mutants; its absence in cells expressing N773;E775A, T8, or T19, may derive from a stronger basal level of autoinhibition in these mutants. Thus disruption of head-tail regulation leads to both increased association with talin and subsequent alteration of focal adhesion morphology.
DISCUSSION

We have found that the autoinhibitory site in vinculin is bipartite, involving contacts not only between D1 and Vt, as described by Izard et al. (20), but also a previously uncharacterized interaction between D4 and Vt. This additional autoinhibitory site provides binding energy sufficient to account for an approximately hundred fold reduction in the bimolecular $K_d$ for head-tail association over that seen for the D1-Vt complex (see Fig. 8A). Functionally, this increased binding affinity strongly shifts the conformational equilibrium of Vt toward the closed state, thereby suppressing the ability of putative activators, such as talin, to displace Vt from D1 (Fig. 8C). These findings establish a link between vinculin and a select class of autoinhibited cytoskeletal molecules, typified by N-WASP, in which multiple intramolecular contacts work cooperatively to maintain the inactive state.

The presence of a bipartite autoinhibitory site has two key implications for biological function. The first is the ability to build a tightly closed/autoinhibited state from relatively low affinity sites, by thermodynamic coupling of multiple weak intramolecular interactions. Our results show that the $10^{-5}$ M D1-Vt bimolecular complex is transformed to a $10^{-7}$ M Vh-Vt complex by the presence of an auxiliary site, which has as little as $10^{-5}$ M binding (Fig. 8A). This high theoretical value for the $K_d$ of D4-Vt interaction is consistent with the absence of a detectable bimolecular interaction between the isolated D4 and Vt domains in pull-down assays, the small surface area buried in this contact, 540 Å$^2$ (22), and the presence of a limited number of bonding interactions, largely electrostatic in nature (Asp$^{175}$-Arg$^{970}$, Glu$^{775}$-Arg$^{978}$). Nonetheless, in the context of a bimolecular complex of Vh-(1–851) with Vt, once Vt is bound to D1, the local concentration of residues at the D4-T12 interface will be quite high, and thus the auxiliary site serves as an effective intramolecular latch to stabilize the autoinhibited state of the Vh-Vt complex. In the full-length molecule, intramolecular tethering of Vh and Vt is also likely to contribute substantially to enhanced affinity of the autoinhibited interaction (Fig. 8B). Indeed theoretical estimates of the $K_d$ for the head-tail interaction in full-length vinculin are below $10^{-9}$ (22).

The second noteworthy consequence of the D4-T12 interface is that the “molecular logic” encoded by a bipartite autoinhibitory site is fundamentally different from the binary activation switch in molecules that contain a single inhibitory site (39, 40). As illustrated by several experiments on N-WASP, the presence of two inhibitory contact sites requires the combinatorial input of two activators, such as PIP$_2$, and Cdc42 (39, 41), PIP$_2$ and Nck (42, 43), or Cdc42 and Toca-1 (44). In this way, N-WASP functions as a “logical AND gate” which integrates input from two signal transduction pathways into a single output, Arp2/3 driven actin polymerization (45).

In analogous fashion, it is possible to envision two signals converging upon vinculin through ligand binding at both the D1-Vt, and at the D4-Vt interfaces. Integrin-based adhesion can serve to cluster talin and α-actinin (46), both of which have been implicated in binding and activation of the D1 domain of vinculin (20). However, likely candidates to disrupt the D4-Vt interface remain elusive. A strong hypothesis is that RhoA signaling or contractile force may be involved in vinculin activation at the D4-Vt interface. This would have the net effect of restricting vinculin activation to zones of highly concentrated mechanical force at the membrane, a model supported by the linear relationship between vinculin localization and intracellular tension (47, 48). Although PIP$_2$ is an attractive Rho-responsive (49) element proposed to regulate vinculin activation (32, 34, 50), we remain skeptical that an interaction that is displaced by 15 mM NaCl (51) or that otherwise requires micelles of acidic phospholipids containing at least 25% PIP$_2$ (24), is significant in a physiological context. Although PIP$_2$ may function cooperatively with other activators, PIP$_2$ binding sites in talin (52) and α-actinin (53) can allow pure PIP$_2$ micelles to bridge vinculin with ligands in the absence of direct protein-protein interaction. Explicit treatment of this bridging phenomenon is lacking in the literature, and thus the definitive experiments on the role of PIP$_2$ as a co-activator of vinculin remain to be done.

Elucidation of a physiological activation mechanism for vinculin remains an important goal. In light of the findings presented here, it is critically important that competitors for the head-tail interaction be evaluated in the context of tail binding to full-length Vh-(1–851), and tested for efficacy in full-length vinculin. Elegant work on the interaction of the talin VBS3 peptide with the vinculin D1 domain (1–258) has provided both the biochemical proof (25, 20) and the structural basis (20) for the mutual exclusivity of talin versus Vt binding to D1. However, the weak D1-Vt interaction permits competition by talin VBS3, whereas autoinhibition in full-length vinculin renders the protein refractile to talin or pVR (a talin-related peptide) binding as shown here and in previous work (18, 22, 24). In disrupting the D4-Vt interface, the T12 and N773E775A mutants show a gain of function activity for talin binding. These findings illustrate that the inability of talin rod to activate wild-type vinculin is not merely a consequence of enhanced binding of D1 and Vt in an intramolecular context, but rather results from the bipartite nature of the head-tail interaction. Thus, studies that represent the autoinhibitory interaction exclusively as the D1-Vt bimolecular complex are likely to overestimate the ability of potential activators to induce conformational changes in full-length vinculin.

Although the precise nature of a role for talin in activation of vinculin remains controversial, there is little doubt that if vinculin incorporates into focal adhesions in an activated conformation, it will be competent to associate with talin. In light of this, the ability of Vt to disrupt existing complexes of Vh with talin rod becomes significant in terms of the in vivo function of head-tail interaction. If the VBS3-D1 paradigm held in focal adhesions, activation of vinculin would be extremely stable, because Vt cannot compete for VBS3-bound D1 (20). Disruption of vinculin-talin complexes would require active cellular mechanisms, independent of autoinhibitory contacts, to dissociate
these proteins during adhesion plaque remodeling. However, in the context of the bipartite autoinhibitory interaction found in full-length vinculin, Vt can effectively disrupt the interaction with talin; thus inactivation of vinculin through reassociation of the head-tail complex may be an important mechanism for regulating vinculin activities in the focal adhesion. This inactivation may be antagonized or delayed by post-translational modification of Vt, such as the Src-dependent phosphorylation of the head-tail complex may be an important mechanism for regulating vinculin activities in the focal adhesion. This inactivation may be antagonized or delayed by post-translational modification of Vt, such as the Src-dependent phosphorylation that disrupts the bipartite head-tail interaction (54). The change in focal adhesion morphology in cells expressing the head-tail interaction mutants suggests that disruption of head-tail interaction traps vinculin in complexes with ligands. Indeed, preliminary results indicate that increased association of vinculin and focal adhesion components interferes with turnover (exchange of bound and free pools) of vinculin, which ultimately leads to hypertrophy of the adhesion (55).

In summary, we have proposed that spatial clustering of ligands at focal adhesions provides a mechanism for combinatorial activation of vinculin if the thermodynamic additivity of binding energies exceeds the affinity of the intramolecular head-tail complex (22). Here we provide compelling biochemical evidence for that model by demonstrating that high affinity autoinhibition of vinculin is achieved by a bipartite autoinhibitory site comprised of D1-Vt and D4-Vt contacts. Most importantly, we show that the D4-Vt interface functions to mask the D4-Vt interface is largely accessible to barriers to binding the closed conformation of vinculin. Thus, it is noteworthy that the D4-Vt interface is largely accessible to solvent; its orientation appears to lie directly opposite to the hinge domain, and it is not sterically occluded by other regions of Vt (22). In light of these findings, we believe that the most likely mechanism for combinatorial activation of vinculin will involve coordination of specific competitors of the D1-Vt and D4-Vt autoinhibitory sites.

Acknowledgments—We thank David Shortle for use of the spectrofluorometer and Drs. Robert Liddington, Tina Bakolitsa, and Tina Izard for stimulating discussions on vinculin structure and biochemistry. Special thanks to Luu Pham for initial data on the binding of D1 and Vt, and construction of pET15b/V1–258.

REFERENCES

1. Xu, W., Baribault, H., and Adamson, E. D. (1998) Development 125, 327–337
2. Zemljic-Harpf, A. E., Penruntana, S., Avalos, R. T., Jordan, M. C., Roos, K. P., Dalton, N. D., Phan, V. Q., Adamson, E. D., and Roos, R. S. (2004) Am. J. Pathol. 165, 1033–1044
3. Barstead, R. J., and Waterston, R. H. (1991) J. Cell Biol. 114, 715–724
4. Alenghat, F. J., Fabry, B., Tsai, K. Y., Goldmann, W. H., and Ingber, D. E. (2000) Biochem. Biophys. Res. Commun. 277, 91–99
5. Goldmann, W. H., and Ingber, D. E. (2002) Biochem. Biophys. Res. Commun. 290, 749–755
6. Coll, J. L., Benz-Ze'ev, A., Ezell, R. M., Rodriguez, F. J. L., Baribault, H., Oshima, R. G., and Adamson, E. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9161–9165
7. DeMali, K. A., Barlow, C. A., and Burridge, K. (2002) J. Cell Biol. 159, 881–891
8. Szabo, M. J., Pertz, O., Adamson, E. D., Turner, C. E., Junger, S., and Hahn, K. M. (2003) J. Cell Biol. 165, 371–381
9. Burridge, K., and Mangeat, P. (1984) Nature 306, 744–746
10. Belkin, A. M., and Koteliansky, V. E. (1987) FEBS Lett. 220, 291–294
11. Kromer, M., Rudiger, A. H., Jockusch, B. M., and Rudiger, M. (1994) FEBS Lett. 353, 259–262
12. Turner, C. E., Glenney, J. R., and Burridge, K. (1990) J. Cell Biol. 111, 1059–1068
13. Brindle, N. P., Holt, M. R., Davies, J. E., Price, C. J., and Critchley, D. R. (1996) Biochem. J. 318, 753–757
14. Kioka, N., Sakata, S., Kawasumi, T., Amachi, T., Akiyama, S. K., Okazaki, K., Yama, C., Yamada, K. M., and Aota, S. (1999) J. Cell Biol. 144, 59–69
15. Mandai, K., Nakanishi, H., Satoh, A., Takahashi, K., Satoh, K., Nishioka, H., Mizoguchi, A., and Takai, Y. (1999) J. Cell Biol. 144, 1001–1017
16. Jockusch, B. M., and Isenberg, G. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3005–3009
17. Johnson, R. P., and Craig, S. W. (1995) J. Cell Biol. 129, 479–488
18. Johnson, R. P., and Craig, S. W. (1994) J. Biol. Chem. 269, 12611–12619
19. Huttelmaier, S., Mayhoroda, O., Harbeck, B., Jarchau, T., Jockusch, B. M., and Rudiger, M. (1998) Curr. Biol. 8, 479–488
20. Izard, T., Evans, G., Borgon, R. A., Rush, C. L., Bricogne, G., and Bois, P. R. (2004) Nature 427, 171–175
21. Borgon, R. A., Vonrhein, C., Bricogne, G., Bois, P. R., and Izard, T. (2004) Structure 12, 1189–1197
22. Bakoliitsa, C., Cohen, D. M., Bankston, L. A., Bobkov, A. A., Cadwell, G. W., Jennings, L., Critchley, D. R., Craig, S. W., and Liddington, R. C. (2004) Nature 430, 583–586
23. Izard, T., and Vonrhein, C. (2004) J. Biol. Chem. 279, 27607–27678
24. Steinle, P. A., Hoffert, J. D., Adey, N. B., and Craig, S. W. (1999) J. Biol. Chem. 274, 18414–18420
25. Bass, M. D., Patel, B., Barak, I. G., Fillingham, I. J., Mason, R., Smith, B. J., Bagshaw, C. R., and Critchley, D. R. (2002) Biochem. J. 362, 761–768
26. Couet, M. D., and Craig, S. W. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8535–8539
27. Siliiano, J. D., and Craig, S. W. (1986) Methods Enzymol. 134, 78–85
28. Xing, B., Jedaduanyanaya, A., and Lam, S. C. (2001) J. Biol. Chem. 276, 44373–44378
29. O'Halloran, T., Molony, L., and Burridge, K. (1996) Methods Enzymol. 134, 69–77
30. Spudich, J., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
31. MacLean-Fletcher, S., and Pollard, T. D. (1980) Biochem. Biophys. Res. Commun. 96, 18–27
32. Bakolitsa, C., de Pereda, J. M., Bagshaw, C. R., Critchley, D. R., and Liddington, R. C. (1999) Cell 99, 603–613
33. Miller, G. J., Dunn, S. D., and Ball, E. H. (2001) J. Biol. Chem. 276, 11729–11734
34. Weekes, J., Barry, S. T., and Critchley, D. R. (1996) J. Biol. Chem. 271, 3005–3009
35. Groesch, M. E., and Otto, J. J. (1990) Cell Motil. Cytoskeleton. 15, 41–50
36. Gilmore, A. P., Wood, C., Ohanian, V., Jackson, P., Patel, B., Rees, D. J., Hynes, R. O., and Critchley, D. R. (1993) J. Cell Biol. 122, 337–347
37. Calderwood, D. A, Tai, V., Hynes, R. O., and Critchley, D. R. (2004) J. Biol. Chem. 279, 28889–28895
38. Lee, H. S., Bellin, R. M., Walker, D. L., Patel, B., Powers, P., Liu, H., Garcia-Alvarez, B., de Pereda, J. M., Liddington, R. C., Volkman, N., Hanein, D., Critchley, D. R., and Rosbon, R. M. (2004) J. Mol. Biol. 343, 771–784
39. Prehoda, K. E., Scott, J. A., Mullins, R. D., and Lim, W. A. (2000) Science 290, 801–806
40. Dueber, J. E., Yeh, B. J., Chak, K., and Lim, W. A. (2003) Science 301, 1904–1908
41. Rohatgi, R., Ho, H. Y., and Kirschner, M. W. (2000) J. Cell Biol. 150, 1299–1310
42. Rohatgi, R., Nollau, P., Ho, H. Y., Kirschner, M. W., and Mayer, B. J. (2001) J. Biol. Chem. 276, 26448–26452
43. Benesch, S., Lommel, S., Steffen, A., Stradal, T. E., Scaplehorn, N., Way, M., Wehland, J., and Rottner, K. (2002) J. Biol. Chem. 277, 37771–37776
44. Ho, H. Y., Rohatgi, R., Lebensohn, A. M., Li, L., Gygi, S. P., and Kirschner, M. W. (2004) Cell 118, 203–216
45. Prehoda, K. E., and Lim, W. A. (2002) Curr. Opin. Cell Biol. 14, 149–154
46. Miyamoto, S., Akiyama, S. K., and Yamada, K. M. (1995) Science 267, 883–885
47. Balaban, N. Q., Schwarz, U. S., Rivelin, D., Golchberg, P., Tzur, G., Sabanay, I., Mahalu, D., Sassa, N., Bershadsky, A., Addadi, L., and Geiger, B. (2001) Nat. Cell Biol. 3, 466–472
48. Tan, J. L., Tien, J., Pirone, D. M., Gray, D. S., Bhadriraju, K., and Chen, C. S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1484–1489
49. Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994) Cell 79, 507–513
50. Gilmore, A. P., and Burridge, K. (1996) Nature 381, 531–535
51. Johnson, R. P., Niggli, V., Durrer, P., and Craig, S. W. (1998) Biochemistry 37, 19211–19222
52. Isenberg, G., Niggli, V., Pieper, U., Kaufmann, S., and Goldmann, W. H. (1996) FEBS Lett. 397, 316–320
53. Fukami, K., Furuhashi, K., Inagaki, M., Endo, T., Hatano, S., and Takenawa, T. (1992) Nature 359, 150–152
54. Zhang, Z., Izaguirre, G., Lin, S. Y., Lee, H. Y., Schaefer, E., and Haimovich, B. (2004) Mol. Biol. Cell 15, 4234–47
55. Cohen, D. M., Rutscher, B., Chen, H., Murphy, D., and Craig, S. W. (2004) Mol. Biol. Cell 15, 81–82