Spatial distribution and functional significance of activated vinculin in living cells

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Conformational change is believed to be important to vinculin’s function at sites of cell adhesion. However, nothing is known about vinculin’s conformation in living cells. Using a Forster resonance energy transfer probe that reports on changes in vinculin’s conformation, we find that vinculin is in the actin-binding conformation in a peripheral band of adhesive puncta in spreading cells. However, in fully spread cells with established polarity, vinculin’s conformation is variable at focal adhesions. Time-lapse imaging reveals a gradient of conformational change that precedes loss of vinculin from focal adhesions in retracting regions. At stable or protruding regions, recruitment of vinculin is not necessarily coupled to the actin-binding conformation. However, a different measure of vinculin conformation, the recruitment of vinexin β by activated vinculin, shows that autoinhibition of endogenous vinculin is relaxed at focal adhesions. Beyond providing direct evidence that vinculin is activated at focal adhesions, this study shows that the specific functional conformation correlates with regional cellular dynamics.

Introduction

Vinculin is a 116-kD, cytoskeleton-associated protein that is essential for brain and heart development in mice (Xu et al., 1998a) and for muscle contraction in nematodes (Barstead and Waterston, 1991). Vinculin is expressed in most cell types and tissues (Otto, 1990), but its localization in muscle is particularly informative. In skeletal and cardiac muscle, vinculin describes a subsarcolemmal lattice of transmembrane connections or costameres (Craig and Pardo, 1983; Pardo et al., 1983a,b; Shear and Bloch, 1985) that attach the myofibrils to the sarcolemma (Pierobon-Bormioli, 1981) and transduce force laterally through the extracellular matrix to neighboring myocytes (Street, 1983; Ervasti, 2003). Vinculin is also enriched at myotendinous junctions (Shear and Bloch, 1985) and intercalated discs (Koteliansky and Gneushev, 1983; Pardo et al., 1983a), intercellular adhesion structures involved in longitudinal transmission of forces between adjacent muscle cells. Vinculin likely plays a role in muscle structure and stability to mechanical forces. Recent data shows that hearts of vin−/− mice are predisposed to stress-induced cardiomyopathy (Zemljic-Harpf et al., 2004). In several cases, mutations and deletion of metavinculin, the muscle-specific spliceform of vinculin (Byrne et al., 1992), are correlated with idiopathic dilated cardiomyopathy (Maeda et al., 1997; Olson et al., 2002), offering additional support for the importance of these proteins in muscle function.

Evidence from cells in culture suggests that vinculin functions in transducing force across cell membranes (Danowski et al., 1992; Alenghat et al., 2000), in regulating cell adhesion and motility (Rodriguez Fernandez et al., 1993; Xu et al., 1998b; DeMali et al., 2002), in controlling cell survival (Subauste et al., 2004), and in executing rac-mediated signaling events (DeMali et al., 2002; Goldmann and Inger, 2002). In cultured cells, vinculin is enriched at cell–cell and cell–matrix junctions (Geiger, 1979) but is in equilibrium with a large cytoplasmic pool (Lee and Otto, 1997). When cells adhere and spread on ECM, a portion of the cytoplasmic vinculin is recruited to specialized sites on the plasma membrane called focal adhesions and focal contacts. At these sites, dynamic connections are made between the actin cytoskeleton and ECM through transmembrane integrin and syndecan receptors for extracellular matrix molecules. These connections relay force across the membrane (Balaban et al., 2001; Beningo et al., 2001; Galbraith et al., 2002; Tan et al., 2003) and are essential for regulation of cell motility (Palecek et al., 1997; Priddle et al., 1998). Focal adhesions appear to be compositionally, structurally, and functionally analogous to the costameres of skeletal and cardiac muscle and the dense plaques of smooth muscle cells and are...
therefore a good model system to examine the mechanism and significance of vinculin action.

Because vinculin lacks intrinsic enzymatic activity, it must exert its functions through interaction with other proteins. Indeed, multiple proteins, including F-actin, talin, α-actinin, α-catenin, vinexin, VASP, ponsin, CAP, arp2/3 (DeMali et al., 2002), Raver-1 (Huttemaier et al., 2001), PKC (Tigges et al., 2003), and paxillin, interact with specific domains of vinculin in vitro and colocalize with vinculin at ECM contacts in vivo (for reviews see Critchley, 2000; Zamir and Geiger, 2001). However, in contrast to isolated domains of vinculin and to vinculin immobilized on nitrocellulose, full-length vinculin in solution binds poorly or undetectably to many of these ligands (Johnson and Craig, 1994, 1995; Kroemker et al., 1994; Huttemaier et al., 1998; Bakolitsa et al., 2004). The inert state of vinculin is caused by high affinity intramolecular binding between the vinculin head domain (Vh; residues 1–851 or 1–857) and tail domain (Vt; residues 884–1066) of vinculin (Johnson and Craig, 1994). In bimolecular assays, the $K_d$ of the Vh–Vt complex is $50 \text{nM}$ (Johnson and Craig, 1994); in an intramolecular context the interaction is estimated, but not directly measured, to be $<1 \times 10^{-9}$ (Bakolitsa et al., 2004). Thus, it is implicit that disruption of the Vh–Vt interaction is required for vinculin activation and subsequent assembly of vinculin-containing protein complexes at adhesion junctions (Johnson and Craig, 1995).

Nevertheless, the model for vinculin activation and function is supported solely by in vitro biochemistry using purified proteins and their domains. Nothing is known about vinculin conformation in cells, its relevance to focal adhesion composition, or its relationship to cellular dynamics. Here, we present data providing new insights on all three of these issues.

## Results

### Construction of vinculin Förster resonance energy transfer (FRET) probes

To monitor activation of vinculin, we developed FRET probes using CFP and YFP as the donor-acceptor pair (Miyawaki and Tsien, 2000). Because the crystal structure of full-length vinculin was not known, we began by positioning ECFP and EYFP on the NH$_2$- and COOH-terminal residues of vinculin (CVY; Fig. 1 A). In cell lysates prepared from HEK 293 cells transfected with CVY, the corrected FRET emission ratio (see Materials and methods) of CVY was 0.05, only slightly greater than the baseline for CFP alone which was set to zero for the calculation. The calculated FRET efficiency (see Materials and methods) for CVY was only 3%, indicating that the NH$_2$ and COOH termini are not in proximity. In addition, there was little change in FRET of the CVY probe upon activation of vinculin by IpA and binding to actin (unpublished data).

Because CVY was not a suitable FRET probe, we explored internal placements for one of the fluorescent proteins. The intramolecular distance between the NH$_2$ and COOH termini of Vt measured from the crystal structure of Vt (Bakolitsa et al., 1999) is $\sim 14 \text{ Å}$. Thus, we anticipated strong FRET from a construct in which CFP and YFP are positioned at the two ends of Vt. Indeed, an EYFP/Vh/ECFP fusion protein exhibited robust FRET (emission ratio of 1.3; efficiency of 43%) and maintained the ability to bind to Vh (unpublished data). Therefore, to construct a full-length vinculin FRET probe we inserted EYFP into vinculin at the beginning of the tail domain and placed an ECFP at the COOH terminus of vinculin to make the FRET probe, referred to as tail probe (Fig. 1, A and C).

### Characterization of tail probe

Tail probe and various control constructs were expressed in HEK293 cells, and lysates were analyzed by spectrophuorometry. Tail probe exhibited a strong FRET signal (Fig. 1 B), with a corrected emission ratio of 1.48 and an efficiency of 46% (see Fig. 4, A and B). To determine whether or not intermolecular FRET contributes to the FRET measurement, we compared a
lysate containing tail probe at 5, 10, and 20 nM with mixtures of lysates containing vinculin/CFP and vinculin/YFP at 5, 10, or 20 nM each in the mixture. At all concentrations tested, the corrected FRET ratio of the mixed probes was close to that of CFP only, whereas the corrected FRET ratio of tail probe was insensitive to dilution. Thus, the FRET values obtained for tail probe report specifically on intramolecular FRET.

SDS-PAGE of HEK 293 cell lysates and Western blotting revealed that tail probe shows three closely spaced bands near the expected molecular weight. All species were recognized by both antivinculin and anti-GFP, which cross-reacted with CFP and YFP (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200410100/DC1). No evidence of proteolytic cleavage of CFP or YFP was detected in the form of GFP-sized bands. Because there is SDS-resistant structure in the CFP and/or YFP moieties (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200410100/DC1), no evidence of proteolytic cleavage of CFP or YFP was detected in the form of GFP-sized bands. Because there is SDS-resistant structure in the CFP and/or YFP moieties (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200410100/DC1), the heterogeneity in migration of the FRET probe likely represents a combination of the fully denatured form (slowest migrating) and faster migrating, partially folded intermediates.

To determine the conformational state of tail probe and to assess its ability to report on activation of vinculin, we measured FRET response as a function of ligand binding to vinculin. Neither tail probe nor endogenous vinculin cosedimented with F-actin, indicating a conformational change of vinculin in the tail domain. (A) Normalized fluorescence emission spectra of cell lysate from HEK 293 cells transfected with tail probe in the absence or presence of 1 μM IpaA or 5 μM actin or both. Spectra were normalized to the emission of tail probe at 475 nm. (B) Samples from A were spun in an Airfuge [Beckman Coulter] at 25 psi (130,000 g) for 35 min. Equivalent amounts of total sample before spin (T), supernatant (S), and pellet (P) fractions were subjected to SDS-PAGE and immunoblotted with hVIN1 and C4 mAbs (Sigma-Aldrich) to vinculin and actin, respectively.

![Figure 2.](image)

**Figure 2. Response of tail probe to ligands.** The binding of actin filaments to IpaA-activated vinculin tail probe induced FRET loss, indicating a conformational change of vinculin in the tail domain. (A) Normalized fluorescence emission spectra of cell lysate from HEK 293 cells transfected with tail probe in the absence or presence of 1 μM IpaA or 5 μM actin or both. Spectra were normalized to the emission of tail probe at 475 nm. (B) Samples from A were spun in an Airfuge [Beckman Coulter] at 25 psi (130,000 g) for 35 min. Equivalent amounts of total sample before spin (T), supernatant (S), and pellet (P) fractions were subjected to SDS-PAGE and immunoblotted with hVIN1 and C4 mAbs (Sigma-Aldrich) to vinculin and actin, respectively.

![Figure 3.](image)

**Figure 3. Response of the control FRET probe YC-V1-400 to ligands.** (A) Normalized fluorescence emission spectra of cell lysates from HEK 293 cells transfected with the control FRET probe in the absence or in the presence of 1 μM IpaA or 5 μM actin or both. Spectra were normalized to emission at 475 nm of control probe alone. The control probe preserves the IpaA binding site and a focal adhesion targeting signal of vinculin but lacks the actin binding site. It does not display FRET change in response to IpaA binding. (B) Actin cosedimentation assay, performed under the same conditions as in Fig. 2 B, showed that the control probe did not bind to actin filaments under conditions in which tail probe did bind.
and are equally able to rescue spreading defects in vinculin null cells (see Fig. 5).

For a control probe, we constructed an EYFP-ECFP chimera fused in frame to vinculin residues 1–400 (Fig. 1 A). This probe contains the binding site for IpaA (Bourdet-Sicard et al., 1999) and a focal adhesion targeting motif (Bendori et al., 1989) but lacks F-actin binding capacity (Menkel et al., 1994). Control probe had a corrected FRET ratio of 1.4 and a FRET efficiency of 44% (see Fig. 4, A and B). These values are similar, fortuitously, to unstimulated tail probe in cell lysates. This property was useful because it allowed us to use the FRET signal of control probe observed in cells to define the baseline FRET for the closed conformation of tail probe. There was no significant change in FRET for the control probe in cell lysates either before or after treatment with IpaA, actin, or both ligands together (Fig. 3 A; and Fig. 4, A and B); nor did the control probe cosediment with actin (Fig. 3 B). Therefore, we conclude that tail probe reports on conformational changes in vinculin that reflect its activation and binding to actin filaments, whereas control probe is insensitive to F-actin and to IpaA, an activator of the Vh.

When transfected into vinculin null mouse embryo cells (vin−/− MEC; Xu et al., 1998a), both the tail probe and untagged vinculin showed a diffuse cytoplasmic pool and were similarly enriched at focal adhesions (Fig. 5 A). Tail probe and untagged vinculin were equally able to rescue the spreading defect (Xu et al., 1998a,b) and change in cell shape (DeMali et al., 2002) as shown in Fig. 5 (C and E and B and D, respectively). Thus, the vinculin tail FRET probe is suitable for analysis of vinculin conformation in living cells.

Detection of tail probe FRET in living cells
To determine if vinculin conformation correlates with subcellular localization, we transfected vin−/− MEC with tail probe and calculated a FRET image from the digital data as described in Materials and methods. The global emission ratio (averaged over all pixels above threshold) observed in these cells was 1.43 ± 0.18 SD (n = 8, compiled from two experiments), which was distinguishable from the baseline of 0.6 obtained from cells transfected with vinculin/CFP. To confirm that the emission ratio reflects FRET, the average FRET efficiency of tail probe in cells was determined by fluorescence recovery after acceptor photobleaching and found to be ~15% (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200410100/DC1).
Figure 6. Spatial distribution of activated vinculin in living cells. Vin^TF/MEC transfected with tail probe (A–F) or control probe (G–L) were imaged 1 h after plating. (A and G) Localization of tail probe and control probe in MEC imaged through CFP channel. (D and J) Pseudocolored ratio (FRET/CFP) image of the cells shown in A and G, (B, E, H, and K) Enlargement of boxed region in A, D, G, and J, respectively. (C and I) The average FRET ratio measured from segmented regions of cytoplasm or focal adhesions; all segmentable focal adhesions were included. (F and L) Histograms of FRET ratios measured from the segmented focal adhesions and cytoplasm. Notably, the tail probe gave a much lower average FRET ratio (corresponding to actin-binding conformation of vinculin) in focal adhesions (B and E, boxed region) than in cytoplasm even though not all focal adhesions are distinguishable from cytoplasm. The control probe did not distinguish between the two locations (H and K, boxed region). Similar results were obtained by analysis of three other cells from a separate experiment.
Spatial resolution of vinculin conformations in living cells

When focal adhesions were examined in vin−/− MEC transfected with tail probe, we found that the average FRET ratio is significantly lower in focal adhesions than in cytoplasm (Fig. 6, A–F), indicating enrichment of the actin-binding conformation in focal adhesions. In contrast, in vin−/− MEC transfected with the control probe (YC/V 1–400), the FRET ratio is similar in focal adhesions and in cytoplasm (Fig. 6, G–L). The FRET ratio of tail probe in cytoplasm is comparable to the global FRET ratio of control probe (Fig. 6, D, F, J, and L), indicating that vinculin is in the nonactin binding conformation in the cytoplasm. Although the actin-binding conformation of vinculin is enriched in focal adhesions, there were regions of the cell in which the conformation of vinculin in the focal adhesions was not readily distinguished from that in cytoplasm (Fig. 6, compare A with D). Similar results were obtained from analysis of three other cells in separate experiments.

Correlation of vinculin conformation with cellular dynamics

To explore the heterogeneity of vinculin conformation in focal adhesions, we asked whether or not the average conformation

Figure 7. Recruitment of vinculin to the peripheral belt of adhesion puncta during cell spreading is associated with conformational activation of vinculin. Vin−/− MECs transfected with tail probe were replated into a dish (Bioptechs) coated with 20 μg/ml fibronectin heated at 37°C. (A and B) Images of a representative cell at the initial attachment stage ~5 min after plating. (C–F) Images of two representative cells at ~45–60 min after plating. (A, C, and E) Localization of tail probe in each Vin−/− MEC imaged through CFP channel. (B, D, and F) Pseudocolored ratio (FRET/CFP) image of the cells shown in A, C, and E. Notably, the adherent rounded cell (A and B) gave a high FRET ratio, similar to that of cells containing control probe (Fig. 6), indicating that vinculin was largely closed in conformation at the earliest stages of spreading. During spreading, tail probe showed lower FRET ratios correlating with actin-binding conformation in adhesion structures (C–F).

Figure 8. The conformation of vinculin during focal adhesion dynamics. 24 h after plating, a fully spread smooth muscle cell was imaged at time 0, 10, 40, and 45 min. Images were corrected for photobleaching before calculation of the FRET ratio image. (A) The positions of the cell at later time points (green) relative to 0 time point (red) were displayed as color joins of CFP images. (B) Enlargement of the retraction zone from region 1 in A. Notably, as mature focal adhesions disassemble, vinculin loses the actin-bound conformation in a gradient from the tip to the base of the focal adhesions. (C) Enlargement of the focal adhesion assembly zone from region 2 in A. As focal adhesions mature, recruited vinculin does not always adopt the actin-bound conformation.
correlated with cellular activity. In vin−/− MECs that have attached to fibronectin but have not initiated spreading, vinculin is uniformly in the nonactin binding conformation (Fig. 7, A and B). At early phases of isotropic spreading, vinculin is recruited to puncta in the peripheral adhesion ring and to short central adhesions where it is largely in the actin-binding conformation (Fig. 7, C–F). Some cells (Fig. 7, C and D) showed the nonactin binding conformation of vinculin in a segment of the adhesion belt puncta (see bottom edge of cell in Fig. 7, C and D). Unfortunately, phototoxicity associated with acquiring the FRET image precluded correlating these asymmetric regions with subsequent events in cell spreading.

Fully spread smooth muscle cells were more photoreistant, enabling limited time-lapse analysis in cells that had spread for 24 h and were undergoing localized, asymmetric cell shape changes. We found that in retracting/contracting regions of the cell there is loss of the actin-binding conformation before loss of vinculin from focal adhesions (Fig. 8, A and B, region 1, compare 0- and 10-min time points). Interestingly, a gradient of vinculin conformation can be observed in which the actin binding conformation is found at the proximal edge of the gliding or disassembling focal adhesion even out to 45 min.

In contrast, a different region of the same cell shown in Fig. 8 showed recruitment of vinculin to growing focal adhesions (Fig. 8, A and C, region 2). The recruited vinculin was not in the actin-binding conformation at the times observed. This result indicates an additional basis for the heterogeneity of the vinculin FRET ratio in fully spread MECs and also indicates that vinculin recruitment and conformational activation are separate processes.

**Activation of vinculin is required for assembly of vinculin-vinexin complexes in focal adhesions**

The images of the FRET probe in living cells indicate that vinculin can exist in a conformationally open state in focal adhesions. To confirm that the FRET probe reports faithfully on the conformation of native, endogenous vinculin at focal adhesions, we took advantage of our finding that vinexin fails to target to focal adhesions in vinculin null cells (Fig. S4, available at http://...
Vinexin β uses its first and second SH3 domains to bind to the proline-rich region of vinculin (Kioka et al., 1999). However, we found that, in vitro, full-length vinculin binds poorly to the SH3(1–2) of vinexin β. Upon addition of IpaA, the amount of vinculin bound to vinexin increased linearly until binding of IpaA to vinculin reached saturation at ~40% of the vinculin (Fig. 9, A and B). Thus, the activated conformation of vinculin is required to bind vinexin β. We then used this property to confirm the presence of the activated conformation of vinculin in focal adhesions and to establish its functional relevance.

Using a permeabilized cell model prepared from vin−/− MEC, we found that recruitment of exogenous vinexin β to focal adhesions was dependent on the presence of vinculin in the focal adhesions (Fig. 10, A and B). Consistent with a direct interaction between the two proteins, recruitment of vinexin β to focal adhesions depends on the presence of the vinexin binding site because Vh1-851, which lacks the site (Kioka et al., 1999), was unable to recruit vinexin (Fig. 10, C and D). Because vinculin must be activated to bind vinexin β (Fig. 9, A and B), we conclude that some of the endogenous vinculin at the focal adhesions must be in the open or activated conformation and that one functional consequence of this activated conformation is assembly of vinculin–vinexin β complexes at focal adhesions.

**Discussion**

**Development of FRET probes for vinculin conformation illustrates the value of modular protein structure**

FRET efficiency (E) declines rapidly as the inverse of the sixth power of the distance between the chromophores (r) according to the relationship $E = 1 / [1 + (r^6/R_0^6)]$, in which $R_0$ is the calculated Förster distance for a particular donor and acceptor chromophore pair (Clegg, 1992). Therefore, to construct a FRET probe with a high FRET efficiency, the chromophores of CFP and YFP need to be positioned within 2$R_0$ of each other, where $R_0$ is the distance between donor and acceptor at which the FRET efficiency is 50%. The calculated $R_0$ for the CFP/YFP pair is 49 Å (Patterson and Piston, 2000), assuming random orientation between donor emission and acceptor absorbance dipoles.

Initially we placed the donor and acceptor GFP variants at NH$_3$ and COOH termini of vinculin to monitor the conformation of the whole molecule. This construct (CVY) gave a barely detectable FRET signal due to long distance or unfavorable angle (or both) between donor and acceptor chromophores. The GFP proteins are β barrels with dimensions of ~40 × 30 Å and the chromophore sits in the middle of the barrel that has a radius of ~15 Å (Ormo et al., 1996; Yang et al., 1996). In a FRET pair dimer, the two chromophores are separated by ~30 Å, reducing maximum FRET efficiency to 95%. Based on recent crystal structures of intact vinculin (Bakolitsa et al., 2004; Borgon et al., 2004) and vinculin domains (Izard et al., 2004), there is ~40 Å from the amino- to carboxy-terminal end of vinculin. The very low FRET of the CVY construct is consistent with the possibility that as much as 70 Å could separate the chromophore centers. Thus, the FRET of CVY indicates that the crystal structure is a good representation of the inactive conformation of vinculin in solution phase.

Because vinculin is a modular protein (Coutu and Craig, 1988; Price et al., 1989), it offers the possibility that self-folding, single-domain proteins such as the GFP variants could be inserted between modules of the protein, as was done for fibronectin (Ohashi et al., 1999). The success of this approach is facilitated by the fact that the NH$_2$– and COOH-terminal ends of GFP are close to each other and contain short unstructured regions that can serve as linker sequences (Fig. 1 C). Our data show that it is possible for GFP modules to be inserted in the loop between two functional domains of a protein, with appropriate spacers, without interfering significantly with the functions of the molecule. Such probes should be especially useful for monitoring signal-induced functional changes in molecules that lack enzymatic activity, such as the structural cytoskeletal proteins and extracellular matrix molecules. With appropriately characterized probes it may be possible to study directly how cellular mechanical activity affects the structure and function of the ECM and cytoskeletal proteins in living cells, and indeed some pioneering work in this vein has been done (Ohashi et al., 1999).

**The conformation of vinculin in cytoplasm versus focal adhesions: validation of the recruitment and activation hypothesis**

Vinculin is proposed to modulate the junction between ECM and the actin cytoskeleton by linking an integrin and talin complex to the actin network (Horwitz et al., 1986). Because biochemical data show that vinculin can bind neither talin nor actin unless the intramolecular interaction between head and tail is released, the prediction is that vinculin at focal adhesions must adopt an active conformation. Our live cell data shows a striking concentration of the actin-binding conformation of vinculin in focal adhesions as compared with cytoplasm, supporting the central prediction of the aforementioned model for vinculin recruitment and activation. For cytoplasmic vinculin, the FRET data allows us to conclude that the conformation of cytoplasmic vinculin is inactive, at least with respect to its actin-binding potential.

**The conformation of vinculin in focal adhesions: new insights**

Detailed inspection of the cellular FRET data show that the aforementioned model of recruitment and activation of vinculin at focal adhesions is oversimplified. Specifically, in some cells, there was polarity in distribution of the actin-binding conformation of vinculin at focal adhesions. To explore this heterogeneity we asked whether the average conformation of vinculin in an adhesive structure correlated with membrane dynamics. Although our ability to do sequential FRET captures on a cell is limited by phototoxicity and photolability issues, we were able to do limited time-lapse sequences on smooth muscle cells transfected with tail probe. These images revealed that the heterogeneity of vinculin conformation in focal adhesions correlated with regional cell dynamics in fully spread...
cells undergoing localized changes in cell shape. Peripheral focal adhesions were enriched for the actin-binding conformation of vinculin, but showed a loss of that conformation upon membrane retraction and focal adhesion disassembly. Interestingly, there was a gradient of vinculin conformation that proceeded from the distal tip toward the proximal edge of the focal adhesions, with the proximal edge being the last to show the nonactin binding conformation. Thus, one source of the heterogeneity of vinculin conformation in focal adhesions is related to regional retraction of the plasma membrane.

When observing retraction events in a cell it is important to consider whether one is simply observing global retraction induced by phototoxicity. Thus, we analyzed only cells that had regions that were stable or protruding at the same time that another region was retracting. This analysis resulted in finding another source of heterogeneity in vinculin conformation. In stable regions of the cell membrane in which vinculin was being recruited to focal adhesions, the vinculin remained in the nonactin binding conformation. Thus recruitment is not necessarily synonymous with actin binding and a second signal or event must be required to link vinculin to actin.

In previous work, it has been observed that recruitment of vinculin is correlated with adhesive strengthening (Galbraith et al., 2002) and with localized application of tension to cell membrane (Balaban et al., 2001), implying vinculin-mediated strengthening of connections to the cytoskeleton. Because recruitment of vinculin to focal adhesions and binding of vinculin to actin are not always coupled events, one can envision that modulating the actin-binding conformation of vinculin may be a cellular response to changes in the amount of tension experienced by a focal adhesion.

Although we were not able to adequately determine FRET in the tiny, very dim focal complexes at the leading edge of lamellipodia, we were able to analyze vinculin conformation in spreading cells. Before initiation of spreading, vinculin is uniformly in the nonactin-binding conformation. But at early stages of spreading, when a band of vinculin-containing puncta circumscribes the edge of the spreading cell, vinculin in the puncta is largely in the actin-binding conformation. To the extent that this spreading edge mimics an advancing lamellipodium, the result suggests that vinculin in focal complexes at the leading edge would be in the actin-binding conformation, as predicted from the work of DeMali et al. (2002).

The significance of vinculin conformation at focal adhesions

We have presented biochemical and cellular evidence that conformational change of vinculin at focal adhesions is functionally correlated with ligand binding. Not only does localization of vinculin β to focal adhesions require vinculin but this recruitment results from selective binding of vincin β to the conformationally open state of vinculin. These data provide direct evidence that the conformation of vinculin regulates focal adhesion plaque composition by direct protein–protein interactions. Moreover, in establishing that endogenous vinculin also exists in a distinct ligand-binding conformation in focal adhesions, these data confirm that the vinculin FRET probe reports faithfully on sites of vinculin activation in living cells.

Although the physiological function of the vinculin–vincin-β complex is unknown, it is interesting that ectopic expression of vincin β stimulates cell spreading in C2C12 cells (Kioka et al., 1999), as does reexpression of vinculin in vin−/− cells (Xu et al., 1998b). Given the requirement for activated vinculin to localize vincin β to adhesion sites, it is intriguing to speculate that integrin-stimulated recruitment and formation of the vinculin–vincin-β complex at focal adhesions may be part of the machinery that links growth factor–stimulated processes to cell adhesion.

In summary, the vinculin FRET probe and the vincin recruitment experiment have enabled us to demonstrate that vinculin becomes activated when it gets recruited to plasma membrane and that activation is required for particular protein–protein interactions at the focal adhesion. These results establish the relevance of the in vitro biochemical insights to actual cellular events. In addition, the FRET analysis reveals that, in vivo, conformational regulation of vinculin is more complex than the original model (Johnson and Craig, 1995). Specifically, vinculin’s conformation varies amongst focal adhesions in a way that correlates with regional membrane dynamics. This result adds another layer to the heterogeneity of focal adhesions; not only do they vary in the amounts and spatial distribution of components (Zamir et al., 1999) but also in the functional conformation of the vinculin that they contain.

Materials and methods

Reagents and proteins

Actin was extracted from chicken skeletal muscle acetone powder, processed through one cycle of polymerization and depolymerization, and gel filtered through a Sephadex G-150 column according to Pardee and Spudich (1982). Recombinant His-tagged chicken vinculin was purified. GST-vincin-β was expressed in bacteria and purified on glutathione agarose (Smith and Johnson, 1998). pCMX encoding murine vinculin was provided by E. Adamson (Burnham Institute, La Jolla, CA). Details on cloning, expression, and purification of IpaA can be found in the online supplemental material.

Construction of FRET probes

To generate vinculin tail probe, first a 9-bp fragment encoding a Nott site was introduced by mutagenesis into pEFP-C1/vinculin cDNA (chicken) immediately after the codon for aa 883 (Coutu and Craig, 1988) using the Quick Change kit (Stratagene). The cDNA of EYFP (CLONTECH Laboratories, Inc.) minus the stop codon and flanked by Nott was inserted into NotI-digested vinculin. pECPF-N3 vector was constructed as an intermediate vector for generating tail probe. ECFP was PCR amplified with 5′-GGT-ACGcattggcgaaggccg-3′ and 5′-GGCGCCGCTttactgtagctc-3′ to generate 3′ KpnI and 3′ NotI. The product was subcloned into TOPO pCRII and sequenced. The KpnI and NotI fragment of pCRII/ECFP was used to replace the EGFP fragment of pEFP-N3 to generate pEFP-N3. Finally, the EcoRI–Sall fragment containing vinculin-1-883-YFP-vinculin884-1066 was subcloned into pECFP-N3 to generate tail probe (p ECFP-N3/V 1-883 GGR-V 884-1066/VDG). To make the control FRET probe pEYFP-C1/CFPV1,400, a HindIII site was engineered before the ATG site of pET51b(CFPV1,439) and a KpnI site after codon 400 by PCR amplification with 5′-CAAGCTTCGatggtgag-3′ and 5′-GGTGACCTCCGtacagctc-3′. The PCR product was introduced into TOPO pCRII and sequenced. The HindIII–KpnI fragment of CFP-vinculin-1,400 was subcloned into pEYFP-C1 to generate the control plasmid pEYFP-CFPV1,400.

Cell culture and transfection

Cells were cultured on 0.1% gelatin-coated tissue culture plates in DMEM with high glucose and glutamine (MediaTech) supplemented with 10% FCS in a 5% CO2 incubator at 37°C. For cell imaging and FRET analysis, vin−/− MECs, isolated from embryo 5.5−/−, were cultured with home-
made phenol red-free DME (same as aforementioned DME except phenol red-free, 4750 mg/ml NaCl, 370 mg/l NaHCO3, 5958 mg/l Heps, and one-fourth the concentration of vitamins). These media modifications reduced background and autofluorescence. HEK 293 cells were seeded on 0.1% gelatin-coated 100-mm dishes at 3 million per plate; transfection was performed the next day with 3 μg of plasmid DNA using LipofectAMINE/Plus reagent (Invitrogen). HEK 293 cells were lysed 2 d after transfection. Vin−/+ MECs were seeded on 20 μg/ml of fibronectin-coated 35-mm tissue culture dish at 120,000 cells; transfection was performed the next day with 1 to ~1.5 μg of plasmid DNA using LipofectAMINE/Plus reagent.

Cell spreading assay

Vin−/+ MEFs were transfected with tail probe, vinculin, or CFP-YFP chimera. 24 h after transfection, ~400,000 cells were seeded on coverslips coated with polylysine and 20 μg/ml of human fibronectin and incubated in 10% FCS/90% DME (MediaTech) at 37°C for 2 h. Cells transfected with tail probe and CFP-YFP chimera were fixed in 4% PFA in PBS for 20 min, washed twice with PBS, and mounted on a slide with ProLong Gold antifade reagent (Molecular Probes). For cells transfected with untagged vinculin, coverslips were fixed and immunostained with Vin11-5 (Sigma-Aldrich) and rhodamine-conjugated donkey anti–mouse IgG (Jackson ImmunoResearch Laboratories). Axial ratios and cell areas of transfected cells were measured using the segmentation and quantitation tools in IPLab (Scanalytics). Multinucleated cells were excluded from the analysis.

FRET assay of cell lysates

HEK 293 cells were detached with 1 mM EDTA in calcium- and magnesium-free PBS at 37°C after 20 min. The pelleted cells were resuspended in ice-cold hypotonic buffer (20 mM Tris, pH 7.5, 2 mM MgCl2, 0.2 mM EGTA, 0.5 mM ATP, 0.5 mM DTT, and 2× protease inhibitor cocktails I and II (Siliciano and Craig, 1986)) at a density of 2 to ~4 × 105 cells/ml incubated on ice for 20 min, and homogenized manually for 5 min in a DUAL1 21 conical ground glass homogenizer ( Kontes Glass Co.). The lysate was cleared by centrifugation at 4°C, 16,000 g for 10 min. The hypotonic lysate was supplemented with KCl to a final concentration of 100 mM for fluorimetric and actin sedimentation assays. The emission spectrum of fluorescent proteins in the lysate was acquired with a Fluoromax-3 spectrofluorimeter (Jobin Yvon). CFP emission was traced from 460 to 600 nm with excitation at 440 nm, and YFP emission was traced from 510 to 600 nm with excitation at 490 nm. The increment was 1 nm and integration was 0.2 s. The excitation and emission slit widths were 3 and 5 nm, respectively. Lysate from an equal number of untransfected HEK293 cells was used to obtain a background emission spectrum. After subtraction of background, the spectra comprising a single experiment were normalized to the CFP emission of a reference spectrum, as specified in the figure legends.

Determination of the corrected FRET emission ratio and FRET efficiencies

To obtain a number for the corrected FRET emission ratio (a value related to FRET efficiency by Eq. 5) and an estimate of the FRET efficiency itself (as reported in Fig. 4), the sensitized YFP emission (SE) due to FRET was extracted from the raw FRET signal. The raw FRET signal is the EYFP emission (peak at 525) stimulated by excitation of ECFP at 440 nm. It consists of the SE, the emission from direct excitation of EYFP by 440 nm, and the overlap of the ECFP emission spectrum with the EYFP emission spectrum (Erickson et al., 2001). The latter two components of the raw FRET signal are referred to as "spectral cross talk." To determine the amount of spectral cross talk contributed by direct excitation of YFP by excitation at 440 nm, the value RY was determined from a sample containing just YFP by the ratio of the emissions at 525 nm after excitation at 440 and 490 nm. RY was 0.11 in this study. The EYFP emission at 525 nm of the FRET probe after excitation at 490 nm was then multiplied by RY to obtain the YFP cross talk component. The direct excitation YFP cross talk at 525 nm was subtracted from the 525 nm FRET emission at 525 nm to correct for direct excitation of EYFP in the FRET probe by 440-nm excitation.

To determine the contribution of CFP emission to the signal at 525 nm after excitation of the FRET probe at 440 nm, the value RC was determined from a sample containing just CFP by ratioing the emission at 525 nm to that at 475 nm after excitation at 440 nm. RC was 0.43 in this study. The corrected FRET emission ratio (ER in Eqs. 4 and 5) is the emission at 525 nm/emission at 475 nm, after correcting for the EYFP and ECFP cross talk. Corrected FRET emission ratio is SE/FDA. Corrected FRET emission ratio correlates with FRET efficiency; the higher the corrected FRET emission ratio, the stronger the FRET. The expression for FRET efficiency (E%), E% = (F0 − FDA/F0) (Miyawaki and Tsien, 2000), is transformed to Eqs. 1–5 to express FRET efficiency in terms of SE, FDA, and the quantum efficiencies of EYFP and ECFP, which are the experimentally determined parameters.

\[
E% = \frac{nF}{nF + F_{DA}} 
\]

\[
= \frac{SE \times (Q_C/Q_Y)}{SE \times (Q_C/Q_Y) + F_{DA}} 
\]

\[
= \frac{SE \times (Q_C/Q_Y)}{SE \times (Q_C/Q_Y) + F_{DA}} + 1 
\]

\[
= \frac{ER \times (Q_C/Q_Y)}{ER \times (Q_C/Q_Y) + 1} 
\]

\[
= \frac{ER}{ER + (Q_C/Q_Y)} 
\]

F0 and FDA are the donor ECFP emission in the absence or presence of acceptor EYFP, respectively. Because F0 = FDA/QC = SE/QC, the net FRET (nF) = F0 − FDA can be approximated as SE × QC/QY. For the fluorescein of the donor, is approximated as nF + FDA and QC are the quantum efficiencies of EYFP and ECFP. QC is 0.7 and QC is 0.4 (Griesbeck et al., 2001). The quantity SE/FCF is measured as the corrected emission ratio (ER) described above.

Fluorescence microscopy and image processing

2 d after transfection, vin−/+ MECS were detached with trypsin and reseeded on a 20 μg/ml of fibronectin-coated delta T dish (Biopheks) equilibrated to 37°C. Images were captured at 37°C with a fluorescence microscope (model Axiovert 135TV; Carl Zeiss Microimaging, Inc.) equipped with a stage and objective heater (Biopheks), a Cool Snap HQ camera (Photometrics), and Chroma filters. We used a 100× Plan Neofluor objective (Carl Zeiss Microimaging, Inc.) with an NA of 1.3 and collected the images with 2 × 2 binning. The excitation filter used for CFP was D436/10 nm. The emission filter used for CFP was D470/30 nm. The emission filter used for YFP was HG535/30 nm. The beam splitter used was JP4 PC. FRET images were captured with the CFP excitation filter and YFP emission filter. Manipulations of numerical files were done using IPLab software (Scanalytics). Background images of CFP and FRET channels were captured from areas lacking cells on the experimental dish, using the same exposure times as for acquisition of the cell images.

The matched background images were subtracted from the fluorescent images to remove background and uneven illumination. After registration of the images, an empirically determined arithmetic averaging filter (blurring) was applied to the CFP and FRET images. This was done to minimize the presence of artificially high pixel ratios at the edges of focal adhesions. This artifact arises in part because the Airy disk of the CFP image is 11% larger than that of the CFP image. The pixel size on the CoolSnap HQ camera is 6.45 μm. In a 2 × 2 binned image, the diffraction limited spots at 100× for the objective are 3.9 pixels for YFP image and 3.4 pixels for the CFP image; these values are close to the width of the focal adhesions. Thus, a 3 × 3 or 5 × 5 pixel averaging filter was used to minimize the artifact generated by ratiing two different sized images (the FRET and the CFP image) of the same focal adhesions. Empirical selection of the filter was made by comparing the histograms of line segments drawn perpendicular to the long axis of the same focal adhesion region in both the CFP and FRET images. The averaging filters were adjusted such that the shapes of these histograms were as closely congruent as possible.

After the aforementioned manipulations, an image of the FRET ratio at each pixel (FR; Miyawaki and Tsien, 2000) was obtained by arithmetic manipulations of the CFP and FRET images according to the equation FR = lCFP(probe)/lYFP(probe). lCFP(probe) and lYFP(probe) denote the fluorescent intensity of CFP and YFP images at each pixel.

To determine the position of the cell edge, a threshold was selected empirically in the registered CFP image such that it included most of the cell boundary but excluded stray light at the cell edges. The segmentation and analysis tools in IPLab were used to estimate this threshold from the infection point in the slope of a plot of pixel intensity versus pixel number along a line segment made perpendicular to the cell membrane. The sub-
threshold region will appear white in a pseudocolor ratio image, the same as background. The segmented image was converted to a binary image with a threshold region assigned a value of 0 and all those below were assigned a value of zero. The FRET ratio image was multiplied by the binary mask to remove pixels that had an artificially high ratio. The final ratio image was pseudocolored by assigning color values to the ratios. A linear scale from blue to red was used to assign colors to the ratio of vinculin to green/red (high ratio and closed vinculin) to construct a ratio of 0.75 was applied to the display. The background and sub-threshold regions are color-coded white in the final FRET images.

To differentiate cytoplasm from focal adhesions for separate quantitation, two segmented images were generated, one for cytoplasm and one for focal adhesions. Image segmentation was performed in the registered CFP image. Each segmented image was converted to a binary image with the segmented region assigned a value of 1 and nonsegmented region assigned a value of zero. The FRET ratio image was then multiplied by each binary mask image to generate a FRET ratio image for focal adhesion or cytoplasm.

Permeabilized cell assay

Vin−/− MECs were cultured on glass coverslides coated with PLL and 20 μg/ml FN. After 16 h growth in 10% FCS/90% DME, the cells were washed briefly in assay buffer (5 mM MES, pH 6.1, 2 mM MgCl₂, 0.5 mM CaCl₂, 137 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO₃, and 0.1% glucose) and extracted for 1 min with ice-cold assay buffer plus 0.05% trypsin (Calbiochem). After a 30-s rinse in ice-cold assay buffer, coverslips were incubated with the indicated proteins for 15 min at 4°C. Vinculin and Vh (residues 1–851) were used at a concentration of 25 μg/ml and GST-vinexin (residues 1–329, encoding full length vinexin) at 10 μg/ml and GST-vinexin (residues 1–329, encoding full length vinexin) was used at 0.5 μg/ml. The coverslips were washed three times with PBS, cells were fixed in 4% PFA in PBS and stained with a monoclonal antivinculin and affinity-purified anti-GST antibodies. Oregon green fluorescent (Oregon green) was used to label vinculin and meta-vinculin are derived from a single gene by alternative splicing of a 207-base pair exon unique to meta-vinculin. J. Biol. Chem. 267:12845–12850.

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