A Conformational Switch in Vinculin Drives Formation and Dynamics of a Talin-Vinculin Complex at Focal Adhesions

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Dynamic interactions between the cytoskeleton and integrins control cell adhesion, but regulatory mechanisms remain largely undefined. Here, we tested the extent to which the autoinhibitory head-tail interaction (HTI) in vinculin regulates formation and lifetime of the talin-vinculin complex, a proposed mediator of integrin-cytoskeleton bonds. In an ectopic recruitment assay, mutational reduction of HTI drove assembly of talin-vinculin complexes, whereas ectopic complexes did not form between talin and wild-type vinculin. Moreover, reduction of HTI altered the dynamic assembly of vinculin and talin in focal adhesions. Using fluorescence recovery after photobleaching, we show that the focal adhesion residency time of vinculin was enhanced up to 3-fold by HTI mutations. The slow dynamics of vinculin correlated with exposure of its cryptic talin-binding site, and a talin-binding site mutation rescued the dynamics of activated vinculin. Significantly, HTI-deficient vinculin inhibited the focal adhesion dynamics of talin, but not Paxillin or α-actinin. These data show that talin conformation in cells permits vinculin binding, whereas the autoinhibited conformation of vinculin constitutes the barrier to complex formation. Down-regulation of HTI in vinculin to $K_d = 10^{-7}$ is sufficient to induce talin binding, and HTI is essential to the dynamics of vinculin and talin at focal adhesions. We therefore conclude that vinculin conformation, as modulated by the strength of HTI, directly regulates the formation and lifetime of talin-vinculin complexes in cells.

Integrins mediate transmembrane connections between the actin cytoskeleton and extracellular matrix (1, 2). These connections are organized into discrete clusters such as focal complexes (3) and focal adhesions (3, 4) in adherent cells. Focal adhesions serve dual, opposing functions in cell motility, acting both in transmission of traction forces from the extracellular matrix to the cytoskeleton and in regulation of focal adhesion structure, and the integrin-cytoskeleton associations contained therein, plays a central role in balancing adhesive and migratory stimuli in the cell.

Two key proteins implicated in the physical connections between integrins and F-actin are the actin-binding proteins talin and vinculin. In vitro, talin binds to β integrin tails through its FERM domain (6) and induces conformational changes in integrin associated with increased binding to the extracellular matrix (7). In cells, exposure of activated epitopes on β1 and β2 integrins and fibronectin binding are strongly inhibited by knockdown of talin expression (8). Moreover, talin-1 null cell lines are deficient in the formation of mechanical linkages between fibronectin, β3 integrin, and the cytoskeleton as shown by loss of a transient molecular slip bond that can sustain up to 2 piconewtons of force (9).

Interestingly, the seminal report that talin binds directly to αβ integrin also demonstrated that integrin, talin, and vinculin form a ternary complex in vitro (10). This observation implicated vinculin as part of the integrin-cytoskeleton linkage. Evidence that vinculin participates in transmembrane connections in vivo is based on defects in fibronectin-based adhesion induced by genetic disruption of vinculin. Vinculin null cells show decreased strength of adhesion to fibronectin surfaces (11, 12), and integrin-cytoskeleton linkages are more easily disrupted by the application of mechanical force in the absence of vinculin (13, 14).

Despite these intriguing findings, there is no direct evidence for a ternary complex of talin-vinculin-integrin in cells, and it is not known how this putative complex is regulated. In vitro, purified vinculin and talin exhibit extremely weak interactions, which can be attributed to an autoinhibitory head-tail interaction (HTI) in vinculin that allosterically blocks the talin-binding site (15). These observations suggest the hypothesis that a conformational switch from an autoinhibited to an activated state of vinculin is required to form talin-vinculin complexes in cells. In vitro structural and biochemical studies have demonstrated that vinculin is tightly autoinhibited ($K_d < 10^{-7}$) by the cooperative interaction of two low affinity intramolecular interfaces (vinculin head D1 domain (Vhd1)-vinculin tail domain (Vt) with $K_d = 10^{-12}$ M and vinculin head D4 domain-Vt with $K_d = 10^{-2}$ to $10^{-3}$ M) estimated from bimolecular interactions (16, 17). These data suggest a model in which two ligands act coordinately to disrupt the two head-tail interfaces and thereby promote vinculin activation.

Alternatively, it has been suggested that activation of cryptic vinculin-binding sites (VBS) in talin is sufficient to disrupt the autoinhibitory HTI in vinculin (18–21). Structural studies have revealed that binding of VBS peptides to Vhd1 (residues 1–258) induces a large conformational change that disrupts a major binding site for Vt (18). Moreover, VBS3 peptide has been reported to bind with similar affinity to either Vhd1 or full-length vinculin (residues 1–1066) when these proteins are adsorbed onto a solid-phase substrate (22). In addition, VBS3 peptide alters the protease sensitivity of full-length vinculin in solution (22). These observations were interpreted to suggest that HTI makes no significant contribution to modulating the affinity of vinculin for the VBS sequence and therefore that the VBS sequences in talin and α-actinin constitute a sufficient mechanism for vinculin activation (19).

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2 The abbreviations used are: HTI, head-tail interaction; Vhd1, vinculin head D1 domain (residues 1–258); Vt, vinculin tail domain (residues 884–1066); VBS, vinculin-binding site(s); Vh, vinculin head domain (residues 1–851); YFP, yellow fluorescent protein; HFFs, human foreskin fibroblasts; MES, 4-morpholineethanesulfonic acid; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein.
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VBS sequences are buried, however, in the context of intact talin (23, 24). Whereas weakly structured fragments of talin show enhanced affinity for Vhd1 compared with intact talin in vitro (25), well folded and measurably more stable domains of talin undergo structural rearrangement associated with VBS exposure only in the presence of Vhd1 (26). As such, it remains controversial as to whether VBS peptides or weakly structured talin fragments reflect bona fide conformations for talin or merely mimic an end state induced by vinculin binding. In contrast, conformational changes in vinculin have been directly visualized and linked to exposure of its actin- and vinexin-binding sites in focal adhesions of live cells (27).

In this study, we have examined the functional relevance of HTI to the formation of a talin-vinculin complex in living cells by testing the extent to which HTI regulates the recruitment of vinculin to talin or talin-integrin clusters in cells. Furthermore, we have explored whether the biochemical constraints imposed by HTI affect the dynamic behavior of vinculin, talin, α-actinin, and paxillin, all of which are potential ligands of vinculin at focal adhesions.

The data demonstrate that intramolecular HTI of vinculin outcompetes binding of talin to vinculin in vivo and that, as a consequence, complexes of vinculin and talin are not detectable in an ectopic recruitment assay unless HTI is partially attenuated. Reducing HTI by 100-fold improves recruitment of vinculin to talin and reduces the requirement for vinculin to interact with talin, and HTI is the major barrier to formation of talin-vinculin complexes. In addition, we have found that vinculin HTI regulates the length of time that vinculin and talin remain associated with focal adhesions (i.e. the exchange rate of these proteins is decreased), whereas the dynamics of two other vinculin ligands in focal adhesions are not affected. We conclude that regulation of HTI can determine the lifetime of specific vinculin-based functional complexes within the longer lived architecture of a mature focal adhesion.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pET30a/talin rod was provided by Dr. Stephen Lam. pET15b/YFP/V1–851 and pET15b/YFP/V1–258 were described previously (17). The A50I mutation was introduced into specified constructs by QuikChange mutagenesis (Stratagene). pEGFPC1/vinculin (17) was modified by QuikChange PCR to facilitate cloning of cDNAs previously in the pET15b vector. The Ndel site present in the cytomagelovirus promoter region was removed (mutating adenine 234 to thymine), and a new Ndel site was introduced into the multiple cloning site immediately before the vinculin start codon. Additionally, the 5′-XhoI site was removed (mutating cytosine 1345 to adenine), and a 3′-XhoI site was introduced 3′ to the vinculin cDNA in lieu of a PstI site. cDNAs encoding the vinculin head domain (Vh; residues 1–851), Vhd1 (residues 1–258), and Vt (residues 811–1066), flanked by Ndel and XhoI sites, were subcloned from pET15b bacterial expression constructs into the multiple cloning site of pET15b vector. Talin was cloned into the Ndel-compatible pEGFPC1 using a linker sequence coding for residues 1064–1066 and terminating in a 3′-Xmal site. In addition to the restriction sites, these primers also contained an ectopic sequence containing a peptide spacer (PRGSPPAAS) designed to prevent deleterious effects of the ActA tag on protein folding. Full-length vinculin cDNA (residues 1–1066) lacking a stop codon was generated by digestion of the vinculin cDNA with NcoI and ligation to a linker sequence coding for residues 1064–1066 and terminating in a Sall site. This cDNA was subcloned into pEFGPC1 using EcoRI and Sall sites. The ActA sequence was then introduced using KpnI and Xmal sites present in the pEFGPC1 vector to yield pEFGP/V1–856/VDGTP/space/ActA. The T12 mutation (17) was introduced into pEFGP/vinculin/ActA by QuikChange mutagenesis. The ActA tag was also subcloned into pEFGP/V1–851/STV/space/ActA. pEFGP/V159–1066/T12/ActA, a vinculin construct deficient in both HTI and the talin-binding domain (18), was generated by replacing the cDNA encoding Vh in pEFGP/V1–851/ActA with a PCR-amplified 2.75-kb product corresponding to T12 vinculin (159–1066). To construct pEFGP/talin/ActA, the stop codon in pEFGP/talin was converted to a serine codon, and a 3′-KpnI restriction site was introduced by QuikChange mutagenesis.

The resulting Ndel/KpnI-flanked cDNA lacking a stop codon was then substituted for vinculin-(1–851) in the pEFGP/V1–851/ActA vector to generate pEFGP/talin/RSVL/space/ActA.

**HTI Mutations**—Characterization of the HTI mutations in vinculin used herein has been reported (17). The specific mutations present in each cluster are as follows: T8, K944A/R945A; T12, D974A/K975A/R976A/R978A; T19, K1047A/R1049A/D1051A; T20, R1057A/R1060A/K1061A; and T8/19, K944A/R945A/D1047A/K1049A/R1051A.

**Talin Rod Binding Assay**—Expression and purification of His-tagged talin rod and His-tagged yellow fluorescent protein (YFP)-vinculin-(1–258) and thrombin digestion of His-tagged YFP-vinculin-(1–258) were carried out as described previously (17). To assay binding, YFP-vinculin-(1–258) (wild-type or A50I) was incubated at 0.2 μM with His-tagged talin rod (0–1.4 μM) for 1 h at room temperature in a 250-μl reaction containing phosphate-buffered saline, 0.1 mg/ml bovine serum albumin, 0.1% Triton X-100, 0.1% β-mercaptoethanol, and 10 mM imidazole (pH 7.8). Talin rod was recovered on nitrilotriacetic acid resin (Qiagen Inc.) with inversion for 30 min at room temperature. YFP fluorescence remaining in the supernatant was assayed on a Fluoromax-3 fluorometer (HORIBA Jobin Yvon). YFP was excited at 490 nm, and peak emission at 527 nm was observed using 3-mm excitation and 5-mm emission slit widths.

**Tissue Culture and Transfections**—Human foreskin fibroblasts (HFFs) were a gift of Dr. Denis Wirtz. HFFs were propagated on 0.1% gelatin-coated 10-cm dishes with 90% high glucose Dulbecco’s modified Eagle’s medium (Mediatech, Inc.) and 10% fetal calf serum (Hyclone) in a 5% CO2 incubator at 37 °C. HFFs were cultured overnight on glass coverslips coated with fibronectin (20 μg/ml) prior to transfection. Transfections were carried out using Lipofectamine Plus (Invitrogen) and serum-free Iscove’s medium (American Type Culture Collection). HFFs were labeled with 200 nM MitoTracker Red CM-H2XRos (catalog no. M-7513, Invitrogen) for 30 min in growth medium prior to fixation. Vinculin null cells (clone 54) were provided by Dr. Eileen Adamson and grown on 0.1% gelatin-coated coverslips in a custom filming medium, which improved live cell imaging. The high glucose Dulbecco’s modified Eagle’s medium formulation (Invitrogen) was modified to exclude phenol red and to reduce sodium bicarbonate to 0.37 g/liter and sodium chloride to 4.75 g/liter. In addition, calcium pantothenate, choline chloride, folic acid, myo-inositol, niacinamide, riboflavin, thiamine hydrochloride, and pyridoxine hydrochloride were reduced to levels present...
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in L-15 medium (25% of the amounts in Dulbecco’s modified Eagle’s medium), HEPES and fetal calf serum were added to 15 mM and 10% final concentrations, respectively. Vinculin null cells were plated on 20 μg/ml fibronectin-coated coverslips or Delta T dishes (Biotech Inc.) for 18 h prior to transfection. Cells were transfected using Lipofectamine Plus and allowed to recover overnight. Transfection particles were removed by extensive washing of the dishes.

Immunofluorescence—HFFs were fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline for 20 min. For experiments examining intracellular epitopes, HFFs were permeabilized in ice-cold 0.1% Triton X-100 dissolved in cytoskeletal buffer (10 mM MES, 138 mM KCl, 3 mM MgCl₂, and 4 mM EGTA (pH 6.1)) for 3 min. Note that in experiments involving dual antibody 12G10/MitoTracker labeling, this permeabilization step was omitted. HFFs or digoxin-permeabilized vinculin null cells were blocked in 2% bovine serum albumin and 2% normal donkey serum in phosphate-buffered saline for 30 min at room temperature. The primary antibodies used for immunofluorescence were as follows: anti-β₁ integrin antibody 12G10 (a generous gift of Dr. Steven Akiyama), monoclonal anti-FLAG M2 and anti-vinculin hVin1 antibodies (purchased from Sigma); anti-talin monoclonal antibody 13820 (obtained from BD Transduction Laboratories), and rabbit anti-talin polyclonal antibody (TnC22 serum; generated in the Craig laboratory). Rhodamine Red-X- or Cy5-conjugated donkey anti-rabbit or anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc.

Fluorescence Recovery after Photobleaching (FRAP) Imaging—A Zeiss Axiovert 135TV fluorescence microscope equipped with both stage and objective heaters (Biotech Inc.) was equilibrated at 37 °C for 1 h before photobleaching experiments began. Dark-field and flat-field images (1 μM enhanced green fluorescent protein in filtering medium) were collected at the beginning of each experiment. Biotech dishes containing transfected cells and 1.4 ml of filtering medium were equilibrated in a normal atmospheric environment for 30 min prior to imaging. Photobleaching was achieved using a Stabilite 2017 argon/krypton 488 nm laser (Spectra-Physics) at 100 milliwatts at exposures ranging from 200 to 400 ms to achieve 40–80% loss of initial green fluorescent protein (GFP) fluorescence. The laser was focused on the specimen in the form of a diffraction-limited slit, measuring 0.5 μm in width. The laser beam was brought to the epi-illuminator of the microscope by beam-shaping lenses, including a spherical lens with a 100-mm focal length and a cylindrical lens with a 300-mm focal length; a beam steerer; two mirrors; and three aperture diaphragms. For photobleach recovery, time-lapse GFP epifluorescence images were collected with a Zeiss Plan Neofluar objective (×100) using mercury lamp illumination with a UV-blocking GG455 filter (Schott) and an infrared-blocking BG38 filter (Schott). Digital images captured by a CoolSNAPHQ camera (Photometrics) were binned at 4 × 4 pixels with exposure times optimized to yield pixel intensity values in the linear range (300–2000 counts). Each photobleach sequence was collected for a period of 4–8 min and contained 25–37 frames representing three sequential time domains (a fast domain, 5–7-s intervals; an intermediate domain, 10–15-s intervals; and a slow domain, 15–30-s intervals). The domain intervals and number of frames/interval were adjusted to avoid over- or undersampling of early versus late phases of the recovery curve (i.e., conditions were chosen to disperse the data as evenly as possible through the recovery curve).

FRAP Analysis—Each FRAP sequence was dark-field- and flat-field-corrected using IPLab software (Scanalytics, Inc.) driven by a LabVIEW (National Instruments) script, "FrapApp" (written by Brett Kutscher). FRAP sequences were also corrected for global fluctuations in fluorescence induced by variations in lamp output by normalizing to background fluorescence levels observed in a region of interest selected outside the cell. Photobleaching regions were then selected for analysis using the IPLab ROI tool. Regions of interests used in these analyses were limited to one or two discrete focal adhesions. Recovery curves were fit using the Levenberg-Marquardt algorithm and a single exponential recovery model following the equation: $F_t = F_0 - (F_i - F_0)e^{-kt}$, where $F_t$ is the fluorescence at time $t$, $F_i$ is the final fluorescence (plateau), $F_0$ is the initial fluorescence (extrapolating to $t = 0$ after photobleaching), and $k = \ln(2)/t_{1/2}$. Movies were excluded from the analysis if they contained significant focus drift, showed focal adhesion growth or decay, or exhibited fluorescence recovery at <20% of the initial fluorescence. Goodness of fit to a single exponential model was assessed by a semilog plot ($ln(F_t - F_i)$ versus $t$), and sequences were truncated at time points following which significant deviation from linearity was observed. Tolerance values for mean squared error for the nonlinear Levenberg-Marquardt fit were set at 250 (i.e. analyses exceeding this value were rejected). Typical mean squared error was below 100 for movies with at least 450 fluorescence units/pixel (mean value in region of interest determining the recovery rate/frame).

Data Processing and Statistical Analyses—Box-plot distributions generated in KaleidaGraph (Synergy Software) were used to summarize the $t_{1/2}$ measurements for each of the vinculin mutants. In these plots, the data are represented in quartiles, with the upper quartile (25% of the data points above the median) and the lower quartile (25% of the data points below the median) representing the top and bottom of the each box, respectively. The median is depicted as a horizontal line within each box. The thin lines extending from the boxes mark the minimum and maximum values of the data set that fall within an acceptable range. Open circles denote outliers, points whose values are either >upper quartile + 1.5 × interquartile distance or <lower quartile − 1.5 × interquartile distance. Appropriate statistical methodology to analyze differences in the mean FRAP $t_{1/2}$ values was used to evaluate the box-plot analyses. For non-normal distributions, as observed for the HTI mutants in Fig. 3C, a two-tailed non-parametric Mann-Whitney test was applied to compare means between two samples. In addition, the entire set of full-length vinculin (wild-type, T20, and HTI mutant) half-time measurements was subjected to analysis of variance using resources at available at www.physics.csbsju.edu/stats/anova.html. For normal distributions, such as those obtained for the GFP adhesion markers, a two-tailed Student’s $t$ test with unequal variance was utilized. $p$ values reported for all of these tests were computed at 95% confidence limits.

RESULTS

Role of Vinculin HTI in Formation of a Ternary Complex with β₁ Integrin and Talin in Cells—To investigate the role of vinculin conformation in assembly of a ternary complex of talin, vinculin, and β₁ integrin in cells, we examined these protein-protein interactions in the context of an ectopic recruitment assay. Targeting proteins away from cell-substrate adhesions to an ectopic location enabled us to evaluate the interaction potential of talin, vinculin, and integrin independently from other focal adhesion proteins. Using the C-terminal ActA tag (28), we mislocalized either vinculin or talin to mitochondria as seen by co-distribution with a mitochondrion-specific dye, MitoTracker (Figs. 1A and 2A).

The GFP-talin-ActA chimera showed extremely poor potential to recruit either endogenous or transfected wild-type vinculin to the mitochondrial surface (Fig. 1, B and D). To test whether autoinhibition of vinculin is limiting to talin-mediated recruitment, we cotransfected
talin-ActA with a FLAG-tagged vinculin mutant, T12, which weakens HTI by 2 orders of magnitude (17). HTI-deficient vinculin was recruited robustly to mitochondria by talin-ActA in contrast to the wild-type vinculin control (Fig. 1, C and D).

Because co-localization of mitochondria and $\beta_1$ integrin was never observed in non-transfected cells or in cells expressing the wild-type vinculin-ActA chimera (Fig. 2C), we interpreted the co-localization of talin-ActA and $\beta_1$ integrin as evidence of complex formation at the plasma membrane. (Note that plasma membrane localization was confirmed by surface labeling with antibody 12G10 in the absence of permeabilization in Fig. 1A.) These structures appear to reflect ectopic protein complexes based on the absence of enrichment of other proteins such as the fibrillar adhesion marker tensin (supplemental Fig. S1).

The association of talin-ActA-tagged mitochondria with activated $\beta_1$ integrin deserves comment because previous findings suggest that talin requires activation to interact with integrin (29, 30). It is possible that talin is activated by the C-terminal ActA tag or that clustering of talin on the mitochondrial surface is sufficient to engage and activate integrin. Mitochondria are motile in the cell and are expected to come into contact with the plasma membrane. Thus, either the derivatized mitochondria activate and cluster surface integrin, or they recruit previously activated surface integrin.

The ability of talin-ActA to support integrin binding is significant in so far as it demonstrates biochemical functionality of mitochondrially targeted talin. Furthermore, it provides important insights into the mechanism of formation of talin-vinculin-integrin complexes in cells. Whereas $\beta_1$ integrin and talin may interact constitutively (at least when
talin is enriched and probably clustered at high concentrations at the mitochondrial surface), autoinhibited vinculin is not recruited to this talin-integrin complex. These data reveal a functional requirement for down-regulation of HTI in vinculin prior to binding of talin in cells. To confirm these observations for endogenous talin, we performed the mitochondrial recruitment assay with ActA-tagged vinculin. GFP-vinculin-ActA did not induce redistribution of talin to mitochondria (Fig. 2C). In contrast, the HTI-deficient vinculin T12 readily recruited talin to mitochondria, often depleting the diffuse cytoplasmic pool of talin (the focal adhesion pool was unaffected) (Fig. 2B). Consistent with the strong propensity for talin-β1 integrin binding in this assay, HTI-deficient vinculin induced the formation of talin-vinculin-β1 integrin ternary complexes at the mitochondrial surface (Fig. 2B). As expected, wild-type vinculin failed to form this ternary complex, and deletion of the talin-binding site in T12 vinculin-ActA disrupted mitochondrial recruitment of both talin and β1 integrin (Fig. 2, C and D). Together, these findings establish that in vitro observations of binary interactions between talin and vinculin and between talin and β1 integrin correctly predict the formation of a ternary complex between these proteins in cells. However, in this sequence of ternary complex formation, activation of vinculin clearly precedes its recruitment to ectopic mitochondrial talin-vinculin-integrin complexes.

**FIGURE 2.** HTI regulates the ability of vinculin to direct assembly of talin-integrin complexes. HFFs expressing GFP-vinculin-ActA (wild-type or T12 mutant) were labeled with MitoTracker and/or immunostained for talin and β1 integrin and visualized by confocal fluorescence microscopy. The boxed regions are enlarged for visualization of marker co-localization. A, T12 vinculin-ActA (T12VincActA) targets to mitochondria as assessed by MitoTracker dye and induces ectopic assembly of integrin. B, T12 vinculin-ActA induces assembly of a ternary complex containing both talin and β1 integrin. C, wild-type vinculin-ActA (WtVincActA) fails to induce ternary complex assembly, as little correlation is observed between GFP staining and organization of talin or β1 integrin. White squiggles are fiduciary markers outlining edges of several mitochondria. These marks were superimposed onto the antibody hVin1 counterstain. D, a T12 deletion mutant, vinculin-(159–1066) (V159–1066 T12ActA), which lacks the talin-binding site, fails to direct assembly of ectopic mitochondrial talin-vinculin-integrin complexes.

**Role of Vinculin HTI in Regulating the Dynamics of Vinculin in Focal Adhesions**—A consequence of HTI deficiency at focal adhesions should be an increase in the affinity of vinculin for its ligands, resulting from the decreased ability of Vt to compete with ligands for Vh. Thus, the expected effect of HTI mutants would be to slow the dissociation of vinculin and/or vinculin-containing complexes from focal adhesions.
To test this possibility, we examined the effect of HTI mutations on the dynamics of vinculin and its focal adhesion ligands by FRAP of GFP-vinculin fusion proteins.

For these studies, we reconstituted vinculin null cells with wild-type vinculin or HTI mutants and assayed the focal adhesion dynamics of vinculin and several of its ligands, including talin, paxillin, and α-actinin. GFP-tagged adhesion proteins were characterized under conditions of relatively static adhesion to fibronectin to minimize the contributions of focal adhesion growth or decay to the fluorescence measurements. Under these conditions, the cells did not actively translocate, and membrane protrusion and retraction occurred on a slower time scale compared with the FRAP measurements. Additionally, the FRAP studies were limited to large peripheral focal adhesions in low to moderate GFP-vinculin-expressing cells because, under these conditions, HTI mutants of vinculin do not perturb focal adhesion morphology or number, in contrast to the situation in which HTI mutants are highly expressed (17). Cytoplasmic levels of wild-type vinculin varied between cells by 4-fold, but the recovery half-time was independent of this variation. Cytoplasmic levels of the various mutants fell largely in the lower half of the wild-type range (data not shown).

As shown in Fig. 3A, GFP-tagged wild-type vinculin recovered homogeneously within the bleached region through a monophasic recovery process, allowing us to model fluorescence recovery in terms of a single rate-limiting event (31). This exponential recovery is typical of FRAP...
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experiments (32) and reflects the fact that dissociation of the bleached protein from the focal adhesion is the rate-limiting step in recovery. Wild-type vinculin had a mean recovery half-time (time taken to reach 50% of the post-bleach maximum) of 83 s (Fig. 3, B and C), which is comparable with the dynamic behavior of other adhesion markers such as α-actinin in mature focal adhesions (33, 34).

Introduction of mutations shown previously to weaken HTI (17) led to significant delays in the FRAP kinetics of vinculin. The recovery half-time of HTI-deficient vinculin was increased by 2–3-fold for the most severe autoinhibitory mutants (186 s for T12, 174 s for N773A/E775A, and 237 s for T8/19) (Fig. 3, B, C, and E). These mutations selectively target the Vhd1-Vt and vinculin head D4 domain-Vt interfaces, which, acting together, are responsible for the strong autoinhibition of vinculin (16, 17). The specificity of the FRAP phenotype was demonstrated by the control mutant T20 (R1057A/R1060A/K1061A), which does not affect HTI, but contains charge-to-alanine substitutions comparable with many of the HTI mutants (17). T20 vinculin showed no detectable differences in FRAP kinetics compared with the wild-type protein (Fig. 3, C and E). Similarly, a clustered basic charge-to-glutamine mutant of vinculin deficient in phospholipid binding but not lacking in HTI was reported to have no effect on the exchange rate of vinculin at focal adhesions (35). The ability of mutations in either the head domain (N773A/E775A) or tail domain (T8, T19, and T12) to inhibit vinculin dynamics demonstrated that this phenotype is likely caused by loss of HTI as opposed to unforeseen secondary effects of the mutations (e.g., changes in intermolecular ligand binding activities). Moreover, the severity of the FRAP phenotype was proportional to the magnitude of the HTI defect associated with each mutant (Fig. 3E).

In addition to recovery rates, the mobile fraction (the fraction of bleached molecules participating in recovery) was also affected by the HTI mutations. Whereas wild-type vinculin in focal adhesions recovered 50–60% of the photobleached fluorescence, strong HTI mutants such as N773A/E775A and T12 plateaued, on average, at 30–40% recovery (Fig. 3D), indicating a bias toward a non-exchangeable pool in the mutants. These findings support a role for HTI in regulation of focal adhesion residency of vinculin.

Exposure of Vhd1 Determines the Half-time of Vinculin in Peripheral Focal Adhesions—To investigate the relationship between changes in FRAP kinetics and exposure of cryptic ligand-binding sites in the activated conformation of vinculin, we measured the FRAP recovery rates for isolated vinculin domains (Fig. 4A). The actin-binding domain (Vt) showed relatively rapid exchange between the cytoplasmic and focal adhesion pools, with a recovery half-time of 76 s. By comparison, Vh or the minimum talin-binding domain, Vhd1 (residues 1–258), showed relatively slow FRAP kinetics, with recovery half-times of 145 and 140 s, respectively. These observations indicate that the slow FRAP kinetics of the HTI mutants are likely the result of conformational exposure of Vh, particularly Vhd1. To test whether exposure of the Vhd1 domain is important in regulating the focal adhesion residency of vinculin, we assayed the effects of Vhd1 deficiency on the FRAP kinetics of vinculin. We disrupted Vhd1 activity using the A50I mutation, which blocks intermolecular Vhd1-ligand interactions (16). This mutation was also implicated in improving bimolecular HTI (16), and we show here that A50I contributed a modest 2–3-fold decrease in the bimolecular dissociation constant for Vh and Vt (supplemental Fig. S2). In contrast, the A50I mutation in vinculin completely ablated talin binding (Fig. 4B).

Loss of talin binding correlated with significant enhancement of the dynamics of vinculin at focal adhesions (Fig. 4C). A50I vinculin had a recovery half-time of 48 s, which was 58% faster than the mean recovery rate of wild-type vinculin. Consistent with an important role of exposure of Vhd1 in activated conformations of vinculin, the A50I mutation partially rescued the slow FRAP phenotype of Vh and the full-length T12 vincin mutant. A50I Vh had a recovery half-time of 82 s (56% of the t½ for Vh), and T12/A50I vincin had a recovery half-time of 105 s (56% of the t½ for T12).

Because A50I comparably suppressed the slow FRAP kinetics of both HTI-deficient vinculin (T12) and isolated Vh, which lacked Vt entirely, the partial rescue of vinculin dynamics in T12/A50I vincin was unlikely to be caused by a conformational shift toward the autoinhibited state. Rather, the mutual dependence of the recovery half-time of wild-
type vinculin, T12, and Vh on the A50I mutation demonstrates that vinculin dissociation from focal adhesion is rate-limited by Vhd1-ligand interactions. This common requirement for Vhd1 in regulating focal adhesion residency time illustrates that, despite conformational variation between different vinculin mutants, these proteins sample similar ligand interactions in the focal adhesion.

Vinculin HTI Controls the Half-time of Talin, but Not Paxillin or α-Actinin, in Focal Adhesions—Because vinculin is proposed to function as a scaffolding protein, the increased residency time of vinculin might be biologically relevant if the dynamic behavior of its focal adhesion targets is also altered. To address whether HTI-deficient vinculin mutants could alter in trans the FRAP kinetics of focal adhesion ligands, we cotransfected vinculin null cells with non-fluorescent vinculin (wild-type or T12) and GFP-paxillin, GFP-α-actinin, or GFP-talin. In the case of GFP-paxillin, reduction of vinculin HTI had no effect on paxillin residency time (Fig. 5). In the presence of either wild-type or T12 vinculin, GFP-paxillin had a mean FRAP half-time of 41 s, indicating either that paxillin does not directly engage vinculin in this system or that additional pathways (e.g. phosphorylation) induce paxillin dynamics independently of vinculin conformation. In the case of α-actinin, a small delay in FRAP kinetics was observed in the presence of T12, although this was not statistically significant. However, talin showed pronounced stabilization in the presence of T12 versus wild-type vinculin, more than doubling its residency half-time from 50 to 123 s (Fig. 5).

**DISCUSSION**

Conformational Change in Vinculin Regulates Assembly of Vinculin into Complexes with Talin or Talin and Integrin—In this study, we tested the extent to which vinculin conformation regulates the binding of intact vinculin and talin in cells. We found that attenuation of vinculin HTI by 100-fold (to $K_d \approx 10^{-7}$) was sufficient to induce the formation of talin-vinculin-integrin complexes at ectopic sites in cells. In contrast, mislocalization of talin failed to recruit wild-type vinculin, although talin associated with β3 integrin. In the cellular context, it therefore appears that talin conformation (i.e. the cost of VBS exposure) does not provide a substantial bias against vinculin binding, whereas HTI constitutes an all-or-nothing barrier for complex formation. Indeed, although the talin-vinculin complex that forms in our ectopic system may not reflect the maximum affinity that can occur between these two proteins, we feel that it is highly significant that the observed interaction of HTI-deficient vinculin and intact talin exhibits sufficient affinity to direct robust subcellular recruitment of either protein, as might be expected for an interaction in the 0.3 μm range (Fig. 4B).

The results from the ectopic recruitment assay provide an interesting complement to a recent study showing that ectopic clusters of α3β3 integrin and talin triggered by Mn2+ stimulation of integrins also fail to recruit vinculin to sites of talin-integrin accumulation (44). Taken together with our data, these two systems show that talin-integrin engagement is not a de facto signal for vinculin recruitment or (by inference) vinculin activation. A limitation of both systems is that they may not fully recapitulate adhesion-based signals (e.g. phosphorylation, proteolytic cleavage, or mechanical stress) that might play a role in VBS unmasking and thus enhancement of talin affinity for the autoinhibited conformation of vinculin. Although we cannot rule out this possibility, we remain skeptical that VBS peptides reflect a physiological conformation of talin existing independently of or prior to engagement with Vh. In contrast to the talin-only activation model, the ectopic recruitment system provides evidence for the hypothesis that HTI regulates the affinity of vinculin for talin in vivo. Thus, other mechanisms of HTI disruption such as combinatorial ligand activation likely play a prominent role in the activation of vinculin in cells, as hypothesized (16, 17).

HTI Regulates Vinculin Dynamics within Focal Adhesions—Although a detailed mechanism for activation of vinculin at focal adhe-
HTI Regulates Adhesion Dynamics of Vinculin

sions has yet to be fully elucidated, the fact that conformational activation of vinculin occurs at sites of cell-matrix adhesion is widely accepted. Indeed, a conformationally responsive fluorescence resonance energy transfer probe of vinculin has provided direct evidence that vinculin preferentially adopts an open actin-binding conformation at stable peripheral focal adhesions (27). Notably, the conformation of vinculin is far more heterogeneous in more dynamic adhesions such as nascent or sliding adhesions (27). The observation of high fluorescence resonance energy transfer corresponding to a relatively closed, inactive conformation of vinculin in some dynamic structures suggests the intriguing possibility that HTI can be reconstituted even in the specialized context of the focal adhesion. Thus, we used FRAP to test whether intramolecular HTI is relevant to vinculin dynamics in focal adhesions by measuring the exchange rates of GFP-vinculin (wild-type or HTI-deficient).

In the absence of photobleaching, GFP intensity (a measure of protein concentration) was relatively constant in peripheral focal adhesions of cells expressing wild-type or mutant vinculin at low to moderate levels. This observation implies that a “steady-state assumption,” in which the total number of binding sites for a protein of interest is fixed, is valid for this class of adhesions. Given cytoplasmic pools of free protein large enough not to be rate-limiting, recruitment of new protein molecules into the focal adhesion is rate-limited by the dissociation of bound (bleached) molecules from pre-existing binding sites. Thus, FRAP exchange rates provide a window into the dissociation rates of protein-protein interactions at focal adhesions.

FRAP measurements are distinct from measures of focal adhesion turnover. Focal adhesion turnover has been defined as the rate of appearance and disappearance of a particular GFP-labeled protein in a focal adhesion (36) and thus characterizes physiological states far removed from steady state. Although less well studied than focal adhesion turnover (37–40), focal adhesion protein exchange rates are also modulated by physiologically relevant events. For example, a link has been established between decreased exchange times of several focal adhesion proteins and loss of mechanical reinforcement of integrin-cytoskeleton linkages in Shp2 null cells (41). Thus, although typically much faster than the turnover of the adhesion, the exchange rates of focal adhesion components are likely to be relevant to adhesion dynamics.

We tested the effect of HTI deficiency on the exchange rates of vinculin and its ligands to address whether HTI is functionally relevant to regulation of vinculin downstream of activation and/or focal adhesion recruitment. Our data make the case that a conformational switch from an active to inactive state is directly responsible for the release of vinculin from the interactions that tether it to the focal adhesion complex. This case is built on three principal observations in our FRAP studies. First, the residency time of vinculin in focal adhesions is inversely proportional to the strength of HTI. Second, constitutive exposure of the head domain, specifically the talin-binding domain or Vhd1, mimics the increase in focal adhesion residency observed in HTI-deficient vinculin. Third, suppression of intermolecular ligand binding activity in Vh (the A50I mutation) largely rescues the focal adhesion dynamics of HTI-deficient vinculin. Thus, antagonism of intermolecular interactions between Vh and its focal adhesion ligands, whether through an artificial mutation (A50I) or a natural mechanism (intramolecular association with Vt), is the driving force for vinculin dynamics in focal adhesions.

Re-association of HTI is a biochemically valid mechanism for dissociation of vinculin from focal adhesions, as the intrinsic strength of HTI (<10^{-7}) is mutually exclusive to known binding strengths of any single physiological ligand (10^{-7} m or less). Moreover, Vt can displace Vh from a ligand-occupied state such as a preformed talin-Vh complex in vitro (17).

Selective Stabilization of Talin in Focal Adhesions by the Activated Conformation of Vinculin—Although HTI deficiency is anticipated to increase the affinity of vinculin for all its ligand-binding partners, the T12 HTI mutant of vinculin selectively increased the focal adhesion residency of talin, whereas paxillin and α-actinin remained relatively unchanged. Although the lack of an effect of HTI-deficient vinculin on FRAP half-times of α-actinin or paxillin does not preclude vinculin binding per se, it does indicate that vinculin binding is not rate-limiting to the dissociation of these proteins from the adhesion complex under the conditions of these experiments. Thus, either only a small fraction of the total adhesion pool of paxillin or α-actinin binds activated vinculin, or their interactions with vinculin are too transient to change their overall adhesion residency. In contrast, when talin interacts with activated vinculin, its focal adhesion residency increases, suggesting that talin either gains the ability to associate with a more stable compartment in the focal adhesion or undergoes a conformational activation event that enhances its ability to bind other ligands. This latter possibility is especially intriguing given structural studies demonstrating that Vh binding induces large conformational changes in talin that expose previously cryptic amphipathic helices (25, 26).

Although we did not see a change in the residency of α-actinin or paxillin, it is possible that their interaction with vinculin might be detected by FRAP when cells are exposed to specific types of growth factor or mechanical stimuli. For example, vinculin has been implicated in sequestering paxillin during apoptotic stimulation (42).

Broader Implications of the FRAP Experiments—A general conclusion from our study is that FRAP, together with well characterized mutants, can be used to monitor specific, dynamic protein-protein interactions in the context of molecularly complex and long-lived focal adhesions. In the case of vinculin and HTI mutations, the FRAP experiments revealed that in vivo competition between intra- and intermolecular interactions accounts for the behavior of a dynamic pool of vinculin and talin in the focal adhesion. Despite their “stationary” appearance, focal adhesions likely experience continual remodeling of extracellular matrix-actin linkages based on the short lifetimes of individual cytoskeletal constituents (e.g. integrin, talin, vinculin, and actin) relative to the overall turnover rate of the focal adhesion. We envision that specific protein-protein interactions transiently formed within a stationary focal adhesion represent individual nanoscale complexes that mediate or modify connections between the extracellular matrix and actin cytoskeleton. Functional complexes may exist for relatively short periods of time in these stationary adhesions and may even be formed in a polarized fashion within the adhesion. Indeed, activated vinculin is polarized to the inner edge in a sliding adhesion (27), and new integrins are preferentially added at the inner edge of such adhesions (43). Such considerations are likely to be relevant to how and where the actin cytoskeleton engages with the focal adhesion and how it moves with respect to the focal adhesion to generate translocation of the cell.

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