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Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion

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Cell migration involves many steps, including membrane protrusion and the development of new adhesions. Here we have investigated whether there is a link between actin polymerization and integrin engagement. In response to signals that trigger membrane protrusion, the actin-related protein (Arp)2/3 complex transiently binds to vinculin, an integrin-associated protein. The interaction is regulated, requiring phosphatidylinositol-4,5-bisphosphate and Rac1 activation, and is sufficient to recruit the Arp2/3 complex to new sites of integrin aggregation. Binding of the Arp2/3 complex to vinculin is direct and does not depend on the ability of vinculin to associate with actin. We have mapped the binding site for the Arp2/3 complex to the hinge region of vinculin, and a point mutation in this region selectively blocks binding to the Arp2/3 complex. Compared with WT vinculin, expression of this mutant in vinculin-null cells results in diminished lamellipodial protrusion and spreading on fibronectin. The recruitment of the Arp2/3 complex to vinculin may be one mechanism through which actin polymerization and membrane protrusion are coupled to integrin-mediated adhesion.

Introduction

Cell migration is a multistep process that involves protrusion, adhesion to the underlying substratum, contraction and breaking of older adhesions at the cell rear (Heath and Holifield, 1991; Condeelis, 1998; Kaverina et al., 2002). In order to give rise to productive cell motility, these processes must be coordinated, and perturbation of any of these events has consequences on a cell's ability to migrate. For example, membrane extensions that adhere to the substratum during the extension process, can develop into a dominant lamellipodium and give rise to forward cell movement. However, if the membrane protrusions fail to adhere to the substratum, the extension will fold back upon itself, forming a membrane ruffle that will not support cell migration. Better understanding of how these processes are coordinated is essential for understanding cell migration (Condeelis, 1998).

The ability to extend protrusions is driven by actin polymerization. Proteins that regulate the assembly and disassembly of actin have been localized to the leading edge of migrating cells (for review see Borisy and Svitkina, 2000; Cooper and Schafer, 2000; Pollard et al., 2000; Condeelis, 2001). A key component regulating nucleation of polymerization is the Arp2/3 complex, which is comprised of seven subunits, actin-related protein (Arp)*2, Arp3, p41-Arc, p34-Arc, p21-Arc, p20-Arc, and p16-Arc in mammalian cells (Welch et al., 1997). The Arp2/3 complex becomes activated downstream from Cdc42 and Rac1. GTPases that induce filopodial and lamellipodial extension, respectively. Rac1 activates the Arp2/3 complex via the Scar/WAVE proteins (Machesky and Insall, 1998; Miki et al., 1998), whereas Cdc42 binds and activates Wiskott-Aldrich Syndrome protein (WASP) or N-WASP via a conserved acidic domain (Rohatgi et al., 1999). Activated WASP binds and induces a conformational change in the Arp2/3 complex that permits the Arp2 and Arp3 subunits of the complex to form the template for the daughter filament (Robinson et al., 2001). Nucleation of actin polymerization in this fashion triggers the addition of actin monomers close to the membrane. In addition, the Arp2/3 complex also binds to the sides of existing filaments (Svitkina and Borisy, 1999). This binding to F-actin activates the Arp2/3 complex in a manner coopera-

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*Abbreviations used in this paper: Arp, actin-related protein; ECM, extracellular matrix; EGFR, EGF receptor; FAK, focal adhesion kinase; FN, fibronectin; HLA, human leukocyte antigen; MEF, mouse embryo fibroblast; PI3K, phosphatidylinositol-3-kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; VASP, vasodilator stimulated phosphoprotein; WASP, Wiskott-Aldrich Syndrome protein.

tive with the activation by the WASP/Scar family of proteins (Higgs and Pollard, 2001). Nucleation of existing filaments in this manner forms a highly branched, dendritic network (Mullins et al., 1998; Svitkina and Borisy, 1999; Blanchoin et al., 2000; Pantaloni et al., 2000; Bailly et al., 2001), and this branching has been shown to be essential for lamellipodial extension (Bailly et al., 2001). The continued addition of actin monomers in this branched network is thought to provide the driving force for the extension of membrane that occurs during lamellipodial protrusion.

Another group of proteins localized and activated in extending lamellipodia are the integrins, cell surface receptors that serve as transmembrane links between the extracellular matrix on the outside and cytoskeleton on the inside of the cell (Nishizaka et al., 2000; Kiosses et al., 2001). The integrins bind a number of cytoskeletal proteins, including talin, α-actinin, and filamin, all of which bind F-actin (Hemler, 1998; for review see Critchley, 2000; Liu et al., 2000; Zamir and Geiger, 2001a,b). Additionally, both talin and α-actinin bind vinculin, another actin-binding protein (Jockusch and Isenberg, 1981; Johnson and Craig, 1995a). Together, these observations have led to the idea that multiple protein complexes mediate attachment of integrins to actin filaments.

In order for cells to migrate effectively, it is important that protrusive activity is linked to adhesion. Potential mechanisms by which this may occur are via integrin engagement activating Rac1 (Clark et al., 1998; Price et al., 1998; Del Pozo et al., 2000) or recruiting activated Rac1 to these sites (Del Pozo et al., 2002). The local activation of Rac1 at the leading edge of cells (Kraynov et al., 2000) is thought to stimulate the Arp2/3 complex in this region, thereby triggering actin polymerization and growth of the dendritic network of actin filaments in the lamellipodium. It seemed to us that besides this signaling pathway linking adhesion to actin polymerization, there may also exist a structural link between integrin engagement and actin polymerization, so that newly engaged integrins might couple to the Arp2/3 complex. Here, we have identified an interaction between vinculin and the Arp2/3 complex that occurs transiently in response to signals that trigger membrane protrusion. This interaction is regulated by phosphatidylinositol-3-kinase (PI3K) and Rac1 activity, and is sufficient to recruit the Arp2/3 complex to new sites of integrin clustering.

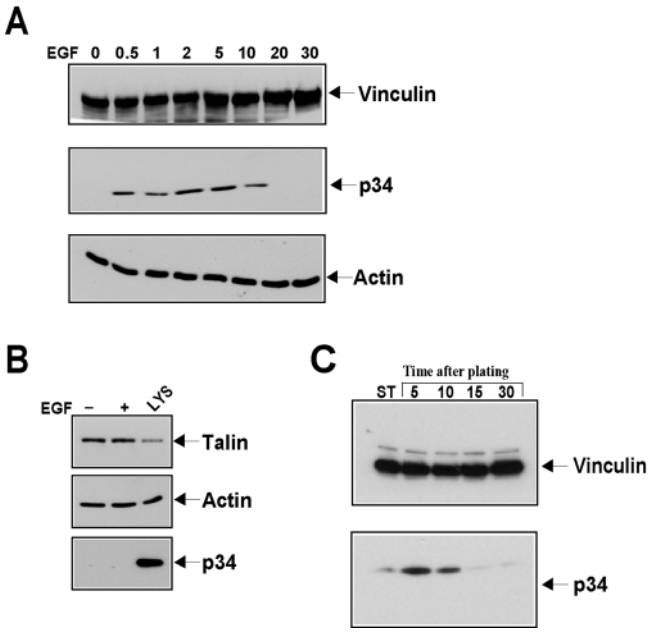


Figure 1. **Factors that trigger membrane protrusion induce the association of the Arp2/3 complex with vinculin.** (A and B) Growth factor treatment of cells. Serum-starved A431 cells were stimulated with buffer (0) or human EGF (100 ng/ml) for the times indicated (min). The cells were lysed and vinculin or talin was immunoprecipitated. 20% of resulting immunoprecipitates were subjected to Western blot analysis with antibodies against vinculin (A) or talin (B), 10% was used for the actin blots (A and B, bottom), and 70% was used for the blots of p34 subunit of the Arp2/3 complex (p34, bottom). LYS denotes a sample of whole cell lysate. (C) Cells spreading on FN. Hs68, human foreskin fibroblasts, were plated into each well of a FN-coated 6-well plate in serum-free DME. The cells were centrifuged onto the dish and allowed to recover at 37°C for the times indicated. The cells were lysed, vinculin was immunoprecipitated, and cells were blotted as described in A and B. ST, stationary cells that had been plated for 4 h on a similar concentration of FN.

Results

Transient binding of the Arp2/3 complex to vinculin

Migration of many vertebrate cells requires both actin polymerization and adhesion to the substratum. In this study we examined whether there is a possible association of the Arp2/3 complex with one of the proteins found at the cytoplasmic face of matrix adhesions (for review see Critchley, 2000). In preliminary work, we looked for possible coelution of Arp2/3 with talin, vinculin, or α-actinin in column fractions of platelet lysates. We observed coelution of the Arp2/3 complex with vinculin and found Arp2/3 cosedimenting with vinculin immunoprecipitated from these fractions (unpublished data).

To explore the interaction of vinculin with Arp2/3, we examined their coimmunoprecipitation from cultured fibroblasts. Only low levels of association were detected (unpublished data). To examine whether the interaction might be stimulated when cells are induced to extend membrane ruffles and lamellipodia, we looked for coimmunoprecipitation from A431 cells, which form large membrane ruffles when stimulated with EGF (Brunk et al., 1976; Diakonova et al., 1995). EGF induced a transient association of the Arp2/3 complex with vinculin. This peaked within 30 s and was diminished by 15–20 min, depending on the experiment (Fig. 1 A). The time at which the Arp2/3 complex was bound to vinculin is similar to the time frame for induction of ruffles by EGF in A431 cells (unpublished data). In addition, recruitment of the Arp2/3 complex to vinculin did not correlate with actin binding, which was constant with it being independent of actin binding. This interaction was specific for vinculin, because talin, another actin binding protein, was unable to recruit the Arp2/3 complex in response to EGF stimulation (Fig. 1 B). Membrane protrusion is also stimulated by cell spreading on extracellular matrix (ECM). Using Hs68 cells, we investigated the association of the Arp2/3 complex with vinculin that was immunoprecipitated from stationary cells or from those spreading on a fibronectin (FN) matrix. For this experiment, adhesion and spreading were ac-

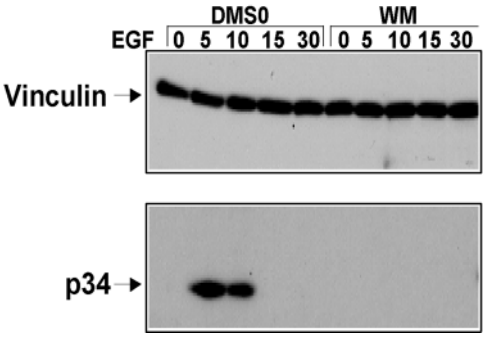


Figure 2. **PI3K is required for recruitment of the Arp2/3 complex to vinculin.** Effect of inhibition of PI3K by wortmannin (A) on recruitment of the Arp2/3 complex to vinculin. Serum-starved A431 cells were pretreated with DMSO or 100 nM wortmannin (WM) for 30 min and then left resting (0) or stimulated with EGF for the times indicated (min). Vinculin was immunoprecipitated and blotted for vinculin or p34-Arc as described in Fig. 1.

celerated by centrifuging the cells onto FN-coated coverslips. A low amount of the Arp2/3 complex was detected associating with vinculin in stationary cells, and this was greatly increased as cells spread on FN (Fig. 1 C). Similar to the EGF-treated cells, the interaction was transient and over by 15–20 min, at which time spreading was largely complete.

Binding of the Arp2/3 complex to vinculin is regulated by PI3K and Rac1

These results suggest that there is a signal that induces binding of the Arp2/3 complex to vinculin. One signaling pathway activated downstream of both EGF stimulation and integrin-mediated adhesion is the PI3K pathway (Vanhaesebroeck and Waterfield, 1999). To address whether PI3K activity affects the interaction of the Arp2/3 complex with vinculin, we explored the effects of PI3K inhibitors, wortmannin and LY294002, on EGF-induced association of these proteins. We found that 100 nM wortmannin (Fig. 2 A) or 50 nM LY294002 (unpublished data) blocked EGF-dependent recruitment of the Arp2/3 complex to vinculin.

PI3K has been identified as an upstream activator of the small GTP-binding protein Rac1 (Ma and Abrams, 1999), and Rac1 becomes activated in response to growth factor stimulation (Hawkins et al., 1995; Sander et al., 1999; Liu and Burridge, 2000; Zondag et al., 2000) and integrin-mediated adhesion (Price et al., 1998). Additionally, Rac1 stimulates actin polymerization via the Arp2/3 complex (for review see Higgs and Pollard, 2001). We explored the role of Rac1 activation in recruitment of the Arp2/3 complex to vinculin. EGF induced an increase in Rac1 activity that persisted for the first 10–15 min of EGF stimulation, coincident with the Arp2/3 complex binding to vinculin (Fig. 3 A). Expression of dominant negative Rac1 in A431 cells abolished Rac1 activation by EGF and prevented binding of the Arp2/3 complex to vinculin induced by EGF (Fig. 3 A). Similarly, dominant negative Cdc42 impaired binding of the Arp2/3 complex to vinculin, albeit to a lesser extent (unpublished data). The observation that Rac1 activates Scar/WAVE, itself an activator of the Arp2/3 complex (Machesky and Insall, 1998; Miki et al., 1998, 2000), is consistent with

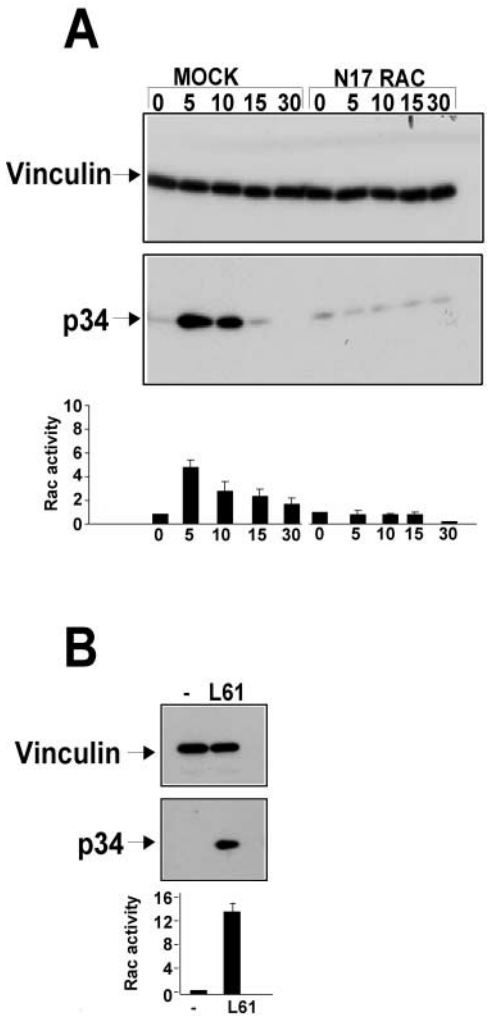


Figure 3. **Rac1 is necessary and sufficient for recruitment of the Arp2/3 complex to vinculin.** (A) Effect of dominant negative Rac1 on the recruitment of proteins to vinculin. A431 cells were transiently transfected with vector alone (MOCK) or myc-tagged N17Rac1 (N17Rac1), allowed to recover for 12 h, and then serum starved for an additional 18–24 h in DME. Cells were stimulated with 100 ng/ml of EGF as in Fig. 1 for the times given (min), and vinculin immunoprecipitated. (B) Effect of constitutively active Rac1 on the recruitment of proteins to vinculin. A431 cells were transiently transfected with AU-tagged L61Rac1 (L61), allowed to recover for 12h, and then serum starved for an additional 18–24 h in DME. Vinculin immunoprecipitates were blotted for p34. The graphs beneath each panel indicate the levels of GTP-bound Rac1. Each number represents the mean fold activation ± SEM in Rac1 activity over unstimulated cells from three independent experiments.

the Arp2/3 complex needing to be activated to bind vinculin. Expression of constitutively active Rac1 (L61Rac1) or loading of cells with GST-L61Rac1 triggered association of the Arp2/3 complex with vinculin (Fig. 3 B). These results suggest that in response to stimuli that trigger membrane protrusion, the Arp2/3 complex is recruited to vinculin in a PI3K- and Rac1-dependent fashion.

PI3K and Rac1 trigger numerous downstream signaling events. At least two of the events downstream of Rac, PIP_2 production and Scar/WAVE activation, potentially could regulate the association of the Arp2/3 complex with vinculin. PIP_2 has been shown to induce a conformation change

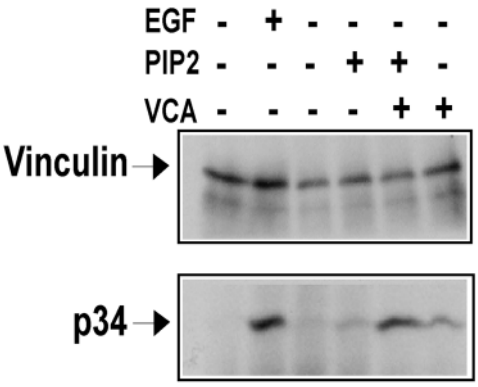


Figure 4. **PIP_2 and GST-VCA induce binding of the Arp2/3 complex to vinculin.** Serum-starved A431 cells were stimulated with buffer alone or with 100 ng/ml EGF for 5 min. The cells were lysed in 500 μl vol as described in the text, and the lysates were incubated in the presence of no exogenous proteins or 30 μg of GST, 10 mM PIP_2, 10 mM PIP_2 +30 μg GST–VCA, or 30 μg of GST–VCA for 20 min at 4°C. Vinculin immunoprecipitates were prepared and analyzed as described above. In three independent experiments, compared with the level of Arp2/3 binding to vinculin in response to 5 min EGF stimulation (100%), the level of Arp2/3 complex recruitment in response to the various stimuli was 13% ± 7% (PIP_2), 27% ± 15% (GST–VCA), and 94% ± 11% (PIP_2 + GST–VCA).

in vinculin that allows for increased association with binding partners such as talin and VASP (Gilmore and Burridge, 1996; Weekes et al., 1996; Huttelmaier et al., 1998). Scar/WAVE activation exposes its COOH-terminal VCA domain. In turn, this domain binds to the Arp2/3 complex, thereby activating it (Miki et al., 1998; Machesky et al., 1999). To address whether PIP_2 production or Arp2/3 complex activation is important for binding of the Arp2/3 complex to vinculin, we immunoprecipitated vinculin from cell lysates incubated in the presence or absence of exogenous PIP_2 or GST-VCA domain. We observed that binding of the Arp2/3 complex to vinculin was stimulated slightly by PIP_2 and more by the VCA domain (Fig. 4). Only in the presence of both PIP_2 and GST-VCA was the recruitment of the Arp2/3 complex to vinculin restored to the level seen in an EGF-stimulated cell. An alternative hypothesis is that these two pathways are needed to give rise to an accumulation of F-actin that would then potentiate the interaction of the Arp2/3 complex with vinculin. We examined the effect of latrunculin treatment on the recruitment of the Arp2/3 complex to vinculin. We found that the latrunculin treatment of cells disassembled stress fibers but had no effect on Arp2/3 complex recruitment to vinculin (unpublished data). Taken together, these results suggest that both activation of the Arp2/3 complex and the conformational change in vinculin contribute to the interaction of these two proteins.

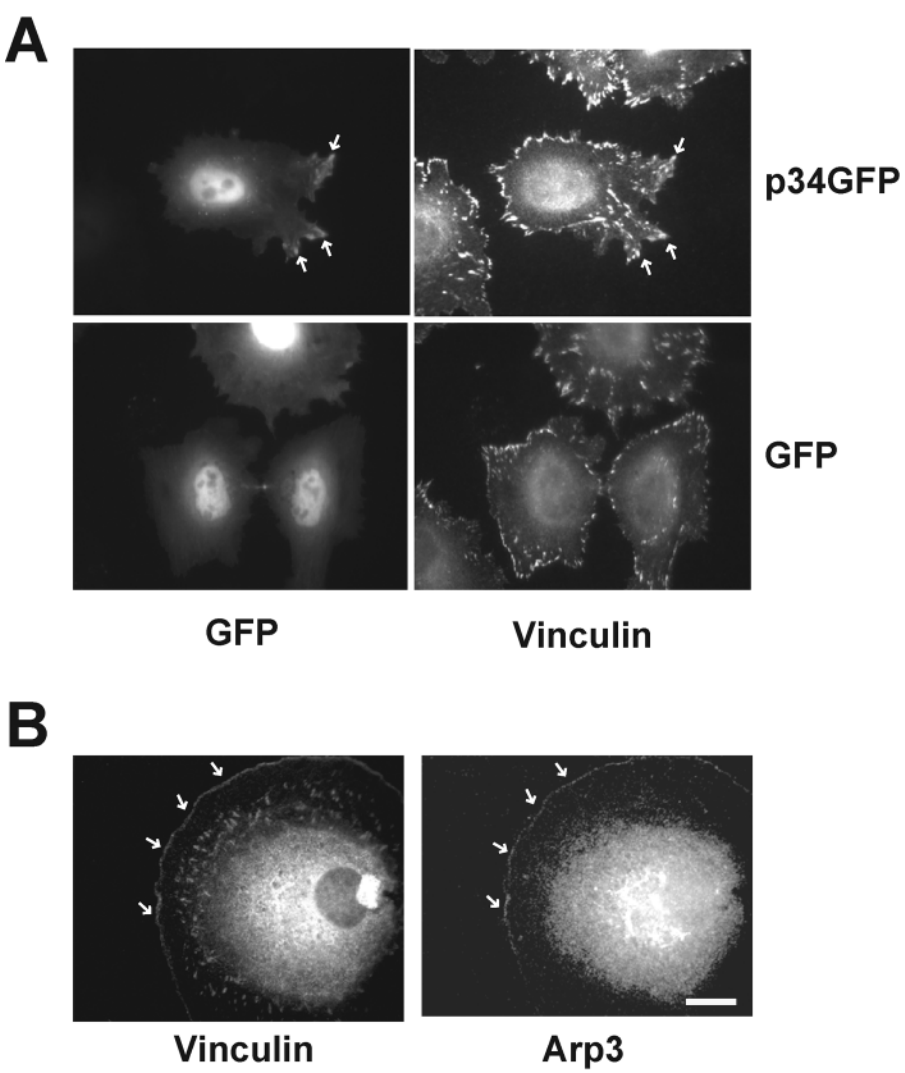
Recruitment of the Arp2/3 complex to early sites of integrin engagement

To explore whether vinculin provides a link between integrins and the Arp2/3 complex, we investigated whether the Arp2/3 complex localizes to sites of integrin aggregation. For these experiments, we used a GFP fusion protein of the p34-Arc subunit of the Arp2/3 complex (p34GFP) and antibodies against vinculin. In cells plated on a FN-coated surface, we did not detect the Arp2/3 complex in focal adhesions revealed by staining for vinculin (Fig. 5 A). However, p34GFP and vinculin colocalized in structures that we interpret as focal complexes at the leading edge (Fig. 5 A, arrows). Focal complexes are the small adhesive structures induced by Rac or Cdc42 activation, that develop at the cell periphery and that often mature into focal adhesions (Nobes and Hall, 1995). Microinjection of cells with 61LRac and the Rho inhibitor, C3, revealed colocalization of Arp3 with vinculin in small structures at the cell periphery (Fig. 5 B). These observations support the biochemical data indicating that the association of the Arp2/3 complex with vinculin is transient and only seen in the most recently formed sites of adhesion at the cell periphery.

The transient association of the Arp2/3 complex with sites of integrin engagement was investigated further using FN-coated beads applied to cells at different time points. Recruitment of the Arp2/3 complex to beneath the beads was visualized again using p34GFP. Early after depositing FN-coated beads on cells, 74% of the beads recruited p34GFP beneath the beads (Fig. 5 C). When GFP alone was expressed, this showed no significant accumulation beneath FN-coated beads. Recruitment of p34GFP did not occur to beads coated with polylysine and the effect was specific for the β1-integrins, as human leukocyte antigen (HLA) was not recruited to the beads. We tested if the recruitment of the Arp2/3 complex to β1-integrins could potentially be mediated by vinculin by examining if the recruitment was transient or PI3K dependent. We found that recruitment of p34GFP was decreased to background levels (~10%) at 35 min after adhesion of the beads to cells (Fig. 5 D). Treatment of cells with 100 nM wortmannin prevented the recruitment of the Arp2/3 complex to the FN-coated beads (Fig. 5 D). Together, these results suggest that a transient association of the Arp2/3 complex with vinculin recruits this complex to new sites of integrin engagement, but not to regions where integrins have been engaged for a long time such as focal adhesions. Further evidence supporting this idea comes from experiments using a vinculin mutant unable to bind the Arp2/3 complex (see below; see Fig. 10).

The Arp2/3 complex binds directly to vinculin amino acids 850–881

To explore the role of this interaction between the Arp2/3 complex and vinculin, we considered generating a mutant form of vinculin that could not bind the Arp2/3 complex. This would allow us to examine the phenotype of this vinculin when expressed in vinculin-null cells. We mapped the Arp2/3 binding site on vinculin using vinculin fragments expressed as GST fusion proteins (Fig. 6 A). Several overlapping fragments bound to the Arp2/3 complex and identified amino acids 850–881 as the critical domain (Fig. 6 B). Because these experiments were carried out using crude platelet extracts, we determined whether the association of Arp2/3 complex depended on the ability of vinculin to recruit actin. We examined whether the Arp2/3 complex was retrieved from platelet extracts using a vinculin fusion protein containing an intact actin binding site (Vin 881–1066), but lacking an Arp2/3 binding site. The converse experiment was also performed with a vinculin fusion protein contain-



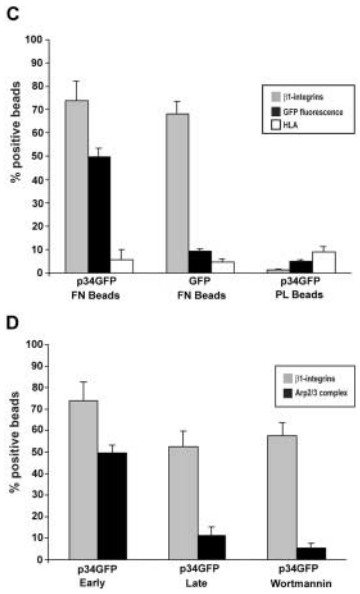


Figure 5. **Recruitment of the Arp2/3 complex to sites of integrin aggregation.** (A) REF52 cells expressing GFP or p34GFP were plated on FN-coated coverslips for 1.5 h at 37°C. (B) Serum-starved Swiss3T3 cells were plated on FN-coated coverslips, and microinjected with 61LRac and C3. The cells were examined by immunofluorescence using antibodies against vinculin and the Arp3 subunit of the Arp2/3 complex. Sites of colocalization of the Arp2/3 complex with vinculin (focal complexes) are indicated with arrows in A. Bar, 20 μm. (C and D) Hela cells expressing GFP or p34GFP were plated on collagen and presented with beads coated with fibronectin (FN) or poly-lysine (PL beads) for 20 min (C and D marked early) or 35 min (D, late). Cells were stained for β1 integrins or HLA. The percentage of beads able to recruit the β1 integrins (gray bars), the GFP fusion proteins (black bars), and/or HLA (white bars) was scored using immunofluorescence. In some experiments, cells were preincubated with 100 nM wortmannin (D, wortmannin) for 30 min before incubation with the beads.

ing an intact Arp2/3 binding site (Vin 811–881), but lacking intact actin binding site. Fusion proteins that bound actin were not able to recruit the Arp2/3 complex and vice versa (Fig. 6 C). We conclude that actin binding to vinculin is not required for binding of the Arp2/3 complex.

We next examined whether the association of the Arp2/3 complex to vinculin was direct using purified proteins. Two GST fusion proteins containing vinculin residues 1–811 and 811–881 were incubated with pure Arp2/3 complex. These experiments demonstrated direct binding between the Arp2/3 complex and residues 811–881 of vinculin (Fig. 7).

To define further which amino acids within this sequence of vinculin were important for binding of the Arp2/3 complex, we made a number of mutations in evolutionarily conserved residues in this region. Two of these mutants, P876A and P878A, were unable to bind the Arp2/3 complex but were able to recruit ponsin, vinexin and VASP, other proteins that have been shown to bind to this region of vinculin (Fig. 6 D).

To ensure that the mutation was capable of interferring with binding of the Arp2/3 complex in the context of full length vinculin, we subcloned the mutation into full length vinculin and assessed its ability to recruit the Arp2/3 complex in vinculin immunoprecipitates. In response to FBS stimulation, the mutant was unable to recruit the Arp2/3 complex to wildtype levels (Fig. 6 E). This impaired binding was not due to differences in the levels of expression as both the WT and mutant vinculin were expressed at similar levels (unpublished data).

We next examined whether the mutant could carry out functions normally ascribed to vinculin. We examined whether the mutant vinculin was able to localize to and concentrate in focal adhesions when expressed in the vinculin-null cells. We found a normal distribution of vinculin in focal adhesions in cells reexpressing the WT or mutant vinculin (Fig. 8). No vinculin staining was detected in the vinculin null cells transfected with the vector alone.

Recruitment of the Arp2/3 complex to vinculin is important for some integrin-mediated events

Previous studies demonstrated that fibroblasts isolated from embryos lacking vinculin have reduced adhesion, spreading

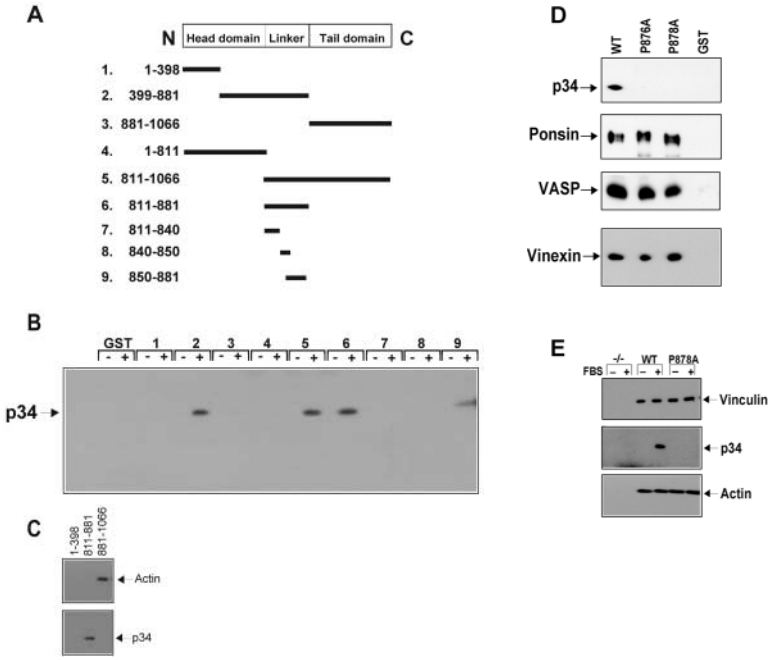


Figure 6. **Mapping the Arp2/3 complex binding site of vinculin.** (A) Linear schematic is shown of vinculin and the fragments of vinculin as fusion proteins of GST (GST–VIN). (B and C) Association of the Arp2/3 complex with GST–VIN proteins. Thirty micrograms of fusion protein attached to beads were incubated for 1.5 h in the absence (−) or presence (+) of 10 μl of a crude platelet fraction. The beads were sedimented, washed, and subjected to Western blot analysis as described. Note that in C, the binding of the Arp2/3 complex to vinculin fragments does not involve binding to actin. (D) Substitution of proline 878 or 876 with alanine prevents binding of the Arp2/3 complex to vinculin in vitro. 30 μg of GST or GST fusions of VIN 811–881, VIN 811–881 with a proline to alanine mutation at 876 (P876A) or at 878 (P878A) were examined for their ability to retrieve the Arp2/3 complex, ponsin, vinexin, or VASP from cell lysates obtained from platelets, MDCK, C2C12, or A431 cells, respectively. Different cell types were used for preparing the lysates because of failure to detect the antigens in some cell types. (E) Full-length vinculin harboring a proline to alanine mutation at 878 prevents recruitment of the Arp2/3 complex, but has no effect on actin binding. Serum-starved, Vin−/− MEFs expressing an empty vector (−/−), full-length vinculin (WT), or full-length vinculin with a P878A substitution (P878A) were left resting (−) or stimulated (+) with 10% FBS for 5 min, vinculin immunoprecipitates were obtained and analyzed.

and formation of lamellipodia on FN, but migrated twofold faster compared with WT cells (Xu et al., 1998b). We assessed the ability of vinculin-null mouse embryo fibroblasts (MEFs) reexpressing WT, mutant vinculin, or vector alone to form lamellipodia in response to plating cells on fibronectin for 45 min. We found that the cells reexpressing WT vinculin were able to form robust lamellipodia. In contrast, both the control and mutant vinculin-reexpressing cells showed fewer, smaller lamellipodial extensions and appeared less spread than the WT reexpressers (unpublished data). By 2 h after plating on FN, the cells reexpressing WT vinculin were broad and flat while both the null and mutant reexpressing cells were more elongated and thin (Fig. 8 A). To quantify the difference, we measured the length/width ratio in the three cell types. This data confirmed that the null and mutant vinculin-reexpressing cells were significantly impaired in their ability to spread to WT levels (Fig. 8 B).

Because vinculin has also been implicated in cell adhesion and migration, we examined if the mutant vinculin was impaired in its ability to regulate these events. We assessed the ability of the vinculin-null, WT, or mutant reexpressing cells to adhere to FN. We observed that cells reexpressing both the WTand mutant vinculin adhered to similar extents, which were 1.9- and 1.7-fold more than the vinculin-null cells, respectively (Fig. 9 A). Migration assays were performed using Transwell filters in which the lower surface of the filter was coated with FN. Migration was allowed to proceed for 6.0 h. Consistent with previous findings, we found that the vinculin null cells migrated faster than the cells reexpressing WT vinculin (Xu et al., 1998b). Furthermore, the mutant vinculin lacking an intact Arp2/3 complex binding site was indistinguishable from WT cells. Thus, ablation of the binding site for the Arp2/3 complex on vinculin had no effect on cell adhesion or migration through a Transwell filter, although it did affect development of lamellipodia and cell spreading.

We next wanted to examine if the integrin-mediated defects we observed were due to impaired recruitment of the Arp2/3 complex to sites of integrin clustering. Using p34GFP, we assessed the distribution of the Arp2/3 complex beneath FN-coated beads adhering for 15 min onto vinculin-null cells reexpressing WT or mutant vinculin. We observed that 59% of the beads recruited WT vinculin, and that 33% of these beads were positive for the Arp2/3 complex (Fig. 10). In contrast, cells expressing recruited vinculin at a similar level to the beads, but only 6% of the total beads recruited the Arp2/3 complex. Preventing binding of the Arp2/3 complex to vinculin significantly decreased recruit-

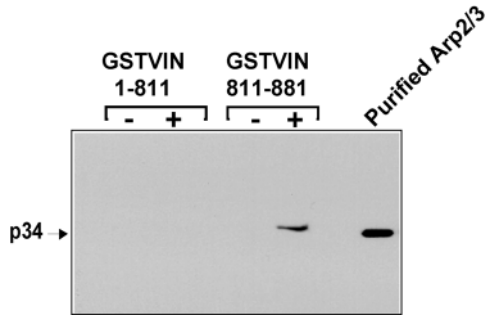
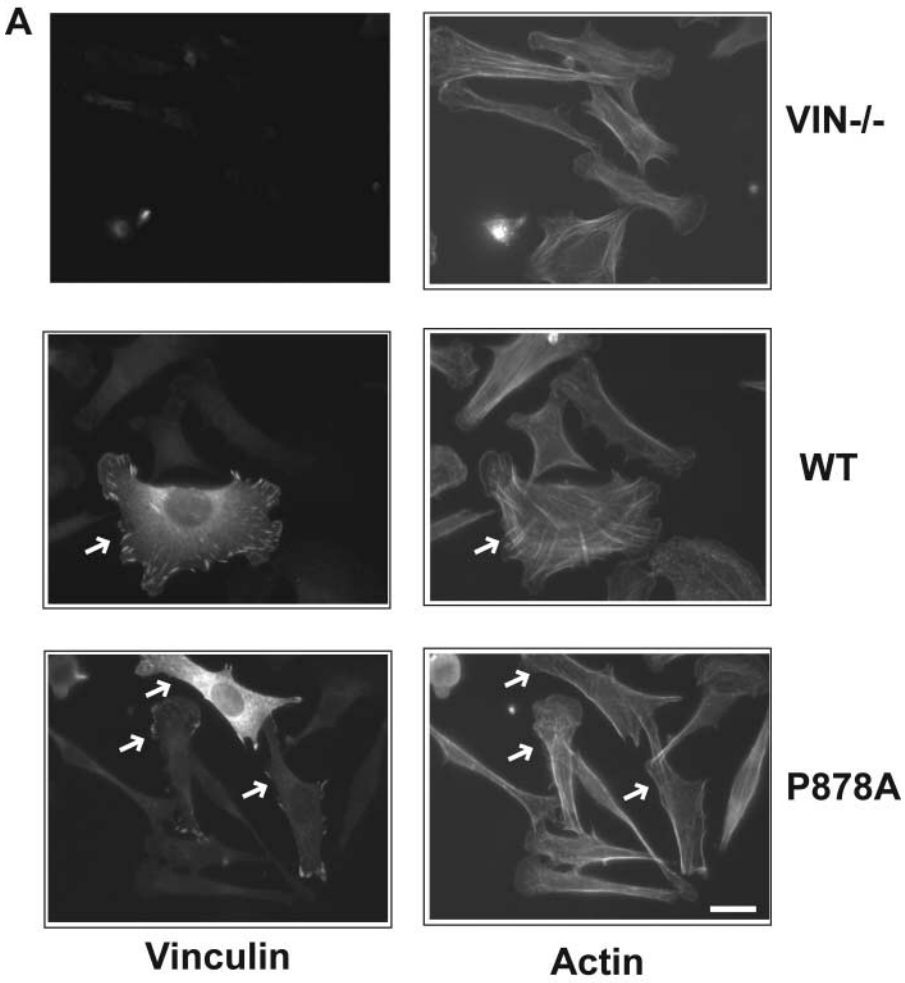


Figure 7. **Binding of the Arp2/3 complex to vinculin is direct**. Vinculin fusion proteins attached to beads were incubated with 2 μg of purified, human platelet Arp2/3 complex for 1.5 h at 4°C. The beads were washed and the pellets were examined for the ability of the fusion proteins to bind the Arp2/3 complex. Purified Arp2/3 denotes a sample of Arp2/3 complex purified from platelets.



B

Cell Type	Ratio of length:width
VIN-/-	3.6 ± 1.1
WT	1.7 ± 0.4
P878A	4.7 ± 1.5

Figure 8. **A vinculin mutant unable to associate with the Arp2/3 complex is impaired in lamellipodial extension and spreading on fibronectin.** Vinculin null cells reexpressing vector alone (Vin−/−), WT or mutant vinculin (P878A) were allowed to spread onto 50 μg/ml of FN for 120 min (A) and then examined by immunofluorescence with antibodies against vinculin or using Alexa-conjugated phalloidin. The white arrows indicate the transfected cells. Bars, 20 μm. (B) the extent of spreading was quantified by expressing the ratio ± SD of the length/width of 50 cells at the 120-min time point. The WT reexpressing cells are significantly more spread than either the null or mutant reexpressers ($P < 0.001$).

ment of the Arp2/3 complex to sites of integrin aggregation. These results support the idea that binding to vinculin serves to localize the Arp2/3 complex to sites of new β1-integrin engagement.

Discussion

Two of the important steps in the complex process of cell migration are adhesion to the underlying substratum and membrane protrusion. Membrane protrusion is driven by the Arp2/3 complex nucleating actin polymerization, whereas adhesion to ECM occurs largely through the integrin family of ECM receptors. These two components of cell migration, adhesion and protrusion, are functionally linked. For example, it has been known for a long time that plating cells on an ECM-coated surface stimulates membrane protrusion as cells spread. Moreover, integrin-mediated adhesion has been shown to activate Rac1 (Clark et al., 1998; Price et al., 1998; Del Pozo et al., 2000, 2002). Rac1 is known to signal to the Arp2/3 complex via the Scar/WAVE family of proteins (Takenawa and Miki, 2001), and to stimulate formation of lamellipodia and membrane ruffles (Ridley et al., 1992; Nobes and Hall, 1995). Recently, evidence for integrin-mediated release of activated Rac1 from the sequestering protein, RhoGDI, has been provided (Del Pozo et al., 2002). Additionally, Sturge and coworkers (Sturge et al., 2002) have described an integrin-dependent mechanism for activation of N-WASP, an activator of the Arp2/3 complex that nucleates actin polymerization. Together these observations indicate that integrin-mediated adhesion can signal to the Arp2/3 complex, thereby promoting membrane protrusion.

At the outset of this work, we were motivated to look for proteins that might directly couple matrix adhesion to nucleation of actin polymerization. We have found here a transient interaction of the Arp2/3 complex with vinculin that is stimulated by matrix adhesion and by growth factor treatment. The interaction of the Arp2/3 complex with vinculin only occurs at sites of newly engaged integrins and is not detected in mature adhesive structures, such as focal adhesions. Together these observations lead us to suggest that this interaction is one way that the actin nucleating machinery that

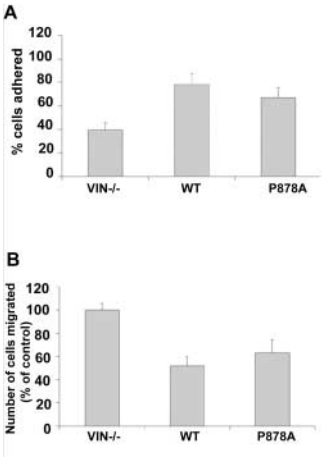


Figure 9. **Recruitment of the Arp2/3 complex to vinculin is not required for cell adhesion or cell migration.** Vinculin-null cells reexpressing vector alone (Vin−/−), WT vinculin, or mutant vinculin (P878A) were examined for the ability to adhere to 50 μg/ml FN (A) or migrate through Transwell filters (B). The mean number of cells expressing vector alone that migrated through the filter was set to 100%. The relative number of cells/field ± SEM was calculated from the means of three independent experiments.

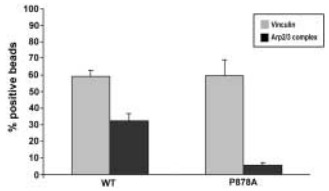


Figure 10. **Mutation of the proline 878 ablates recruitment of the Arp2/3 complex to FN-coated beads.** Vinculin-null cells reexpressing WT vinculin or vinculin with a mutated Arp2/3 complex binding site (P878A) were presented with beads coated with FN (50 μg/ml) for 15 min. The percentage of beads able to recruit vinculin (light gray) and p34GFP (dark gray) was scored by immunofluorescence microscopy.

drives membrane extension can be coupled to new sites of cell–ECM adhesion. Further evidence supporting this idea comes from our studies of vinculin-null cells reexpressing either WT vinculin or a mutant form of vinculin that does not bind Arp2/3. Whereas the WT vinculin restores normal formation of lamellipodia, the mutant unable to bind Arp2/3 does not (see below).

Vinculin is a prominent component of focal adhesions and it is notable that the Arp2/3 complex has not been detected in these structures either in previous localization studies (Welch et al., 1997) or in this study using a p34GFP chimera. Many vinculin-binding proteins have been identified, and so far the Arp2/3 complex is unusual among them in not colocalizing with vinculin in focal adhesions. The Arp2/3 complex is prominent in the leading edge of cells, where vinculin is also found, but much less prominently than its localization in focal adhesions. This implies that most of the vinculin in the cell is not associated with the Arp2/3 complex, which is what we have found biochemically. In quiescent, stationary cells, the amount of Arp2/3 coprecipitating with vinculin is very small and often difficult to detect. Indeed, we would probably have missed this association if we had not first observed it occurring in crude platelet fractions. With hindsight, we suspect that some of the platelets used to prepare these fractions had become activated thereby stimulating this interaction between vinculin and the Arp2/3 complex.

The transient nature of the interaction of the Arp2/3 complex with vinculin is striking. We observed that it is induced by both adhesion to ECM and by stimulating with EGF. Pursuing the pathways leading to this association, we determined that it was blocked by agents that inhibit PI3K or Rac1 activity. The interaction of the Arp2/3 complex with vinculin could be triggered by activated Rac1. In many systems, PI3K is upstream of Rac1 activation and Rac1 has been shown to stimulate the Arp2/3 complex via the WASP/Scar family of proteins (Aspenstrom et al., 1996; Symons et al., 1996; Miki et al., 1998). Upon activation, the WASP/Scar proteins undergo a conformational change exposing the COOH-terminal VCA domain that binds and activates the Arp2/3 complex. Thus, one way that Rac1 might promote binding of the Arp2/3 complex to vinculin would be through activation of the Arp2/3 complex. We tested this possibility by adding the recombinant VCA domain to cell lysates before immunoprecipitating vinculin. We found that this significantly increased the amount of Arp2/3 binding to vinculin, confirming that the interaction required activated Arp2/3 complex. However, we also found that the binding could be stimulated even further by addition of PIP_2 to the lysates. PIP_2 binds to vinculin (Johnson and Craig, 1995b), inducing a conformational change that dissociates the head domain from the tail domain (Johnson and Craig, 1995b; Gilmore and Burridge, 1996; Weekes et al., 1996; Steimle et al., 1999). This exposes binding sites on vinculin for several of its binding partners. Together, these results suggest that the interaction of the Arp2/3 complex with vinculin requires both activation of Arp2/3 and the open vinculin conformation.

We were curious whether the interaction of vinculin with Arp2/3 stimulated its actin nucleating activity. However, using actin polymerization assays, we found no evidence that vinculin is able to induce nucleation via the Arp2/3 complex. Furthermore, vinculin does not bind the Arp2/3 complex via an acidic domain like other Arp2/3 activators. We conclude that the interaction serves to localize the activated Arp2/3 complex to sites of new integrin engagement. The transient nature of the interaction also raises the question of how this association is turned off. One possibility is that the Arp2/3 complex is displaced by another protein that binds to vinculin in the same region. Several proteins (VASP, vinexin, and ponsin) have been identified that, like the Arp2/3 complex bind to the hinge region of vinculin (Huttelmaier et al., 1998; Kioka et al., 1999; Mandai et al., 1999). Currently, we know little about the function of ponsin and vinexin and even less about how their interaction with vinculin may be regulated. In future work, we hope to explore whether any of these proteins displace the Arp2/3 complex from vinculin and thus confine the interaction to the newest sites of adhesion. Presumably, whatever mechanism dissociates the Arp2/3 complex from vinculin also prevents recruitment of the Arp2/3 complex to focal adhesions.

Vinculin-null cells have provided clues to the function of vinculin in cells. Although the vinculin knockout mice are embryonic lethal (Xu et al., 1998a), the fibroblasts from these mice are surprisingly normal. Interestingly, the vinculin-null cells are less adherent to ECM and show reduced rates of spreading when plated on ECM-coated surfaces (Coll et al., 1995; Xu et al., 1998a,b). Contrary to many expectations, these cells can develop focal adhesions and are able to migrate. Indeed, migration is enhanced relative to cells expressing vinculin (Coll et al., 1995; Xu et al., 1998a,b). Presumably, this reflects that the migration rate of these cells is limited by the strength of their adhesions. To explore specifically the role of the interaction of the Arp2/3 complex with vinculin, we generated a point mutation in the binding site on vinculin for Arp2/3. This mutant vinculin was still able to bind to the other proteins that also bind to the hinge region of vinculin, indicating that the mutation was not disrupting vinculin's conformation and affecting multiple interactions. The mutant vinculin targeted to focal adhesions and restored the adhesive properties of the cells to the same extent as the WT vinculin. Whereas reexpression of WT vinculin restored cell spreading and lamellipodia formation to normal levels, the mutant vinculin did not. In these characteristics, the cells resembled the null cells. As noted above, these observations support the idea that the binding of the Arp2/3 complex to vinculin functions to promote the extension of lamellipodia and cell spreading.

We were interested in comparing the mutant and WT vinculin-expressing cells with respect to migration. Our initial expectation was that because the mutant vinculin-expressing cells showed diminished extension of lamellipodia, that these cells would migrate more slowly than the cells expressing WT vinculin. However, we found little difference in the rate of migration of the two cell types and that both migrated more slowly than the null cells. The cells reexpressing WT or mutant vinculin have increased adhesion to the substratum and we conclude that this retards their rate of migration. This result suggests that the rate limiting step in these cells is the release of adhesions rather than protrusion at the leading edge.

It is clear that the vinculin-null MEFs can generate lamellipodial protrusions, albeit less well than cells in which WT vinculin has been expressed. However, there are vinculin-deficient F9 embryonic carcinoma cells that have also been studied. Like the MEFs devoid of vinculin, these cells also reveal reduced adhesion and spreading on FN. In contrast to the vinculin-null MEFs, these F9 cells lacking vinculin were unable to develop lamellipodia (Coll et al., 1995), but continued to develop filopodia. Recent work from Goldmann and Ingber has found that these F9 cells deficient in vinculin fail to develop lamellipodia in response to Rac activation (Goldmann and Ingber, 2002). This is restored by reexpression of WT vinculin, leading to the conclusion that vinculin is critical in lamellipodial extension. However, because MEFs lacking vinculin can generate lamellipodia, we conclude that multiple mechanisms are involved. For some cells (e.g., the F9 cells) vinculin appears to be critical for this activity, whereas for others, such as the fibroblasts used here, the coupling of Arp2/3 to vinculin provides just one of possibly several mechanisms that contribute to lamellipodial extension.

Materials and methods

Cell lines

The vinculin-null mouse embryo fibroblasts (Vin−/−; Xu et al., 1998a) were a gift of E. Adamson (Burnham Institute, La Jolla, CA). Hs68 (American Type Culture Collection), A431, HeLa, REF52, and Vin−/− cell lines were maintained in DME + 10% FBS.

Constructs

The GST–vinculin fusion proteins Vin 1–399, 399–881, 881–1066 were provided by D. Critchley (University of Leicester, Leicester, UK). All other fusion proteins containing fragments of vinculin were constructed by PCR amplification of the DNA of interest and subcloning the resulting DNA into pGEX-T. To obtain GST–P876A and GST–P878A, site-specific mutagenesis was carried out using the GST–VIN 811–881 template according to the Quickchange Manual (Stratagene). These mutants were subcloned into full-length vinculin in pCMV-myc (CLONTECH Laboratories, Inc.) with a modified multiple cloning site. pGEX-VCA domain was a gift from H-Y. H. Ho and R. Rohatgi (Harvard Medical School, Boston, MA). The GFP fusion protein, p34GFP, was constructed by RT-PCR amplification a *Sal-BamH1* p34-Arc subunit of the Arp2/3 complex cDNA from mRNA obtained from HeLa cells and subcloning the resulting DNA into pEGFP-N3 (CLONTECH Laboratories, Inc.).

Reagents and antibodies

Wortmannin (Sigma-Aldrich) and LY294002 (Alexis) were resuspended according to the manufacturer's specifications. Human FN was prepared (Ruoslahti et al., 1982) or purchased from GIBCO-BRL. PIP_2 was a gift from Andrew Morris (University of North Carolina, Chapel Hill, NC). The GST–VCA protein was prepared as described (Rohatgi et al., 2000). The Arp2/3 complex for the actin polymerization studies was purified as described (Welch and Mitchison, 1998). The Arp2/3 complex used in the binding studies was provided by D. Yaarar (University of California, Berkeley, CA). The Arp2/3 complex was blotted using a rabbit antibody raised against a peptide that included amino acids 179–204 of the p34-Arc subunit of the Arp2/3 complex. Vinculin was immunoprecipitated and blotted using a rabbit antibody raised against purified chicken gizzard vinculin. Antibodies against VASP and Rac1 were obtained from Transduction Labs, against HLA from Pharmingen, and against integrins (TS2/16) from American Type Culture Collection. Affinity-purified, anti-vinexin antibody was a gift from N. Kioka (Kyoto University, Kyoto, Japan). The ponsin antibody (Mandai et al., 1999) was a gift from Y. Takai (Osaka University School of Medicine, Osaka, Japan).

Bead experiments were carried out as previously described with minor modifications (Miyamoto et al., 1995). Latex beads (3.0 μm) were coated with 50 μg/mL human plasma FN or 100 μg/mL of poly-lysine for 1.0 h at 37°C and then blocked using 10 mg/mL BSA. HeLa or Vin−/− cells transiently expressing p34GFP or GFP were plated on collagen-coated coverslips for 1.0 h at 37°C, washed in PBS, and 1.0×10^6 beads were incubated with the coverslips for the times indicated. The coverslips were fixed, permeabilized, and stained with antibodies against vinculin, β1-integrins or HLA, followed by a Texas red–conjugated secondary antibody. For each experiment, ~150 beads were scored for the presence or absence of fluorescence around the beads. Values are number of positive beads ± SEM from at least three independent experiments.

Rac1 activity

To measure Rac1 activity, A431 cells were left resting or stimulated with EGF at specified time points, cells were washed, lysed, and active GTP-bound Rac1 was precipitated using GST-PBD beads, as described (Bagrodia et al., 1998). Densitometric analysis of films was performed as described (Liu and Burridge, 2000).

Immunoprecipitation and Western blot analysis

For the EGF stimulation experiments, subconfluent A431 cells were starved for 24 h in DME + 0.1% FBS and then stimulated with human EGF in 2 mM acetic acid, 10 mg/mL BSA at a concentration of 100 ng/mL. Control cells were treated with an equivalent amount of 2 mM acetic acid, 10 mg/mL BSA. For the cell spreading on FN experiments, cells were trypsinized, washed three times in DME, and plated onto FN-coated coverslips submerged in a 6-well plate at a density of 1.33×10^5 cells. The

plates were centrifuged at 300 *g* for 5 min and allowed to spread for the times indicated at 37°C. Subsequent to stimulation of cells by EGF or spreading on FN, the cells were washed in HBS (20 mM HEPES, pH 7.4, 150 mM NaCl + 2 mM Na_3VO_4) and lysed in ice-cold EB (1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% BSA, 20 μg/mL aprotinin, 1 mM PMSF, 2 mM Na_3VO_4). For the experiments addressing whether PIP_2 and/or GST–VCA can induce binding of the Arp2/3 complex to vinculin, the Triton X-100 cleared lysates were incubated with 10 mM PIP_2 and/ or 30 μg GST–VCA. Vinculin was immunoprecipitated with a polyclonal antibody raised against chicken gizzard vinculin, the immunoprecipitates were washed four times in ice-cold EB, fractionated by SDS-PAGE, transferred to PVDF membranes, and subjected to Western blot analysis. 20% of the immunoprecipitate was analyzed for vinculin recovered, 10% was used for the actin blots, and between 70 and 80% was analyzed for p34-Arc coprecipitation. The blots were developed with Western blot detection reagents (ECL; Amersham Biosciences), and the signal was detected on x-ray film.

Migration assays

Migration assays were performed using Transwell cell culture chambers containing polycarbonate membrane inserts with 8.0 μm pores (Becton-Dickinson) as previously described (Xu et al., 1998a). The filters were processed for immunofluorescence as described above and stained with DAPI (Sigma-Aldrich) to stain nuclei.

Adhesion assays

Cells were plated on coverslips coated with 50 μg/mL of human FN, allowed to adhere for 45 min at 37 °C, rinsed three times with PBS, and then processed for immunofluorescence and stained with DAPI (Sigma-Aldrich).

Immunofluorescence

Cells were fixed, permeabilized, and washed as described (Liu and Burridge, 2000). Cells were incubated with primary antibodies for 1.0 h at room temperature, washed, and incubated with secondary antibody for 1.0 h at room temperature. Vinculin and myc monoclonal antibodies (Sigma-Aldrich) were used at 1:50 and 1:1,000, respectively. Alexa fluoro 594-conjugated phalloidin (Molecular Probes) was used to visualize actin at 1:200. Images were obtained as described (Liu and Burridge, 2000).

Spreading assays

Cells were seeded onto coverslips coated with 50 μg/mL of human FN and incubated for 45 or 120 min at 37°C. The cells were then processed for immunofluorescence.

Microinjection

Cells were cultured and microinjected as previously described (Nobes and Hall, 1995).

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