Protein Kinase C Regulates αvβ5-dependent Cytoskeletal Associations and Focal Adhesion Kinase Phosphorylation

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Abstract. Integrins αvβ3 and αvβ5 both mediate cell adhesion to vitronectin yet trigger different postligand binding events. Integrin αvβ3 is able to induce cell spreading, migration, angiogenesis, and tumor metastasis without additional stimulators, whereas αvβ5 requires exogenous activation of protein kinase C (PKC) to mediate these processes. To investigate this difference, the ability of β3 or β5 to induce colocalization of intracellular proteins was assessed by immunofluorescence in hamster CS-1 melanoma cells. We found that αvβ5 induced colocalization of talin, α-actinin, tensin, and actin very weakly relative to αvβ3. αvβ5 was able to efficiently induce colocalization of focal adhesion kinase (FAK); however, it was unable to increase phosphorylation of FAK on tyrosine. Activation of PKC by adding phorbol ester to αvβ5-expressing cells induced spreading, increased colocalization of α-actinin, tensin, vinculin, p130^{cas} and actin, and triggered tyrosine phosphorylation of FAK. Unexpectedly, talin colocalization remained low even after activation of PKC. Treatment of cells with the PKC inhibitor calphostin C inhibited spreading and the colocalization of talin, α-actinin, tensin, and actin for both αvβ3 and αvβ5. We conclude that PKC regulates localization of cytoskeletal proteins and phosphorylation of FAK induced by αvβ5. Our results also show that FAK can be localized independent of its phosphorylation and that cells can spread and induce localization of other focal adhesion proteins in the absence of detectable talin.

Integrins mediate adhesion of cells to extracellular matrix proteins and to other cells. During adhesion, they also form linkages to the cytoskeleton and regulate intracellular signaling pathways, thereby coordinating attachment to cell architecture and gene expression (Burridge et al., 1988; Hynes, 1992; Miyamoto et al., 1995; Schwartz et al., 1995). Despite significant progress towards understanding these phenomena, much remains to be learned about how integrin-dependent events control cell functions.

Integrins are heterodimers, and binding specificity is generated by varied combinations of the 14 α and 8 β subunits identified to date (Hynes, 1992). Binding specificities show a high degree of redundancy, however, such that both integrins and their ligands bind multiple partners. Evidence is emerging that individual receptors mediate distinct functions and trigger distinct signaling pathways (Hynes, 1992; Sastry and Horwitz, 1993; Schwartz et al., 1995).

After initial ligand binding, integrins recruit a number of signaling and cytoskeletal proteins to sites of adhesion (Burridge et al., 1988; Miyamoto et al., 1995). While the precise organization of these structures remains to be determined, α-actinin and talin have been shown to bind directly to sequences in the β1 cytoplasmic domain (Horwitz et al., 1986; Otey et al., 1990). Similar sequences are present in several other integrin β chains, suggesting that these interactions are conserved. Both α-actinin and talin have been reported to bind vinculin (Belkin and Koteliansky, 1987; Wachsstock et al., 1987), which can associate with both tensin and paxillin (Wilkins et al., 1986; Turner et al., 1990). Focal adhesion kinase (FAK)[1] has been reported to bind to peptides from the integrin β1 cytoplasmic domain, to talin, to paxillin, and to p130^{cas} (Polte and Hanks, 1995; Bellis et al., 1995; Chen et al., 1995; Schaller and Parsons, 1995; Schaller et al., 1995). There is evidence that these linkages are subject to regulation, although the details are not well understood.

Integrin function is regulated by cytokines and other soluble factors in a variety of systems. In most cases, these factors modulate the avidity of integrins for their ligands, by altering either the conformation of the receptor or the lateral distribution in the plasma membrane (for review see Schwartz et al., 1995). Integrin αvβ5 represents an interesting exception to this rule, since it binds very well to its ligand vitronectin (VN), but in the absence of exogenous soluble factors, it fails to promote cell spreading or migration (Wayner et al., 1991; Klemke et al., 1994)[2]. By

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1. *Abbreviations used in this paper*: FAK, focal adhesion kinase; FN, fibronectin; PKC, protein kinase C; VN, vitronectin.

2. Brooks, P.C., R.L. Klemke, S. Schön, J.M. Lewis, M.A. Schwartz, and D.A. Cheresh. Manuscript submitted for publication.

contrast, the β3 subunit, which is 56% homologous to β5, also pairs with αv and binds VN but induces spreading and migration in the same cells in the absence of cytokines (Wayner et al., 1991; Filardo et al., 1995). However, upon stimulation with either phorbol ester or cytokines to activate protein kinase C (PKC), αvβ5 acquires the ability to mediate cell spreading and migration in vitro (Klemke et al., 1994; Yebra et al., 1995). This PKC-dependent activation correlates with the ability of αvβ5 to promote angiogenesis by endothelial cells and metastasis by tumor cells in vivo (Friedlander et al., 1995).[2]

The aim of this study was to investigate how PKC regulates the function of αvβ5. While previous studies suggested that PKC may alter connections to the cytoskeleton,[2] the molecular mechanism explaining this observation remains unclear. We therefore assayed the ability of αvβ5 to induce localization of a number of cytoskeletal and signaling proteins before and after activation of PKC. Our results show that PKC does regulate linkages to specific cytoskeletal proteins. We also find that FAK colocalized with αvβ5 independent of PKC but that its phosphorylation is PKC dependent.

Materials and Methods

Cell Culture

CS-1 melanoma cells transfected with cDNAs encoding either the integrin β3 subunit or the integrin β5 subunit have been described previously (Filardo et al., 1995). Cells were maintained in RPMI media supplemented with 10% FBS (GIBCO BRL, Gaithersburg, MD).

Reagents

Human plasma VN was purified as previously described (Yatohgo et al., 1988). Fibronectin (FN) was prepared from human plasma by affinity chromatography on gelatin Sepharose (Miekka et al., 1982). Rhodamine phalloidin was purchased from Molecular Probes (Eugene, OR). All other chemicals, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO).

Antibodies

The monoclonal antibodies LM609 (αvβ3) and P1F6 (αvβ5) have been described previously (Cheresh, 1987; Wayner et al., 1991). Monoclonal antibodies directed against vinculin and talin were purchased from Sigma Immunochemicals (St. Louis, MO), as was a rabbit polyclonal antibody directed against α-actinin. A rabbit polyclonal antibody directed against tensin was a generous gift of Dr. Lan-Bo Chen. Rabbit antibodies directed against $p130^{cas}$ were a gift from Amy Bouton (University of Virginia, Charlottesville, VA), and a polyclonal rabbit antiserum directed against talin was a gift from Keith Burridge (University of North Carolina, Chapel Hill, NC). Two antibodies directed against FAK, the rabbit polyclonal antibody BC3 and the mouse monoclonal antibody 2A7, were generous gifts from Dr. J. Thomas Parsons. The mouse monoclonal antibody 4G10, directed against phosphotyrosine, was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Fluorescein- or rhodamine-conjugated, affinity-purified secondary antibodies were purchased from Cappel Laboratories (Organon-Teknika, West Chester, PA).

Adhesion Assays

24-well tissue culture cluster plates (Costar Corp., Cambridge, MA) were coated overnight at 4°C with 10 μg/ml VN in PBS, pH 7.4. Before use, wells were blocked with 10 mg/ml heat-denatured BSA. Cells were detached with trypsin and washed once in RPMI containing 1% nuclease- and protease-free BSA, 10 μg/ml aprotinin and 10 μg/ml leupeptin (RB), and 250 μg/ml soybean trypsin inhibitor. Cells were resuspended in RB and plated at 10^5 cells/well. At times indicated, adherent cells were washed once with medium and three times with PBS. Cells were incubated with 200 μl of a solution containing 50 mM sodium acetate, pH 5.0, 0.4% Triton X-100, and 3 mg/ml nitrophenylphosphate (No. 104, Sigma Chemical Co.) for 1 h at room temperature. After the addition of 50 μl 1M NaOH to each well, samples were transferred to 96-well plates and the OD at 405 nm was determined. Assays were performed in triplicate.

Bead Conjugation

VN at 1.5 mg/ml final concentration or FN at 0.5 mg/ml, in 50 mM borate, pH 9.5, with 10 μg/ml aprotinin and 10 μg/ml leupeptin, was conjugated to 2×10^8 tosyl-activated 4.5-μm polystyrene beads/ml (DYNAL, Inc., Great Neck, NY). Samples were incubated for 24 h at room temperature with gentle rotation. Conjugated beads were washed four times with 50 mM Tris-HCl, pH 9.5. Free tosyl groups were blocked in the same buffer plus aprotinin and leupeptin by incubation for 16 h at 4°C with gentle rotation. Beads were washed twice and stored in sterile PBS containing 1% protease-free BSA.

Binding of Beads to Cells

Cells in suspension were incubated with VN- or FN-coated beads as previously described (Lewis and Schwartz, 1995), except that RPMI was used instead of DME, and 10 μg/ml each aprotinin and leupeptin were included as protease inhibitors. Cells and beads were incubated for 1–2 h at 37°C.

For activation studies, cells and beads were incubated for 30 min at 37°C to initiate binding. After the addition of 10 nM PMA, samples were incubated for an additional 30 min. Calphostin C (200 nM) was incubated with suspended cells before the addition of beads, at 37°C for 30 min, under fluorescent room lighting to induce activation.

Cell Spreading on VN

Cells were detached with trypsin, washed in RB containing 250 μg/ml soybean trypsin inhibitor, and allowed to attach in RB to VN-coated glass coverslips for 30 min at 37°C. PMA at 10 nM was added to some samples to induce spreading. After an additional 30 min at 37°C, all samples were fixed with 3.7% formaldehyde in 0.5 M Pipes buffer containing 1 mM $MgCl_2$ and 1 mM EGTA and either photographed or processed for immunofluorescence.

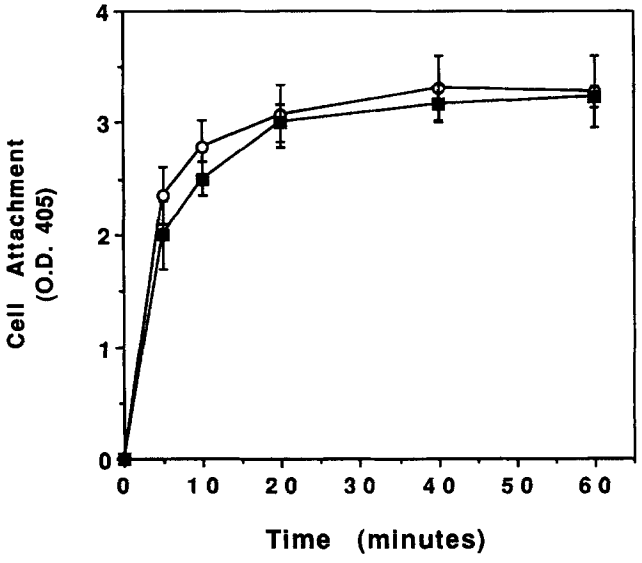


Figure 1. Attachment to VN is equivalent for αvβ3 and αvβ5. CS-1 cells expressing αvβ3 or αvβ5 were incubated for the times indicated in wells coated with 10 μg/ml VN. After adhesion, unattached cells were removed by washing, and the attached cells assayed with the phosphatase substrate nitrophenylphosphate. The OD 405 nm is directly proportional to cell number. Each data point represents the mean ± the standard deviation of triplicate samples.

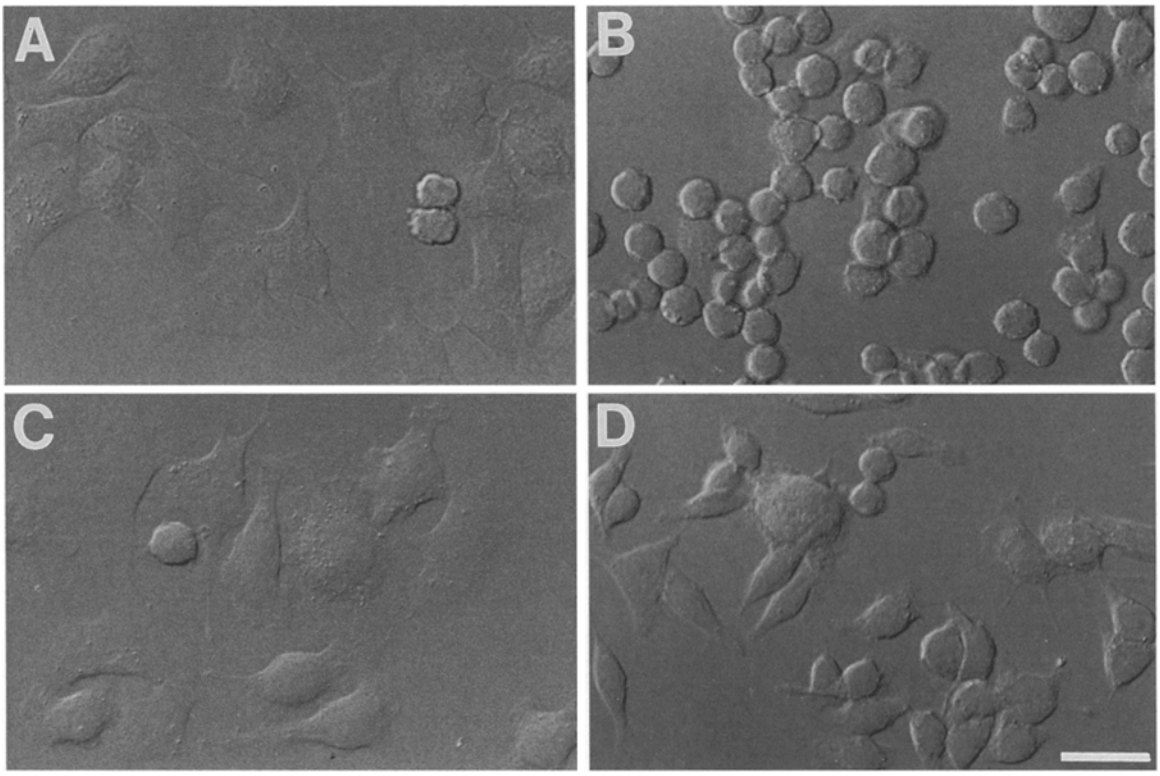


Figure 2. Spreading of αvβ5 CS-1 cells on VN depends on PKC. β3-transfected cells (*A* and *C*) or β5-transfected cells (*B* and *D*) were allowed to attach to glass coverslips coated with 10 μg/ml VN for 30 min at 37°C. PMA at 10 nM was added (*C* and *D*), and all samples were incubated for an additional 30 min. Cells were then fixed and Nomarski micrographs taken using Tmax 400 film. Bar, 20 μm.

Microscopy

Either suspended cells with attached beads or cells spread on VN were processed for immunofluorescence as previously described (Lewis and Schwartz, 1995). Samples were viewed on an inverted fluorescence microscope (Nikon Inc., Melville, NY) equipped with a 60× oil objective (Olympus Corp., Lake Success, NY). Quantitation was performed also as described previously (Lewis and Schwartz, 1995). Nomarski images of attached cells were obtained using a microscope equipped with a 40× objective (model BX60; Olympus Corp.). For confocal microscopy, immunofluorescent samples were scanned with a laser confocal microscope (model MRC 600; Bio-Rad Labs, Hercules, CA) equipped with a 63× objective (Carl Zeiss Inc., Thornwood, NY). Photographs were taken at the cell–substratum interface.

Cell Lysates

VN at 10 μg/ml was coated onto tissue culture plates for 2–3 h at 37°C. Plates were washed twice with PBS, blocked with 10 mg/ml denatured BSA for 5 min, and washed again three times. Suspension culture plates were made by coating tissue culture plates with the denatured BSA. Cells were detached with trypsin, washed once with RB containing 250 μg/ml soybean trypsin inhibitor, and plated in RB containing 10 μg/ml each aprotinin and leupeptin. Cells were incubated at 37°C. At 30 min, 10 nM PMA was added to some samples with gentle swirling to mix. Cells were harvested at 60 min by first placing all dishes on ice and then washing each three times with ice cold PBS. Each 10-cm plate of cells was lysed with 0.5 ml RIPA buffer containing 2 mM sodium vanadate, 1 mM PMSF, and 10 μg/ml each of aprotinin and leupeptin; RIPA buffer consisted of 10 mM Tris-Cl, pH 7.4, with 158 mM NaCl, 0.1 mM EGTA, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS. Cell material was scraped off the plates and allowed to lyse for an additional 30 min on ice. Samples were centrifuged for 30 min at 14,000 *g*, at 4°C, and the supernatants were removed. Protein concentrations were determined using the Pierce BCA assay (Pierce, Rockford, IL).

Immunoprecipitations

For each gel lane, cell lysate containing 750 μg of protein was incubated with 5 μl BC3 antibody for 1 h on ice. After the addition of 20 μl protein G–Sepharose (Pierce), samples were rotated at 4°C for 4 h. Beads were collected by centrifugation for 1 min at 3,000 *g* and washed five times with RIPA buffer containing fresh protease inhibitors and vanadate. Immunoprecipitates were solubilized by dissolving in gel sample buffer and boiled for 5 min.

Immunoblots

Immunoprecipitates were run on a 6% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose paper. Blots were blocked overnight at 4°C in 3% nuclease- and protease-free BSA in pH 7.0 Tris-buffered saline (TBS) for the 4G10 antibody and 5% milk in TBS for the 2A7 antibody. Primary antibody incubations were for 3 h at room temperature with 1:2,000 dilutions of the 4G10 and 1:750 dilutions of the 2A7 in 3% BSA or 3% milk, respectively. Blots were washed three times for 10 min in TBS containing 0.5% Tween 20 and incubated 2 h with a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (BioSource International, Camarillo, CA). Blots were again washed and visualized using chemiluminescence (ECL kit; Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

Results

Attachment of Cells to Vitronectin

Parent CS-1 melanoma cells lack expression of both αvβ3 and αvβ5 and thus fail to bind vitronectin (Thomas et al., 1993). These cells, however, can attach to and spread on FN via integrin α5β1. When CS-1 cells are transfected with

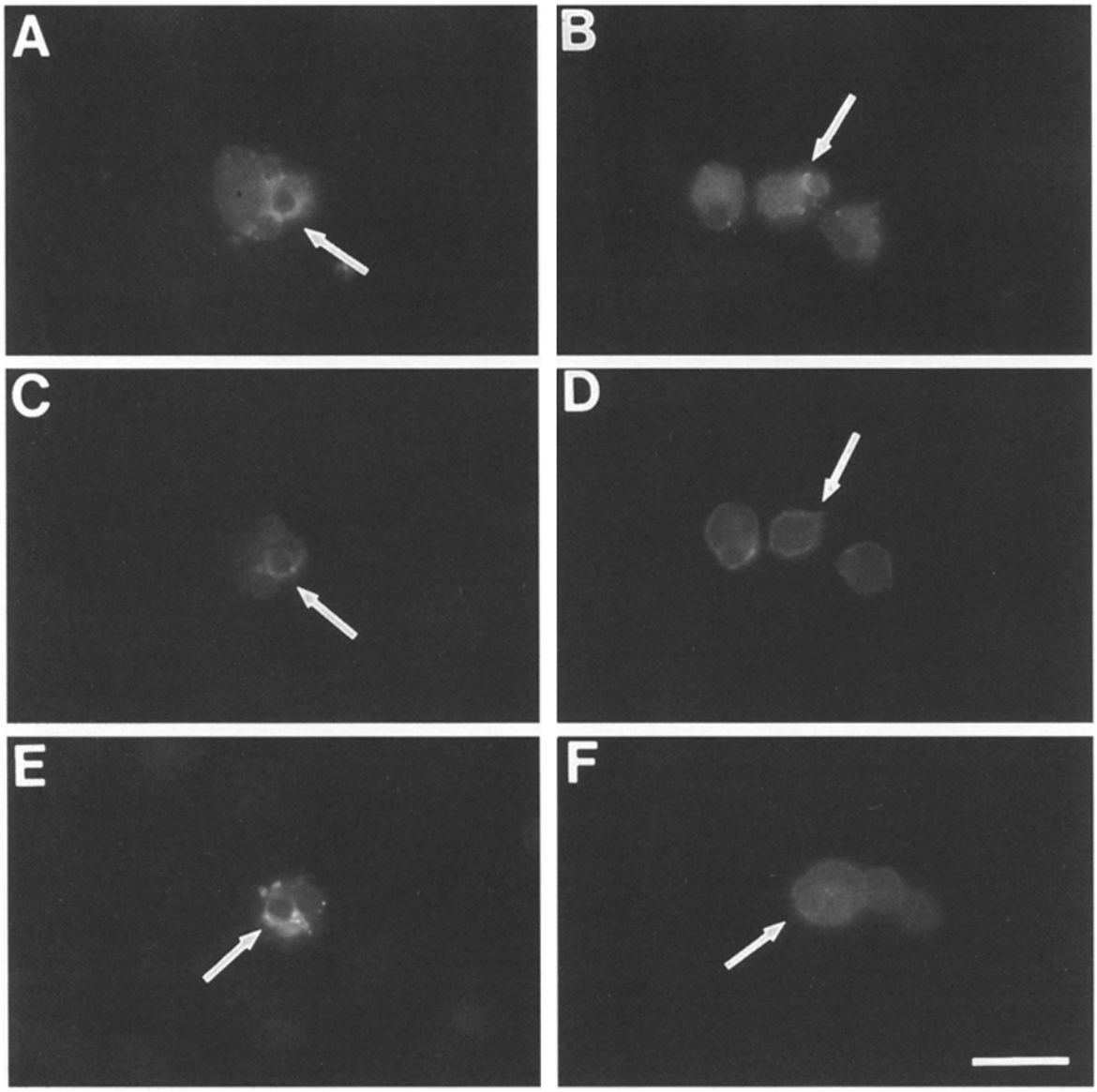


Figure 3. Cytoskeletal recruitment by integrin αvβ3 and αvβ5. VN-coated beads were incubated 60 min with CS-1 hamster melanoma cells expressing either αvβ3 (*A*, *C*, and *E*) or αvβ5 (*B*, *D*, and *F*) and then fixed. Cells in the top four panels were double-labeled with antibodies specific for the integrins (*A* and *B*) and with rhodamine phalloidin to stain f-actin (*C* and *D*). Cells in *E* and *F* were labeled with a single antibody specific for talin. Arrows indicate position of beads. Bar, 20 μm.

expression vectors coding for integrin β3 or β5, they express αvβ3 or αvβ5 on their surfaces and acquire the ability to attach to VN (Filardo et al., 1995). Transfected cell populations were sorted by FACS® to select for equivalent expression. Analysis of these cells lines by FACS® (Filardo and Cheresh, manuscript submitted for publication) has demonstrated that they do express equivalent levels. Therefore, differences observed between β3- or β5-transfected cells to mediate VN-stimulated reorganization of cytoskeletal proteins resulted from properties of αvβ3 or αvβ5 and were not due to differences in general levels of expression.

The ability of these cell lines expressing β3 or β5 to adhere to VN was also assessed. As shown in Fig. 1, both types of cell adhere well to VN with very similar kinetics of binding. Additional experiments demonstrated that parent CS-1 cells showed no adhesion, and that adhesion of αvβ3 and αvβ5 lines were inhibited by blocking monoclonal antibodies to αvβ3 and αvβ5, respectively (Filardo, E.J., and D.A. Cheresh, manuscript submitted for publication). Thus, αvβ3 and αvβ5 are expressed at similar levels and mediate attachment to VN equally well.

Spreading of β3 or β5 Cells on VN

The ability of β3- and β5-transfected cells to mediate spreading on VN was assessed. When plated in culture medium containing serum or on purified VN (Fig. 2 *A*), only the β3-transfected cells spread; β5 CS-1 cells remained adherent but entirely round (Fig. 2 *B*). By contrast, β3- and

β5-transfected cells spread equally well on FN via endogenous integrin α5β1, demonstrating that the inability to spread was a property of the integrin and not the cell line.

Previous work has shown that migration, angiogenesis, and metastasis via integrin αvβ5 can be induced when cells are stimulated to activate PKC (Klemke et al., 1994; Friedlander et al., 1995).[2] To determine whether αvβ5-containing CS-1 cells could spread on VN after PKC activation, phorbol ester was added to cells attached to VN, and the cells were incubated for an additional 30 min. After treatment, β3-transfected cells were unchanged since they spread in the absence of PMA (Fig. 2 *C*). However, after activation for 5–10 min, many of the β5-transfected cells were starting to spread on VN, and by 30 min, most of these cells showed some degree of spreading (Fig. 2 *D*). Additional experiments showed that the anti-αvβ5 monoclonal antibody P1F6 blocked this spreading event (not shown). Additionally, calphostin C, a specific inhibitor of PKC, completely blocked the effects of PMA on spreading mediated by either αvβ3 or αvβ5 (not shown). Thus, αvβ5 is capable of mediating cell spreading on VN, but unlike αvβ3, this spreading seems to require an additional signal from PKC.

Interactions of αvβ5 or αvβ3 with Cytoskeletal Proteins

To determine why αvβ5 fails to mediate cell spreading without exogenous activation of PKC, we investigated the ability of αvβ5 clustered by VN-coated microbeads to induce colocalization of cytoskeletal proteins, as shown previously for β1 and FN (Lewis and Schwartz, 1995; Miyamoto et al., 1995). VN-coated beads did not adhere to untransfected CS-1 cells (not shown) but bound efficiently to either β3- or β5-transfected cells. Binding of VN-coated beads to cells expressing αvβ3 or αvβ5 induced localization of both integrins to the interface with the beads equally well (Fig. 3). As expected, αvβ3 clustered by VN-coated beads also induced colocalization of actin and talin. However, αvβ5 did not induce detectable colocalization of either of these two proteins. Quantitation of results from these and additional experiments revealed that binding of αvβ3 cells to VN beads induced high levels of colocalization of actin, α-actinin, talin, tensin, and FAK, which is consistent with its ability to promote formation of focal adhesions and cell spreading (Fig. 4). Clustering of αvβ5 integrin, however, induced significantly weaker colocalization of actin, α-actinin, talin, or tensin ($P < 0.05$). Therefore, the inability of αvβ5 to mediate spreading of cells on VN correlates with diminished reorganization of key cytoskeletal proteins after binding of VN beads.

Surprisingly, αvβ5 induced colocalization of FAK more effectively than did αvβ3. The staining with the anti-FAK antibody was very bright, which appears to account for the fact that accumulation of FAK exceeded that of αvβ5. As a control for these experiments, beads coated with FN were bound to cells, and localization of the cytoskeletal proteins was examined by immunofluorescence. FN beads induced similar colocalization of actin and talin, as well as FAK, in αvβ3 and αvβ5 cell lines (Fig. 5). Colocalization of α-actinin was somewhat lower in αvβ5 cells. We do not fully understand this result, but it suggests that integrin

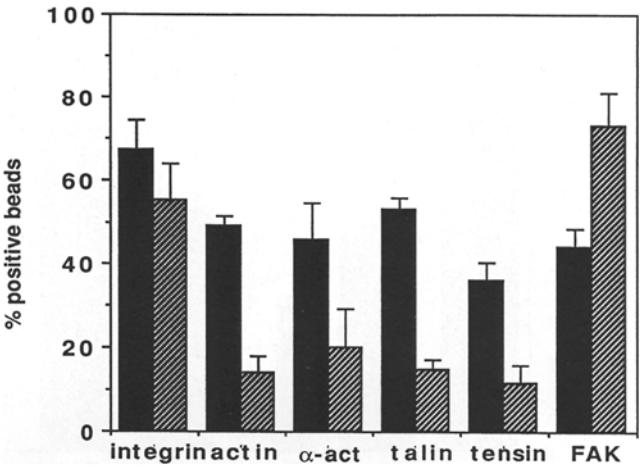


Figure 4. Quantitation of cytoskeletal recruitment. Suspended cells expressing either αvβ3 or αvβ5 were incubated with VN-coated polystyrene beads, processed for immunofluorescence, and scored for colocalization. The ordinate indicates the percentage of beads positive for immunofluorescence for each antigen. Data are expressed as the mean ± standard deviation from three separate experiments. *Solid bars*, β3-transfected cells; *hatched bars*, β5-transfected cells.

αvβ5 may exert some dominant negative effect on this protein. Nevertheless, these results show that the differences in localization of actin, talin, and FAK are due to the different integrins and not to properties of the cell lines.

Additional controls to test the specificity of integrin localization were performed. They showed that FN-coated beads failed to induce detectable accumulation of αvβ3 or αvβ5; conversely, VN-coated beads failed to induce accumulation of α5β1 on either cell type. Finally, activation with PMA (see below) did not trigger any changes in the specificity of integrin localization (data not shown).

Interactions of αvβ5 After Activation of PKC

We next investigated if the conditions that trigger spreading of the β5-transfected cells alter the ability of αvβ5 to induce colocalization of cytoskeletal proteins. Cells in which αvβ5 was clustered with VN-coated beads were stimulated by PMA, and samples were analyzed by immunofluorescence (Fig. 6). Stimulation of the cells with PMA resulted in large increases in the levels of actin, α-actinin, vinculin, tensin, and cas that colocalized with αvβ5 at the bead–cell interface ($P < 0.0005$). Unexpectedly, colocalization of talin with αvβ5 remained low even after stimulation ($P < 0.375$). In contrast, stimulation with PMA did not change the ability of α5β1 clustered with FN-coated beads to recruit both α-actinin and talin (not shown).

Calphostin C inhibited the colocalization of α-actinin and talin with αvβ3 and blocked the increases in colocalization of α-actinin and talin with αvβ5 induced by PMA (Fig. 7). These results support the notion that the effects of PMA on the localization of these proteins are mediated by PKC and indicate that for αvβ3, these cytoskeletal interactions are similarly regulated by PKC.

Analysis of Cells by Confocal Microscopy

Beads mimic the generation of focal adhesion sites and re-

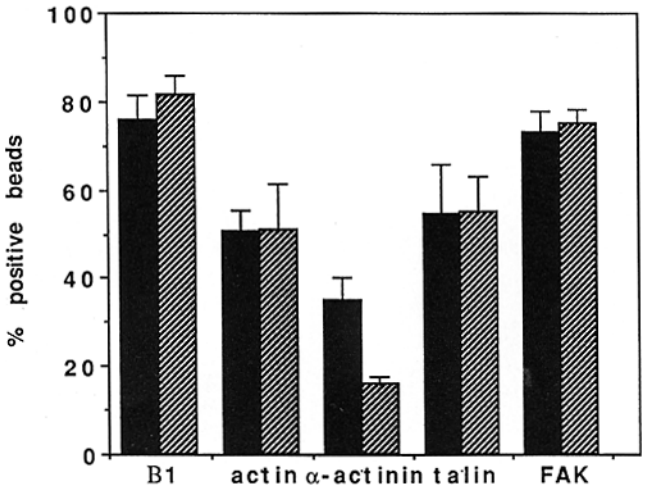


Figure 5. Recruitment of proteins with fibronectin-coated beads. Suspended cells expressing either αvβ3 or αvβ5 were incubated with FN-coated polystyrene beads, processed for immunofluorescence, and scored for colocalization. The ordinate indicates the percentage of beads positive for immunofluorescence for each antigen. Data are expressed as the mean and standard deviation. *Solid bars*, β3-transfected cells; *hatched bars*, β5-transfected cells.

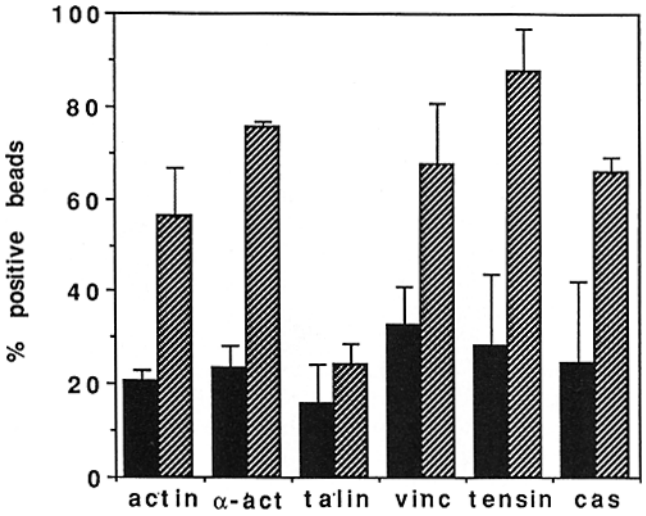


Figure 6. Stimulation of αvβ5 cells with PMA. Suspended cells expressing αvβ5 were incubated with VN-coated polystyrene beads. After attachment of beads to cells, 10 nM PMA was added to some samples. All samples were fixed after further incubation, processed for immunofluorescence, and scored for colocalization. The ordinate indicates the percentage of beads positive for immunofluorescence for each antigen. Data are expressed as the mean ± standard deviation. *Solid bars*, without PMA; *hatched bars*, with PMA.

organization of cytoskeleton necessary for cell spreading. To confirm that 4.5-μm beads provide a suitable model for interactions with planar substrata, we examined the localization of cytoskeletal proteins using confocal microscopy to analyze cells plated on coverslips coated with VN (Fig. 8). Fluorescence in the 1.0-μm section nearest the substratum was analyzed. As expected, the unstimulated, round αvβ5 cells exhibited primarily a diffuse staining pattern for αvβ5, with some concentration of fluorescence at the edges of the cells. Staining for α-actinin, talin, and tensin were also diffuse and showed only slight overlap with αvβ5. After activation of the cells by PMA, both αvβ5 and α-actinin became distinctly polarized and showed a high degree of colocalization. Tensin also colocalized strongly with αvβ5. Talin, however, failed to colocalize with β5 and remained diffuse. These results therefore confirm those obtained using the bead assay.

Phosphorylation of FAK

We next asked if the colocalization of FAK with αvβ5 correlates with changes in FAK phosphorylation. We therefore compared the expression levels and phosphorylation state of FAK for the β3- and β5-transfected cells, with and without activation of PKC (Fig. 9). Both β3- and β5-transfected cells express equivalent amounts of FAK, which is unaffected by adhesion to VN or activation by PMA (Fig. 9 *B*). As expected, phosphorylation of FAK on tyrosine residues increased in αvβ3 cells upon adhesion to VN, and showed no further increase following treatment with PMA (Fig. 9 *A*). For αvβ5 cells, the phosphorylation of FAK remained low after attachment to VN, correlating with their failure to spread. After treatment with PMA to induce cell spreading, the phosphorylation of FAK increased several-fold and was now equivalent to phosphorylation in well-spread αvβ3 cells (Fig. 9 *C*). Therefore, ligand binding to integrin αvβ5 triggers localization of FAK but not its phosphorylation. Only after exogenous activation of PKC does FAK becomes phosphorylated, in parallel with cell spreading.

Discussion

Integrins mediate cell adhesion events that are critical for tissue morphogenesis, wound repair, and cell motility. The biological activity of integrins involves binding to extracellular matrix proteins and consequent reorganization of the actin cytoskeleton and generation of intracellular signals (Burridge et al., 1988; Hynes, 1992; Schwartz et al., 1995). The integrins αvβ3 and αvβ5 both bind VN but have different requirements for mediating subsequent biological events. Induction of cell spreading, migration, angiogenesis, and tumor cell metastasis by αvβ5 in carcinoma and endothelial cells requires additional activation by cytokines, whereas αvβ3 can induce these events without additional activators (Klemke et al., 1994; Friedlander et al., 1995).[2] This observation is interesting in light of the fact that αvβ5 is very widely expressed in normal tissues (Felding-Habermann and Cheresh, 1993). In many of these tissues, unregulated cell spreading and motility would likely be deleterious. In comparison, expression of αvβ3 is highly regulated, appearing primarily on motile cell types in vivo, such as angiogenic endothelial cells, migrating smooth muscle cells, and invasive melanoma cells (Clyman et al., 1992; Brooks et al., 1994; Filardo et al., 1995). Thus, motility mediated by both αvβ3 and αvβ5 is regulated, but by distinct mechanisms.

The regulation of integrins by cytokines ("inside-out sig-

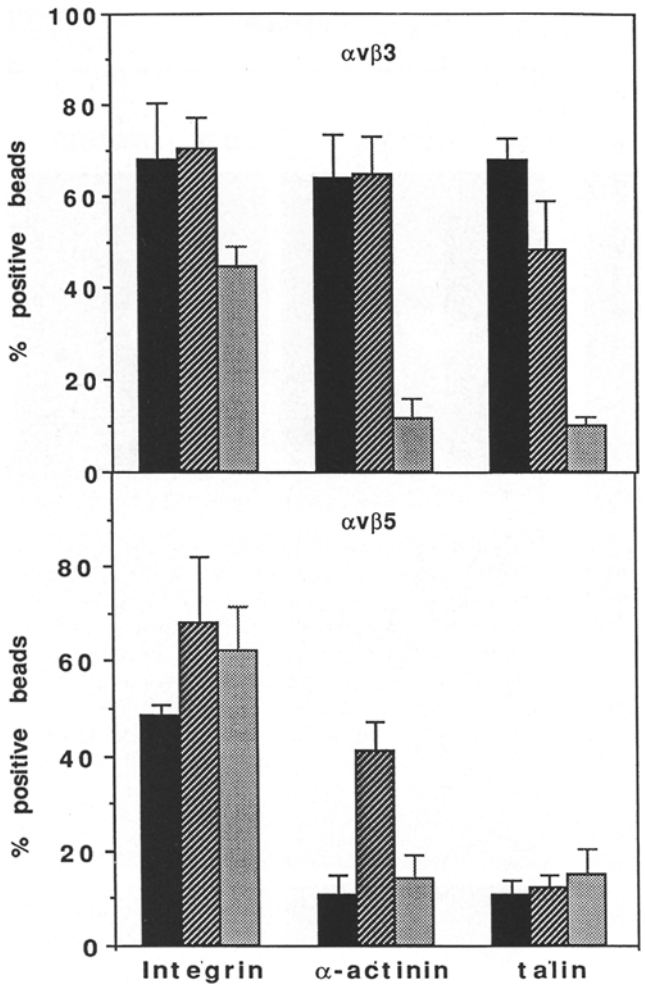


Figure 7. Inhibition of PKC with calphostin C. Suspended cells expressing αvβ3 or αvβ5 were incubated with 200 nM calphostin C for 30 min at 37°C before the addition of VN-coated beads (*stippled bars*). PMA at 10 nM was added 5 min before the addition of the beads (*hatched* and *stippled bars*); samples without any pretreatment are shown in solid bars. All samples were incubated with VN-coated beads, processed for immunofluorescence, and scored for colocalization. The ordinate indicates the percentage of beads positive for immunofluorescence for each antigen. Data are expressed as the mean ± standard deviation.

naling") has been well-studied. The platelet integrin αIIbβ3 is activated by thrombin via PKC, and the leukocyte integrin β2 also requires cell activation. In these cases, cytokine regulation involves modulation of the integrin affinity for its ligand. In other cases, adhesion of cells to immobilized ligand is regulated by changes in integrin clustering without changes in affinity (for review see Schwartz et al., 1995). The unusual feature of cytokine regulation of αvβ5 function is that ligand binding is unchanged, but postligand binding events differ. We therefore designed experiments to investigate the molecular basis for cytokine regulation of αvβ5 function.

These experiments lead to several novel observations. First, upon binding to immobilized VN, unactivated αvβ5 induces the localized accumulation of actin, α-actinin, talin, tensin, p130^{cas}, and vinculin very poorly relative to integrin αvβ3. Upon activation of PKC, αvβ5 acquires the ability to recruit actin, α-actinin, tensin, vinculin, and p130^{cas}, but not talin. This suggests that PKC activation may exert effects on the conformation or phosphorylation state of either the β5 cytoplasmic tail, the cytoskeletal proteins, or both. The induced colocalization of these proteins likely accounts for, on the molecular level, the observation that unlike other integrins, αvβ5 requires exogenous activation of PKC to mediate cell spreading and migration (Klemke et al., 1994).

It may be relevant that cell spreading and formation of focal adhesions via other integrins has been found to require PKC (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993). Adhesion of HeLa cells to collagen triggers release of diacylglycerol and translocation of PKC to the membrane fraction, and adhesion of 3T3 cells to fibronectin also triggers translocation of PKC (Chun and Jacobson, 1992; Vuori and Ruoslahti, 1993; Auer and Jacobson, 1995). In both these systems, inhibition of PKC blocks cell spreading. While these events are due primarily to β1 integrins, αvβ3 is very likely to behave similarly. Taken together, these results suggest that integrins such as αvβ3 or α5β1 trigger PKC themselves and so do not require additional factors. Integrin αvβ5, however, may be unable to trigger activation of PKC and therefore requires exogenous activation. We are currently attempting to test this hypothesis.

The cytoplasmic domain of β5 is structurally unique among the integrin β subunits β1, β2, β3, β5, β6, and β7 (Wayner et al., 1991; Sastry and Horwitz, 1993), which are otherwise quite similar. The cytoplasmic tails of other β subunits have been shown to interact directly with the cytoskeletal proteins talin and α-actinin (Tapley et al., 1989; Otey et al., 1990), and regions have been defined that are critical for these interactions to occur (Reszka et al., 1992; Lewis and Schwartz, 1995; Schaller et al., 1995). These regions include the two "NPXY" sequence motifs that are predicted to form one face of a helix in β1 and other β subunits. These sequences are important for localization of β1 to focal adhesions and for β3-mediated cell migration (Reszka et al., 1992; Filardo et al., 1995). Indeed, for β1, two regions surrounding these motifs have been shown to mediate interactions either with α-actinin, or with talin, actin, and FAK (Tapley et al., 1989; Lewis and Schwartz, 1995; Schaller et al., 1995). It is therefore interesting that the spacing between the two NPXY motifs is conserved in other integrins, but that β5 has an insertion that increases the spacing from 8 to 16 amino acid residues. The unique cytoplasmic tail of β5, when expressed in CHO cells as a chimera with the extracellular and transmembrane domains of β1, has been found to confer properties of β5 and not β1; these properties include increased cell migration and lack of localization to focal adhesions (Pasqualini and Hemler, 1994). It seems likely that this difference in primary structure also accounts for the reduced interaction of αvβ5 with talin. Evidently, however, αvβ5 can still interact with other cytoskeletal proteins to mediate cell spreading.

While it is known that integrins can bind directly to talin and to α-actinin (Horwitz et al., 1986; Otey et al., 1990), exactly how these interactions link integrin to the actin cytoskeleton is still unclear. Our data show that the interaction between αvβ5 and α-actinin appears to be regulated

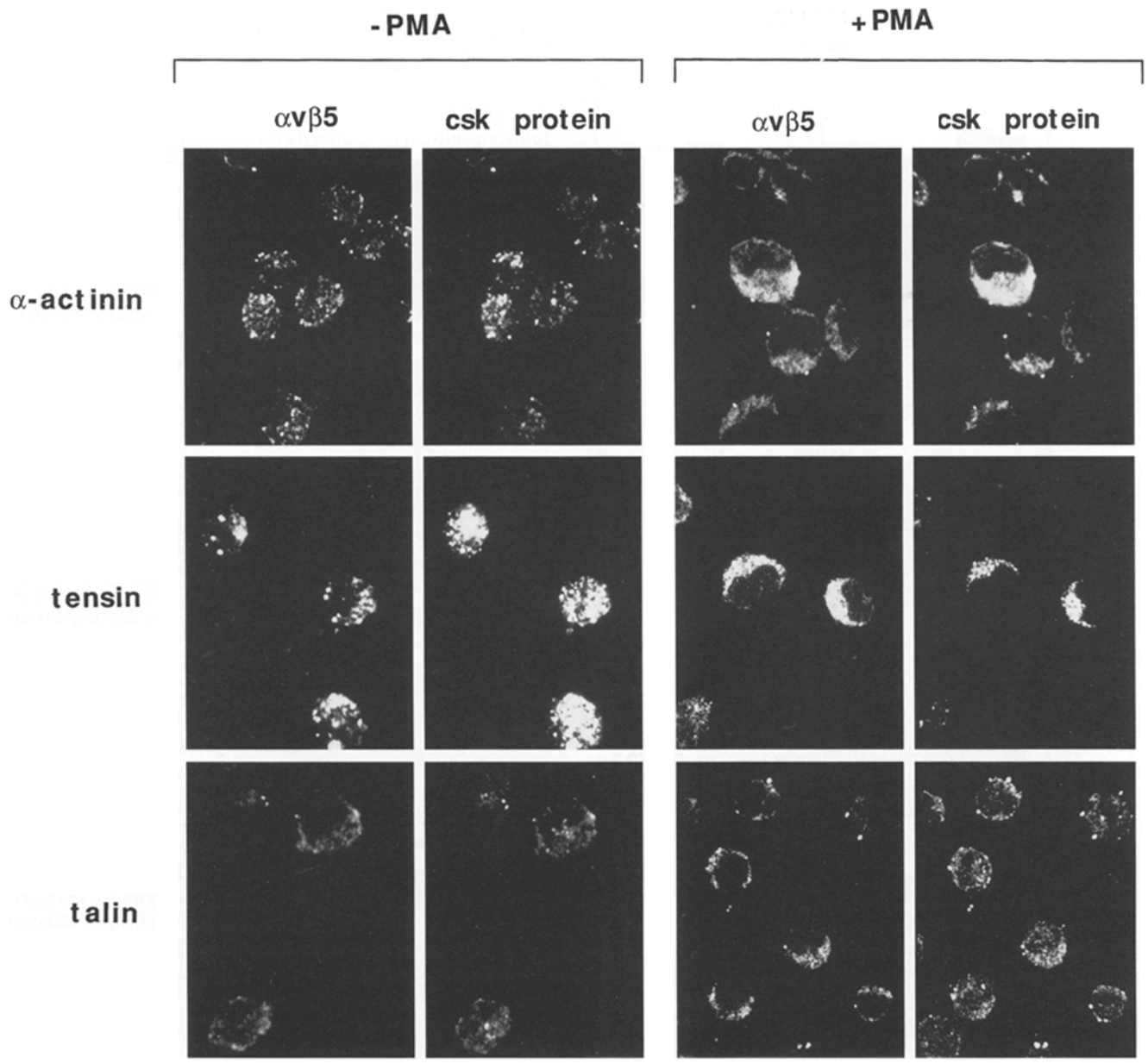
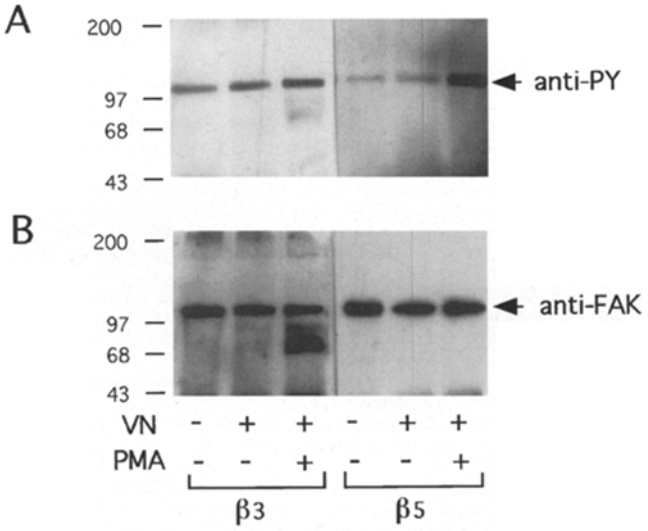


Figure 8. Confocal analysis of cells on coverslips. β5-transfected cells were plated onto glass coverslips coated with 10 mg/ml VN and allowed to attach for 30 min at 37°C. PMA at 10 nM was added to some samples (right half of figure), and all samples were incubated for an additional 30 min. Cells were fixed, processed for immunofluorescence, and double-labeled with antibodies directed against αvβ5 (P1F6) and either α-actinin, tensin, or talin. Samples were scanned with a laser confocal microscope, and photographs were taken of images near the cell–substratum interface. *csk*, cytoskeletal protein (listed to left of each set of photographs).

and to require activation of PKC. This is consistent with studies involving neutrophils, where exogenous activation has been shown to regulate the interaction between α-actinin and β2 (Pavalko and LaRoche, 1993). It is unknown how activation might regulate this binding at the molecular level or if the interaction is regulated for other β subunits. Our data also reveal that αvβ5 is unable to efficiently recruit talin, with or without activation. While cells were able to spread without recruiting talin, our data do not exclude its importance in cell spreading or migration mediated by other integrins.

Our data bear upon a number of other issues related to the organization of focal contacts. For example, FAK has been shown to bind peptides from the integrin β subunit cytoplasmic domain, talin, and $p130^{cas}$ (Chen et al., 1995; Polte and Hanks, 1995; Schaller et al., 1995). It is possible that talin or other proteins are present at undetectably low levels before activation of PKC. It does, however, appear that FAK is present in the absence of detectable levels of talin or $p130^{cas}$ and that upon activation, the level of $p130^{cas}$ increases without any increase in FAK. Thus, within the limits of the assay, it is unlikely that FAK requires talin or cas for its localization. Furthermore, if FAK binds $p130^{cas}$ in focal adhesions, that binding must be regu-



C

VN	-	+	+
PMA	-	-	+
β3	1.0	2.5	2.3
β5	1.0	0.9	2.3

Figure 9. Phosphorylation of FAK requires stimulation by PMA. Cells expressing either αvβ3 or αvβ5 were either kept in suspension or plated onto VN for 30 min; 10 nM PMA was added to some samples, and all samples were incubated for an additional 30 min. Cells were lysed with RIPA buffer, and FAK was immunoprecipitated (*A* and *B*). Samples were resolved on 6% gels, transferred to nitrocellulose, and blots were labeled with antibodies against phosphotyrosine (mAb 4G10; *A*) or FAK (mAb 2A7; *B*). Blots were quantitated by densitometry, with the level of FAK phosphorylation corrected for the amount of FAK immunoprecipitated for each sample (*C*). Fold activation is relative to cells in suspension.

lated. Our results also suggest that the associations of vinculin, tensin, and cas with αvβ5 do not depend on the presence of talin.

To our surprise, FAK was present at high levels in clusters of αvβ5 before activation, but this FAK contained low levels of phosphotyrosine. After activation of PKC, FAK phosphorylation on tyrosine increased substantially, consistent with reports that PKC regulates both αIIbβ3- and α5β1-mediated FAK phosphorylation (Vuori and Ruoslahti, 1993; Haimovich et al., 1996). However, the colocalization of FAK did not increase further. These results lead to the important and novel conclusion that FAK can associate with a component of focal adhesions before and independent of its activation. This conclusion is consistent with a report that FAK can bind to the β1 cytoplasmic region in vitro (Schaller et al., 1995) and with results showing that FAK is localized to clusters of α5β1 in the presence of cytochalasin D or tyrosine kinase inhibitors.

The integrins αvβ3 and αvβ5 have previously been shown to perform different functions. In addition to their differential localization on the cell surface (Wayner et al., 1991), an antibody blocking β3 has been shown to prevent tumor-induced angiogenesis, whereas an antibody against β5 had no effect (Brooks et al., 1994). These two integrins have also been shown to regulate two distinct pathways of angiogenesis (Friedlander et al., 1995). Furthermore, unlike αvβ3, αvβ5 can direct cell migration only after activation of PKC, which requires protein synthesis (Yebra et al., 1995). Taken together, these studies suggest that αvβ3 and αvβ5 integrins initiate distinct events following binding to the same ligand. Our data provide a molecular explanation for these phenomena.

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References

Auer, K.L., and B.S. Jacobson. 1995. β1 integrins signal lipid second messengers required during cell adhesion. *Mol. Biol. Cell.* 6:1305–1313.

Belkin, A.M., and V.E. Koteliansky. 1987. Interaction of iodinated vinculin, metavinculin and α-actinin with cytoskeletal proteins. *FEBS Lett.* 220:291–294.

Bellis, S.L., J.T. Miller, and C.E. Turner. 1995. Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. *J. Biol. Chem.* 270:17437–17441.

Brooks, P.C., R.A.F. Clark, and D.A. Cheresh. 1994. Requirement of vascular integrin αvβ3 for angiogenesis. *Science (Wash. DC).* 264:569–571.

Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: Transmembrane junctions between the extracellular matix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487–525.

Chen, H.-C., P.A. Appeddu, J.T. Parsons, J.D. Hildebrand, M.D. Schaller, and J.-L. Guan. 1995. Interaction of FAK with cytoskeletal protein talin. *J. Biol. Chem.* 270:16995–16999.

Cheresh, D.A. 1987. Human endothelial cells synthesize and express an arg-gly-asp directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc. Natl. Acad. Sci. USA.* 84:6471–6475.

Chun, J.-S., and B.S. Jacobson. 1992. Spreading of Hela cells on a collagen substratum requires a second messenger formed by the lipoxygenase metabolism of arachadonic acid released by collagen receptor clustering. *Mol. Biol. Cell.* 3:481–492.

Clyman, R.I., F. Mayray, and R.H. Kramer. 1992. β1 and β3 integrin have different roles in the adhesion and migration of vascular smooth muscle cells on extracellular matrix. *Exp. Cell Res.* 200:272–284.

Felding-Habermann, B., and D.A. Cheresh. 1993. Vitronectin and its receptors. *Curr. Opin. Cell Biol.* 5:864–868.

Filardo, E.J., P.C. Brooks, S.L. Deming, C. Damsky, and D.A. Cheresh. 1995. Requirement of the NPXY motif in the integrin β3 subunit cytoplasmic domain tail for melanoma cell migration in vitro and in vivo. *J. Cell Biol.* 130: 441–450.

Friedlander, M., P. Brooks, R. Shaffer, C. Kincaid, J. Varner, and D. Cheresh. 1995. Definition of two angiogenic pathways by distinct αv integrins. *Science (Wash. DC).* 270:1500–1502.

Haimovich, B., N. Kaneshiki, and J. Ping. 1996. Protein kinase C regulates tyrosine phosphorylation of pp125FAK in platelets adherent to fibrinogen. *Blood.* 87:152–161.

Horwitz, A., K. Duggan, C. Buck, M. Beckerle, and K. Burridge. 1986. Interaction of plasma fibronectin receptor with talin—a transmembrane linkage. *Nature (Lond.).* 320:531–533.

Hynes, R.O. 1992. Integrins: versatility, modulation and signaling in cell adhesion. *Cell.* 69:11–25.

Klemke, R.L., M. Yebra, E.M. Bayna, and D.A. Cheresh. 1994. Receptor tyrosine kinase signaling required for integrin αvβ5-directed cell motility but not adhesion on vitronectin. *J. Cell Biol.* 127:859–866.

Lewis, J.M., and M.A. Schwartz. 1995. Mapping in vivo associations of cytoplasmic proteins with integrin β1 cytoplasmic domain mutants. *Mol. Biol. Cell.* 6: 151–160.

Miekka, S.I., K.C. Ingham, and D. Menache. 1982. Rapid methods for isolation of human plasma fibronectin. *Thromb. Res.* 27:1–14.

Miyamoto, S., H. Termoto, O.A. Coso, J.S. Gutkind, P.D. Burbelo, S.K. Akiyama, and K.M. Yamada. 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J. Cell Biol.* 131:791–805.

Otey, C.A., F.M. Pavalko, and K. Burridge. 1990. An interaction between α-actinin and the β1 integrin subunit in vitro. *J. Cell Biol.* 111:721–729.

Pasqualini, R., and M.E. Hemler. 1994. Contrasting roles for integrin β1 and β5

cytoplasmic domains in subcellular localization, cell proliferation and cell migration. *J. Cell Biol.* 125:447–460.

Pavalko, F., and S. LaRoche. 1993. Activation of human neutrophils induces an interaction between the integrin β2 subunit (CD-18) and the actin binding protein α-actinin. *J. Immunol.* 151:3795–3807.

Polte, T., and S. Hanks. 1995. Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130cas. *Proc. Natl. Acad. Sci. USA.* 92:10678–10682.

Reszka, A.A., Y. Hayashi, and A.F. Horwitz. 1992. Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117:1321–1330.

Sastry, S.K., and A.F. Horwitz. 1993. Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr. Opin. Cell Biol.* 5:819–831.

Schaller, M.D., and J.T. Parsons. 1995. pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high affinity binding site for crk. *Mol. Biol. Cell.* 15:2635–2645.

Schaller, M.D., C.A. Otey, J.D. Hildebrand, and J.T. Parsons. 1995. Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. *J. Cell Biol.* 130:1181–1187.

Schwartz, M.A., M.D. Schaller, and M.H. Ginsbrg. 1995. Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Biol.* 11:549–599.

Tapley, P., A. Horwitz, C. Buck, K. Burridge, K. Duggan, and L. Rohrschneider. 1989. Analysis of the avian fibronectin receptor (integrin) as a direct substrate for pp60v-src. *Oncogene.* 4:325–333.

Thomas, L., P.W. Chan, S. Chang, and C. Damsky. 1993. 5-Bromo-2-deoxyuridine regulates invasiveness and expression of integrins and matrix-degrading proteinases in a differentiated hamster melanoma cell. *J. Cell Sci.* 105:191–201.

Turner, C.E., J.R. Glenney, and K. Burridge. 1990. Paxillin: a new vinculin-binding protein present in focal adhesions. *J. Cell Biol.* 111:1059–1068.

Vuori, K., and E. Ruoslahti. 1993. Activation of protein kinase C precedes α5β1 integrin-mediated cell spreading on fibronectin. *J. Biol. Chem.* 268:21459–21462.

Wachsstock, D.H., J.A. Wilkins, and S. Lin. 1987. Specific interactions of vinculin with α-actinin. *Biochem. Biophys. Res. Commun.* 146:554–560.

Wayner, E.A., R.A. Orlando, and D.A. Cheresh. 1991. Integrins αvβ3 and αvβ5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *J. Cell Biol.* 113:919–929.

Wilkins, J.A., M.A. Risinger, and S. Lin. 1986. Studies of proteins that co-purify with smooth muscle vinculin: identification of immunologically related species in focal adhesions of nonmuscle and Z-lines of muscle cells. *J. Cell Biol.* 103:1483–1494.

Woods, A., and J.R. Couchman. 1992. Protein kinase C involvement in focal adhesion formation. *J. Cell Sci.* 101:277–290.

Yatohgo, T., M. Izumi, H. Kashiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparan affinity chromatography. *Cell. Struct. Funct.* 13:281–292.

Yebra, M., E.J. Filardo, E.M. Bayna, E. Kawahara, J.C. Becker, and D.A. Cheresh. 1995. Induction of carcinoma cell migration on vitronectin by NF-κB-dependent gene expression. *Mol. Biol. Cell.* 6:841–850.