Restructuring of Focal Adhesion Plaques by PI 3-Kinase: Regulation by PtdIns (3,4,5)-P_3 Binding to α-Actinin

Jeffrey A. Greenwood,* Anne B. Theibert,‡ Glenn D. Prestwich,§ and Joanne E. Murphy-Ullrich*

*Department of Pathology, Division of Molecular and Cellular Pathology, ‡Department of Neurobiology, University of Alabama at Birmingham, Birmingham, Alabama 35294; and §Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84112

Abstract. Focal adhesions are an elaborate network of interconnecting proteins linking actin stress fibers to the extracellular matrix substrate. Modulation of the focal adhesion plaque provides a mechanism for the regulation of cellular adhesive strength. Using interference reflection microscopy, we found that activation of phosphoinositide 3-kinase (PI 3-kinase) by PDGF induces the dissipation of focal adhesions. Loss of this close apposition between the cell membrane and the extracellular matrix coincided with a redistribution of α-actinin and vinculin from the focal adhesion complex to the Triton X-100–soluble fraction. In contrast, talin and paxillin remained localized to focal adhesions, suggesting that activation of PI 3-kinase induced a restructuring of the plaque rather than complete dispersion. Furthermore, phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)-P_3), a lipid product of PI 3-kinase, was sufficient to induce restructuring of the focal adhesion plaque. We also found that PtdIns (3,4,5)-P_3 binds to α-actinin in PDGF-treated cells. Further evidence demonstrated that activation of PI 3-kinase by PDGF induced a decrease in the association of α-actinin with the integrin β subunit, and that PtdIns (3,4,5)-P_3 could disrupt this interaction in vitro. Modification of focal adhesion structure by PI 3-kinase and its lipid product, PtdIns (3,4,5)-P_3, has important implications for the regulation of cellular adhesive strength and motility.

Key words: cell motility • phosphoinositide 3-kinase • PDGF • integrin • vinculin

Introduction

Cell adhesion is an important mechanism by which cells interact with the extracellular environment. Cell-surface receptors bind specific extracellular matrix components, thereby initiating signaling pathways that regulate the organization of the cytoskeleton and gene expression (Burridge and Chrzanowska-Wodnicka, 1996; Gumbiner, 1996). The adhesive state of a cell has significant influence on growth and survival, motility, and signal transduction (Meredith et al., 1996; Howe et al., 1998). Cell adhesion to the extracellular matrix is a continuous process that can be separated into three stages: (1) attachment, (2) spreading, and (3) formation of focal adhesions and stress fibers (Couchman and Woods, 1996; Greenwood and Murphy-Ullrich, 1998). Focal adhesions are the points of closest apposition between the cell membrane and the extracellular matrix substrate (Izzard and Lochner, 1976). These plaquelike structures link bundles of actin microfilaments, or stress fibers, to the extracellular matrix via transmembrane integrin and syndecan receptors, resulting in firm adherence of the cell to the substrate.

Numerous structural and signaling proteins have been localized to the focal adhesions of adherent cells (Miyamoto et al., 1995, 1996; Plopper et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996). α-Actinin, vinculin, and talin are important structural components involved in the formation and stability of focal adhesions. α-Actinin and talin have been demonstrated to link actin filaments directly to integrin receptors (Pavalko et al., 1991; Otey et al., 1993). α-Actinin is also an actin-binding and cross-linking protein that may play an important role in the regulation of stress fibers (Blanchard et al., 1989). Vinculin does not bind directly to integrins, but does link actin filaments to integrin receptors through interactions with other focal adhesion proteins, such as talin (Craig and Johnson, 1996). Although specific protein–protein interactions between

Address correspondence to Jeffrey A. Greenwood, Department of Biochemistry and Biophysics, 2103B Agricultural and Life Sciences, Oregon State University, Corvallis, OR 97331-7305. Tel.: (541) 737-4997. Fax: (541) 737-0481. E-mail: jeffrey.greenwood@orst.edu

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many focal adhesions proteins have been identified, it is not clear how this multimeric complex of proteins organize to form a focal adhesion.

The formation of focal adhesions is initiated by the binding of integrins to specific extracellular matrix ligands and subsequent clustering of these receptors (Burridge and Chrzanowska-Wodnicka, 1996). Strong evidence implicates the small GTP-binding protein Rho in the formation of focal adhesions and stress fibers (Hall, 1998). Activation of Rho during adhesion has been linked to myosin-mediated contractility (Chrzanowska-Wodnicka and Burridge, 1996) and the activation of PI 5-kinase and phosphatidylinositol (4,5)-bisphosphate (PtdIns (4,5)-P_2)[1] production (Chong et al., 1994). PtdIns (4,5)-P_2 appears to be essential for the formation of focal adhesions and stress fibers, potentially by regulating the structure and function of focal adhesion proteins such as α-actinin and vinculin (Fukami et al., 1992; Craig and Johnson, 1996; Gilmore and Burridge, 1996). Both proteins have been demonstrated to bind to different phospholipids and exhibit a high affinity for PtdIns (4,5)-P_2 (Burn et al., 1985; Fukami et al., 1992; Johnson and Craig, 1995; Craig and Johnson, 1996; Gilmore and Burridge, 1996). PtdIns (4,5)-P_2 binding to α-actinin enhances its ability to promote actin polymerization (Fukami et al., 1992). PtdIns (4,5)-P_2 binding to vinculin increases its binding to both actin filaments and the focal adhesion protein talin (Craig and Johnson, 1996; Gilmore and Burridge, 1996). Talin also has been demonstrated to bind PtdIns (4,5)-P_2 (Heraud et al., 1998), however, the functional significance of this interaction is not clear. The understanding of the mechanisms involved in the formation of focal adhesions has advanced dramatically in recent years; however, regulation of the plaque after the initial formation is only beginning to be addressed.

Extracellular factors exist that regulate cell function by stimulating reversal of one or more of the stages of the adhesion process. Modification of the structural link between the cytoskeleton and the focal adhesion plaque is an important target for the regulation of cell adhesiveness. This laboratory and others have demonstrated that the extracellular matrix proteins thrombospondin (TSP; Murphy-Ullrich and Hook, 1989; Murphy-Ullrich et al., 1993), tenascin (Murphy-Ullrich et al., 1991; Chung et al., 1996), and SPARC (Murphy-Ullrich et al., 1995), induce the disassembly of focal adhesions in bovine aortic endothelial (BAE) cells. In previous studies, we demonstrated that alteration of the integrity of focal adhesions by the extracellular matrix protein TSP involved the activation of phosphoinositide 3-kinase (PI 3-kinase) and the generation of its lipid product phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)-P_3; Greenwood et al., 1998). In these studies, we sought to determine how PtdIns (3,4,5)-P_3 acts to modify these structures. We found that the activation of PI 3-kinase by PDGF induces restructuring of focal adhesion plaques in rat embryonic fibroblasts (REF). Furthermore, our results show that PtdIns (3,4,5)-P_3, a lipid product of PI 3-kinase, is sufficient for inducing restructuring of focal adhesion plaques. We also present strong evidence that PtdIns (3,4,5)-P_3 binds to α-actinin in PDGF-treated cells, disrupting its interaction with the integrin β subunit. These results suggest that PtdIns (3,4,5)-P_3, as well as other phosphoinositides, regulate the composition and structure of focal adhesion plaques and, potentially, the adhesive strength of the cell.

[1]*Abbreviations used in this paper:* BAE, bovine aortic endothelial; GST, glutathione-S-transferase; IRM, interference reflection microscopy; PI 3-kinase, phosphoinositide 3-kinase; PtdIns-P, phosphatidylinositol-phosphate; PtdIns (4)-P, phosphatidylinositol (4)-phosphate; PtdIns (4,5)-P_2, phosphatidylinositol (4,5)-bisphosphate; PtdIns (3,4)-P_2, phosphatidylinositol (3,4)-bisphosphate; PtdIns (3,4,5)-P_3, phosphatidylinositol (3,4,5)-trisphosphate; REF, rat embryonic fibroblast; TSP, thrombospondin.

Materials and Methods

Reagents and Antibodies

Phospholipids were purchased from Matreya or Echelon Research Laboratories Inc. Antiphosphotyrosine antibodies (PY20 and RC20) were purchased from Transduction Laboratories. Anti–α-actinin (clone BM75.2), antivinculin (clone VIN-11-5), antitalin (clone 8D4), purified α-actinin, and wortmannin were from Sigma Chemical Co. Antipaxillin (clone Z035) was from Zymed Laboratories. Anti–β_3 integrin IgG (clone F-11) and anti-PDGF receptor (958) were purchased from Santa Cruz Biotechnology, Inc. Anti–β_3 integrin IgM (clone 26) was purchased from Transduction Laboratories; anti–$\alpha_5\beta_1$ integrin was from GIBCO BRL; and human recombinant PDGF-BB was from Upstate Biotechnology. LY294002 was purchased from BIOMOL. All other reagents were purchased from Sigma Chemical Co.

Cell Culture

REFs (Woods and Couchman, 1992; a gift from Dr. Anne Woods, University of Alabama at Birmingham, Birmingham, AL) were cultured in DME containing 4.5 g/liter glucose, 2 mM glutamine, and 10% FBS following previously described procedures (Murphy-Ullrich et al., 1993). BAE cells were isolated and cultured as previously described (Murphy-Ullrich et al., 1993).

Interference Reflection Microscopy (IRM)

Focal adhesion assays were performed as previously described (Murphy-Ullrich et al., 1993). In brief, REFs were grown on glass coverslips in 10% FBS-DME until 80% confluence. The attached cells were rinsed three times with 0.2% FBS-DME and incubated for 18 h in 0.2% FBS-DME. Cells were rinsed three times with warm serum-free DME to remove serum components and incubated in serum-free DME for 1 h at 37°C. Cells were treated with 30 ng/ml PDGF for various times, fixed with warm 3% glutaraldehyde in serum-free DME, and examined by IRM using a Zeiss Axiovert 10 microscope. A minimum of 100 cells/condition was evaluated for the presence of focal adhesions. A cell was scored positive if it contained at least five adhesion plaques.

Fluorescence Microscopy

REFs were stained with rhodamine-phalloidin (Molecular Probes) or antibodies recognizing α-actinin, vinculin, talin, paxillin, and $\alpha_5\beta_1$ integrin. Cells double labeled with rhodamine-phalloidin (1:400) and anti–α-actinin (1:500) were extracted with Triton X-100 buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EGTA, 5 mM EDTA, 100 μM Na_3VO_4, 50 mM sodium pyrophosphate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5% Triton X-100) for 2 min on ice, followed by fixation with 3% formaldehyde (Tousimis) in PBS for 30 min at room temperature. Cells stained for vinculin (1:20), talin (1:20), and paxillin (1:20), were fixed with 3% formaldehyde in Triton X-100 buffer for 30 min at room temperature. After fixation, cells were washed with PBS, blocked with 1% BSA in PBS, incubated with antibodies, and examined using a Zeiss Axiovert 10 microscope. In some experiments, coverslips were coated with vitronectin or fibronectin (Collaborative Biomedical Products) as described previously (Murphy-Ullrich and Hook, 1989).

Immunoprecipitation

Cells were scraped into ice-cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2 mM Na_3VO_4, 1% Triton X-100, 0.5% NP-40, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and precleared by

centrifugation. Supernatants (1 mg/ml) were incubated with 4 μg/ml anti–p85-PI 3-kinase, 10 μg/ml PY20, 10 μl anti–α-actinin, 5 μg/ml anti–β_3 integrin, or 10 μl antitalin for 2 h on ice. 10 μl of either protein A–Sepharose or anti–IgM-agarose, blocked with 2 mg/ml ovalbumin, was added and incubated for 1 h at 4°C with shaking. Immunoprecipitates were washed three times in lysis buffer and assayed for lipid content, PI 3-kinase activity, or immunoblotted. For each procedure, the identity of the immunoprecipitated protein was confirmed by immunoblotting. Protein staining of nitrocellulose blots with Ponceau S was also used to verify that equivalent amounts of the protein was immunoprecipitated from the sample.

Immunoblotting

Protein was eluted from the immunoprecipitates by adding 60 μl of SDS-PAGE sample buffer, followed by incubation at 100°C for 2 min. Samples were resolved by electrophoresis on SDS–polyacrylamide gels and transferred to nitrocellulose. Blots were blocked, probed, and developed by enhanced chemiluminescence (NEN Research Products).

Phosphorus-32 Labeling of REFs and Lipid Analysis

Analysis of PDGF-stimulated incorporation of phosphorus-32 into the lipids in intact cells was performed as described (Greenwood et al., 1998). REFs were grown to ~80% confluency in 100-mm culture dishes. Cells were incubated for 18 h in 0.2% FBS-DME. The attached cells were rinsed three times with phosphate-free DME and incubated for 1 h in phosphate-free DME containing 0.25 mCi/ml $^{32}P_i$ (ICN). Cells were stimulated with 30 ng/ml PDGF and the media were aspirated. To determine the phosphoinositides associated with specific proteins (Chellaiah and Hruska, 1996; Heraud et al., 1998), the cells were lysed with ice-cold lipid lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 1% Triton X-100, 0.5% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 50 mM NaF, and 30 mM sodium pyrophosphate) and immunoprecipitated as described above. After immunoprecipitation, lipids were extracted by adding 160 μl of methanol/chloroform (1:1), followed by the addition of 80 μl of 1 M HCl. Samples were centrifuged to separate the phases, and the lower phase was spotted on Silica Gel 60 plates (Whatman) precoated with 1% potassium oxalate. The TLC plates were developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7) and exposed for autoradiography using BioMax MS film and a TranScreen HE intensifying screen (Amersham Pharmacia Biotech). Isolation of the total lipid from $^{32}P_i$-labeled cells was carried out as previously described (Greenwood et al., 1998). The positions PtdIns-P, PtdIns-P_2, and PtdIns (3,4,5)-P_3 migrated on the TLC plate was visualized by exposing the plate to iodine vapor and identified using purified standards. ^{32}P-labeled standards were also prepared using immunoprecipitated PI 3-kinase (anti-p85 PI 3-kinase; Upstate Biotechnology) to phosphorylated purified phospholipids.

PI 3-kinase Assay

PI 3-kinase activity was measured in antiphosphotyrosine immunoprecipitates using PtdIns (4,5)-P_2 (American Radiolabeled Chemicals) as a substrate exactly as described previously (Greenwood et al., 1998).

Inositol(1,3,4,5)P_4 Affinity Chromatography

20 μg of purified α-actinin, 500 μg REF cell lysate, or 500 μg BAE cell lysate, was incubated with 50 μl of a 1:1 slurry of Affigel-conjugated aminopropyl-inositol(1,3,4,5)P_4 (Hammonds-Odie et al., 1996; Theibert et al., 1997; Blader et al., 1999) for 1 h at 4°C in binding buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EGTA, 0.2 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The beads were washed three times with 1 ml of binding buffer containing 200 mM NaCl. Protein was eluted from the beads by adding 60 μl of SDS-PAGE sample buffer followed by incubation at 100°C for 2 min. Samples were immunoblotted as described above.

Phosphoinositide Disruption of α-Actinin Binding to β_3 Integrin

β_3 Integrin was immunoprecipitated from REF cell lysate as described above. Immunoprecipitates were washed twice with lipid incubation buffer (10 mM Hepes, pH 7.0, and 1 mM EDTA) and incubated with 20 μg of the indicated lipid, which had been sonicated into 500 μl of lipid incubation buffer, for 30 min at room temperature. Immunoprecipitates were washed twice with lysis buffer, protein was eluted by adding 60 μl of SDS-PAGE sample buffer, followed by incubation at 100°C for 2 min, and immunoblotted as described above.

α-Actinin Binding to GST-β1 Cytoplasmic Tail

The construct encoding the glutathione-S-transferase (GST)–β1 cytoplasmic tail was provided by Dr. Fredrick M. Pavalko (Indiana University School of Medicine, Indianapolis, ID). The fusion proteins were expressed and purified as described previously (Sampath et al., 1998). 200 nM of purified α-actinin was incubated with the 200-nM GST-β1 cytoplasmic tail for 1 h at room temperature in PBS. The protein mixture was added to anti–α-actinin, bound to anti–IgM-agarose, and incubated with shaking for 30 min at room temperature. The complex was washed three times with lysis buffer, and the protein was eluted and immunoblotted. In some experiments, the complex was further incubated in the presence of various phospholipids as described above.

Results

Activation of PI 3-kinase by PDGF in REFs

Several studies have demonstrated that PDGF stimulates potent activation of p85/p110 PI 3-kinase in various cell culture systems (Heldin et al., 1998). PI 3-kinase is activated by association of the p85 regulatory subunit with specific tyrosine-phosphorylated residues on the PDGF receptor (Heldin et al., 1998). In REFs, a time and concentration-dependent tyrosine phosphorylation of the PDGF receptor was observed. Tyrosine phosphorylation of the PDGF receptor was observed as early as 2 min after addition of PDGF, peaking at 10 min and decreasing significantly by 60 min (Fig. 1 A). Tyrosine phosphorylation of the PDGF receptor was detected at concentrations of PDGF as low as 10 ng/ml, with maximum activation occurring at 30 ng/ml (Fig. 1 B). Therefore, a concentration of 30 ng/ml PDGF was used for the remaining experiments. The association of tyrosine-phosphorylated PDGF receptor with the p85 regulatory subunit of PI 3-kinase was observed after the same time course (Fig. 1 C). Activation of PI 3-kinase also peaked at 10 min (Fig. 1 D), resulting in an increase in the level of PtdIns (3,4,5)-P_3 (Fig. 1 E). The PDGF-induced increase in PtdIns (3,4,5)-P_3 was inhibited by preincubation with 100 nM wortmannin, a specific inhibitor of PI 3-kinase activity. Basal levels of PtdIns (3,4,5)-P_3 were also decreased in wortmannin-treated cells, however, the levels of phosphatidylinositol (4)-phosphate (PtdIns (4)-P) and PtdIns (4,5)-P_2 were not significantly affected (data not shown). These results demonstrate that PDGF stimulates activation of PI 3-kinase and the production of PtdIns (3,4,5)-P_3 in REFs, which are consistent with previously characterized systems (Heldin et al., 1998).

Activation of PI 3-kinase by PDGF Induces Focal Adhesion Disassembly

Focal adhesions are plaquelike accumulations of proteins linking actin stress fibers to the extracellular matrix via transmembrane receptors, resulting in firm adherence of the cell to the substrate (Greenwood and Murphy-Ullrich, 1998). PDGF is a potent stimulator of cell motility and proliferation (Heldin et al., 1998). These two cellular responses involve changes in cell adhesion. To determine if PDGF modulates the structure of focal adhesions, REFs were examined using IRM. Since focal adhesions are the points of closest apposition, ~10–15 nm, between the cell membrane

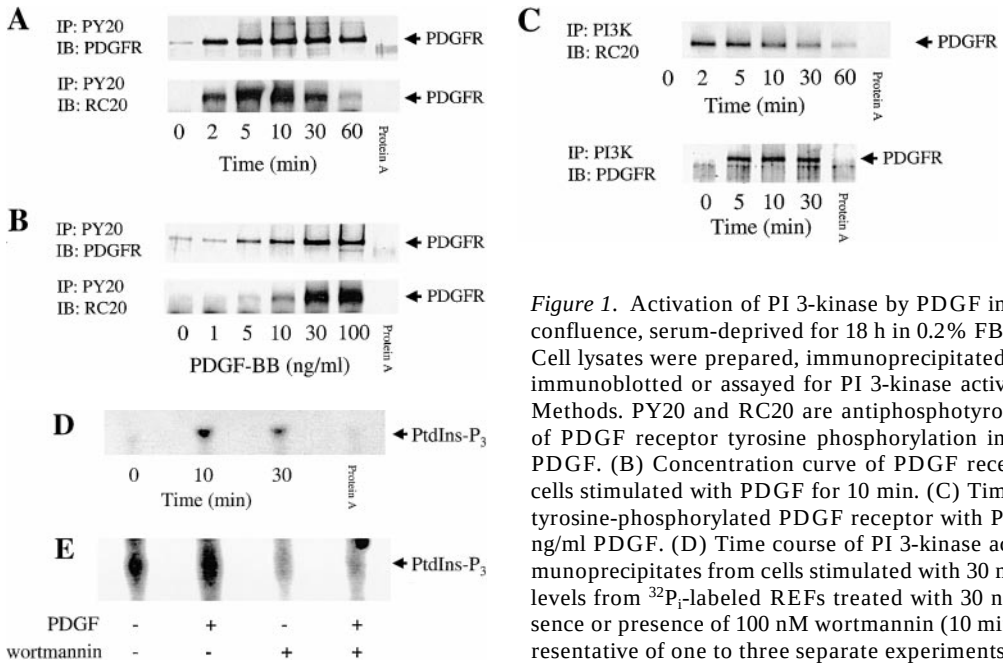


Figure 1. Activation of PI 3-kinase by PDGF in REFs. Cells were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, and treated as indicated. Cell lysates were prepared, immunoprecipitated with the indicated antibody, and immunoblotted or assayed for PI 3-kinase activity as described in Materials and Methods. PY20 and RC20 are antiphosphotyrosine antibodies. (A) Time course of PDGF receptor tyrosine phosphorylation in REFs stimulated with 30 ng/ml PDGF. (B) Concentration curve of PDGF receptor tyrosine phosphorylation in cells stimulated with PDGF for 10 min. (C) Time course of the association of the tyrosine-phosphorylated PDGF receptor with PI 3-kinase in cells treated with 30 ng/ml PDGF. (D) Time course of PI 3-kinase activity in antiphosphotyrosine immunoprecipitates from cells stimulated with 30 ng/ml PDGF. (E) PtdIns (3,4,5)-P_3 levels from $^{32}P_i$-labeled REFs treated with 30 ng/ml PDGF for 10 min in the absence or presence of 100 nM wortmannin (10 min preincubation). Results are representative of one to three separate experiments.

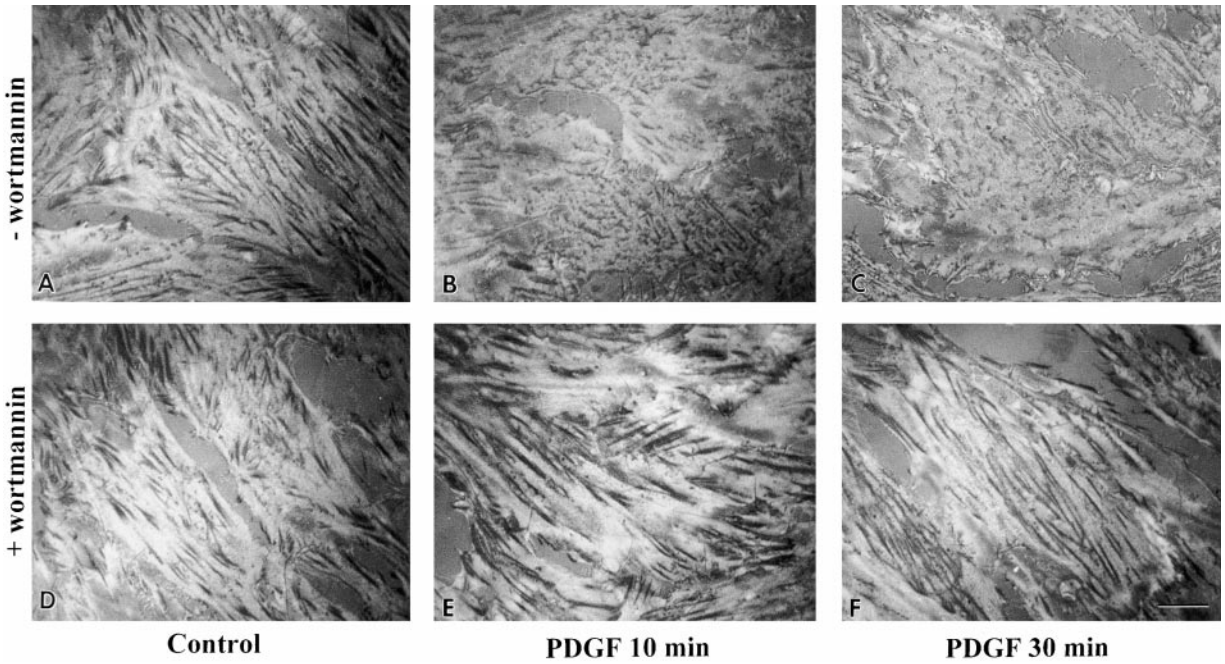
and the extracellular matrix substrate, IRM is an established method for examining the structural integrity of focal adhesions (Izzard and Lochner, 1976; Verschueren, 1985; Greenwood and Murphy-Ullrich, 1998). REFs were grown to 80% confluency and serum-deprived overnight as previously described in Materials and Methods. Before treatment with PDGF, the cells were washed and incubated with serum-free media to remove serum components. Under these conditions, a majority of the cells contained several large and stable focal adhesions, characterized by intense black streaks organized longitudinally throughout the cells (Fig. 2 A). In REFs treated with PDGF for 10 min, the black streaks representing 10–15-nm contacts between the cell membrane and the substrate were largely absent, being replaced by smaller, more diffuse structures (Fig. 2 B). By 30 min of PDGF treatment, focal adhesions were completely lost throughout the cells (Fig. 2 C), however, the cells remained attached and spread. A majority of the cells responded to PDGF treatment as described (Fig. 2 G).

PI 3-kinase is a well characterized mediator of signaling by the PDGF receptor involved in reorganization of the actin cytoskeleton (Heldin et al., 1998). To determine if PI 3-kinase activity is required for the PDGF-induced loss of focal adhesions, REFs were treated after preincubation with wortmannin, an inhibitor of PI 3-kinase activity acting at the lipid-binding site. Wortmannin (100 nM) completely blocked the focal adhesion disassembly induced by PDGF at 10 and 30 min (Fig. 2, E–G). Lower concentrations of wortmannin were also effective with an IC_{50} of ~20 nM (data not shown). 100 nM wortmannin alone did not have any significant effects on the cells (Fig. 2 D). However, it does appear that the focal adhesions of some cells were enhanced in the presence of both PDGF and wortmannin (Fig. 2, E and F). 25 μM LY294002, an inhibitor of PI 3-kinase activity which competes for the ATP-binding site, also blocked the loss of focal adhesions stimulated by PDGF (data not shown).

Activation of PI 3-kinase by PDGF Induces Redistribution of Specific Focal Adhesion Proteins

To understand the mechanism for the loss of focal adhesion structure observed using IRM, the effect of PI 3-kinase activation by PDGF on specific focal adhesion proteins was examined by fluorescence microscopy. α-Actinin is an actin-bundling protein involved in the formation of actin stress fibers and the anchoring of stress fibers to focal adhesions (Pavalko et al., 1991). Previous studies demonstrated that the accessibility of antibodies to α-actinin localized to focal adhesions is limited (Pavalko et al., 1995). Detergent extraction of the cells before fixation increases accessibility, resulting in clear localization of α-actinin to focal adhesions (Greenwood et al., 1998). Extraction removes the Triton X-100–soluble fraction, leaving the insoluble cytoskeleton intact, which can be fixed and probed using specific antibodies. One advantage of the extraction procedure is that it allows the clear identification of proteins redistributing between the two fractions.

REFs were treated in the absence or presence of PDGF for 10 or 30 min, extracted, fixed, and stained for α-actinin as described in Materials and Methods. Cells were double labeled with rhodamine-phalloidin to visualize the actin microfilaments. In control cells, actin stress fibers were observed throughout the cell body, terminating at the focal adhesions (Fig. 3 a). Intense α-actinin staining was localized to the focal adhesions, with a less intense beaded staining pattern observed along the actin stress fibers (Fig. 3 A). When REFs were stimulated with PDGF for 10 min, α-actinin staining was almost completely removed by Triton X-100 extraction (Fig. 3 B). In contrast, the actin cytoskeleton remained insoluble although the microfilament bundles were absent and a finer filamentous phalloidin-staining actin network was observed (Fig. 3 b). Although some α-actinin colocalized with phalloidin-staining membrane ruffles (Fig. 3 c) after 30 min of PDGF treatment, much of the α-actinin



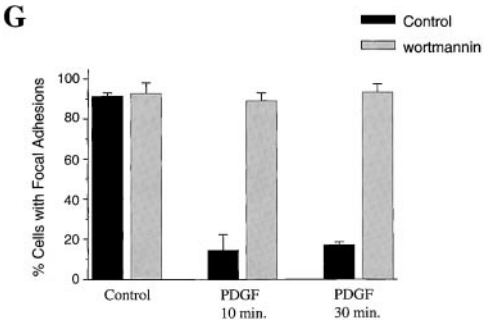
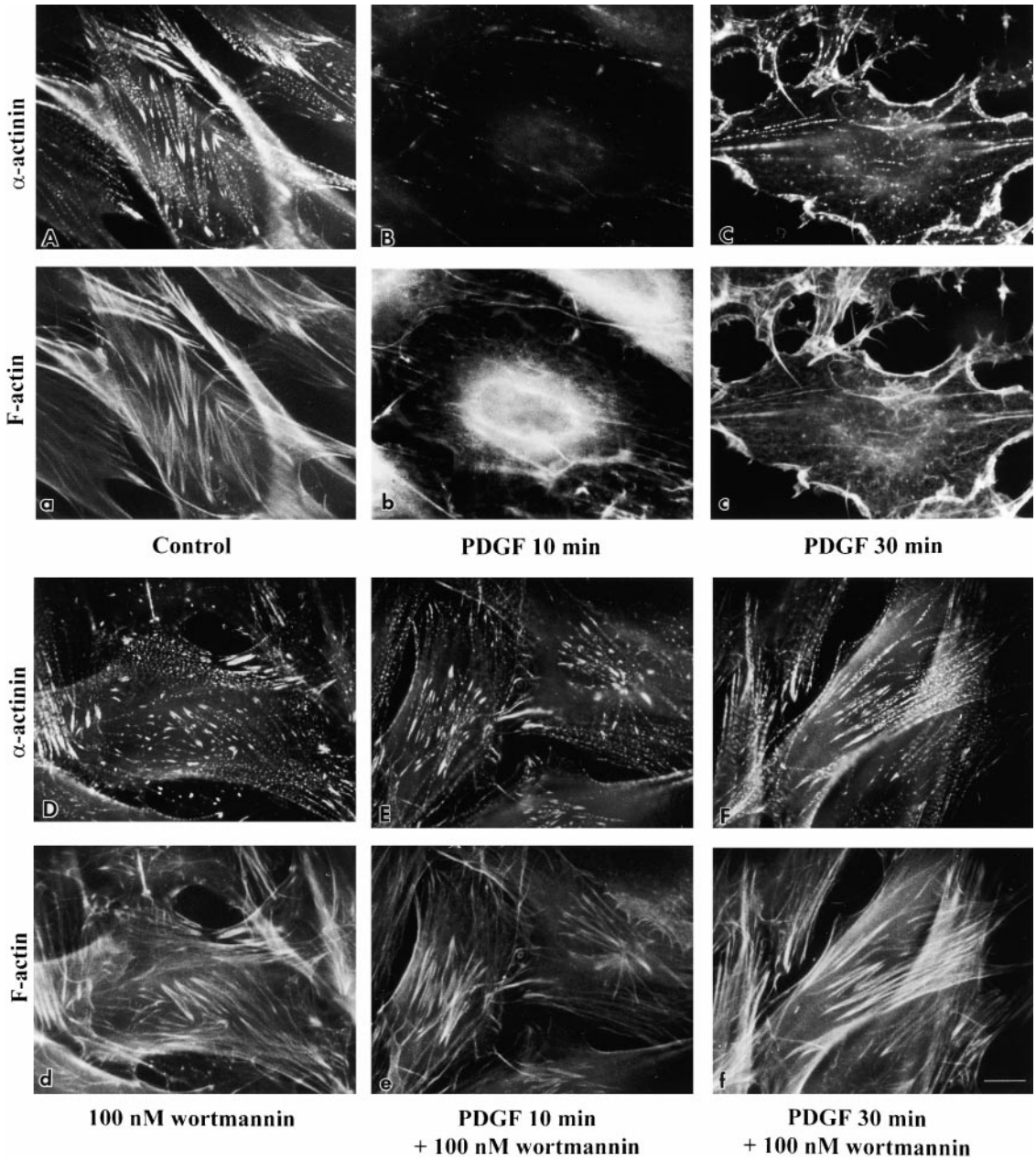


Figure 2. Activation of PI 3-kinase by PDGF induces focal adhesion disassembly. REFs were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, rinsed and incubated in DME for 1 h, treated as indicated, fixed with 3% glutaraldehyde, and examined by IRM as described in Materials and Methods. (A) Control; (B) PDGF 10 min; (C) PDGF 30 min; (D) wortmannin; (E) PDGF 10 min + wortmannin; (F) PDGF 30 min + wortmannin. The concentration of PDGF was 30 ng/ml. Cells were preincubated with 100 nM wortmannin for 10 min. The percent of cells positive for focal adhesions was quantitated for each treatment (G; n = 3–4); error bars represent SEM.

appeared to be lost during extraction (Fig. 3 C). In cells that were fixed without extraction, PDGF induced an increase in diffuse α-actinin staining throughout the cell body (data not shown). Preincubation of the cells with 100 nM wortmannin (Fig. 3 E, e, F, and f) or 50 μM LY294002 (data not shown) completely inhibited the PDGF-induced redistribution of α-actinin and reorganization of the actin cytoskeleton. 100 nM of wortmannin alone did not have any significant effects on α-actinin localization and organization of the actin cytoskeleton (Fig. 3, D and d). To confirm the redistribution of α-actinin from the Triton X-100–insoluble cytoskeleton to the soluble cellular fraction, immunoblot analysis of the soluble and insoluble fractions was performed. In control cells, only a small percentage of the total α-actinin was found in the soluble fraction. However, after treatment with PDGF, an increase in soluble α-actinin was observed (Fig. 3 G). Preincubation of the cells with 100 nM wortmannin inhibited the increase in soluble α-actinin. In addition, a wortmannin-sensitive decrease in insoluble α-actinin was observed after PDGF treatment (Fig. 3 H). These results suggest that the activation of PI 3-kinase by PDGF induces the redistribution of α-actinin from the insoluble focal adhesion plaques and actin cytoskeleton to the soluble cellular fraction.

Vinculin is a structural protein that interacts with other focal adhesion components to form the plaque (Burridge and Chrzanowska-Wodnicka, 1996). Although vinculin can be localized to the focal adhesions in fixed cells, the visualization of vinculin staining in focal adhesions is improved by fixing the cells in the presence of a detergent that removes much of the soluble vinculin as described in Materials and Methods. In control cells, vinculin staining was present in a characteristic focal adhesion pattern (Fig. 4 A). Similar to α-actinin, PDGF treatment of REFs for 10 and 30 min induced an almost complete loss of vinculin staining with no vinculin-staining focal adhesions present (Fig. 4, B and C). Preincubation of the cells with 100 nM wortmannin (Fig. 4, E and F) or 50 μM LY294002 (data not shown) completely inhibited the PDGF-induced loss of vinculin staining. Wortmannin alone did not appear to have any significant effects on vinculin localization (Fig. 4 D). These results suggest that the stimulation of PI 3-kinase activity by PDGF induces the redistribution of vinculin from the Triton X-100–insoluble focal adhesion to the soluble cellular fraction.

Thus far, our results had demonstrated that PDGF activation of PI 3-kinase induced the loss of focal adhesion integrity as determined by IRM, the loss of actin stress fibers, and the loss of α-actinin and vinculin-staining focal adhesions. To determine if PDGF induced complete dissipation of the focal adhesion plaque, the distribution of talin and paxillin was examined. Talin is a well characterized focal adhesion protein



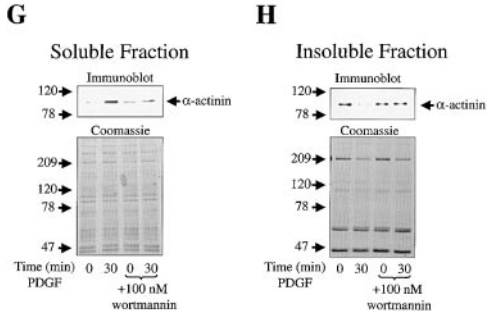


Figure 3. Activation of PI 3-kinase by PDGF induces redistribution of α-actinin. REFs were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, rinsed and incubated in DME for 1 h, treated as indicated, and processed for fluorescence microscopy as described in Materials and Methods. Cells were double labeled with anti–α-actinin (A–F) and phalloidin (a–f). (A) Control; (B) PDGF 10 min; (C) PDGF 30 min; (D) wortmannin; (E) PDGF 10 min + wortmannin; (F) PDGF 30 min + wortmannin. Results are representative of 6–13 separate experiments. The soluble (G) and insoluble (H) fractions, which were extracted with Triton X-100 buffer, were separated by electrophoresis and immunoblotted using anti–α-actinin (1:10,000) or stained with Coomassie blue as described in Materials and Methods. Results are representative of one to three separate experiments. The concentration of PDGF was 30 ng/ml. Cells were preincubated with 100 nM wortmannin for 10 min.

that interacts directly with the β subunit of integrins within the plaque (Burridge and Chrzanowska-Wodnicka, 1996). In control cells, talin staining was localized to the focal adhesions (Fig. 5 A). After a 10- and 30-min treatment with PDGF, talin-staining focal adhesions remained intact (Fig. 5, B and C). Interestingly, the structure of the talin-staining focal adhesions after 30-min PDGF treatment appeared slightly elongated (Fig. 5 C). Paxillin is a highly tyrosine-phosphory-

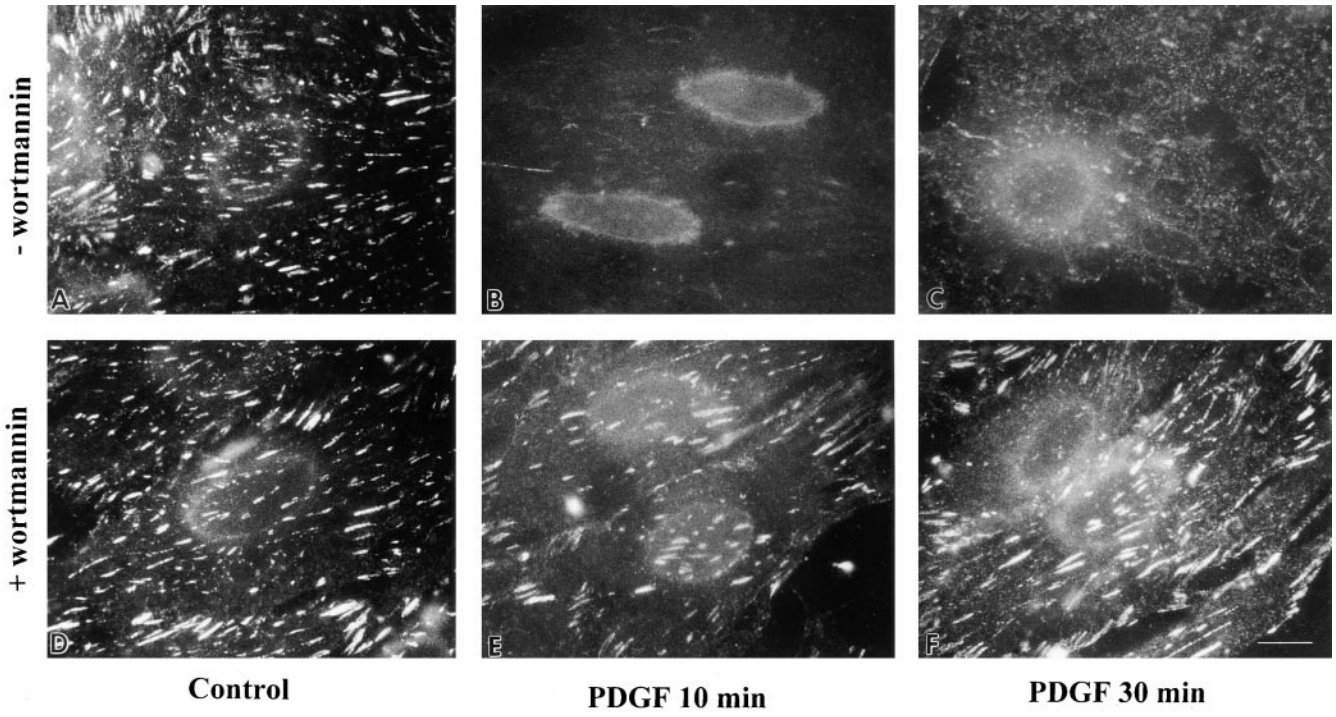


Figure 4. Activation of PI 3-kinase by PDGF induces redistribution of vinculin. REFs were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, rinsed and incubated in DME for 1 h, treated as indicated, and processed for fluorescence microscopy as described in Materials and Methods. (A) Control; (B) PDGF 10 min; (C) PDGF 30 min; (D) wortmannin; (E) PDGF 10 min + wortmannin; (F) PDGF 30 min + wortmannin. The concentration of PDGF was 30 ng/ml. Cells were preincubated with 100 nM wortmannin for 10 min. Results are representative of three to six separate experiments.

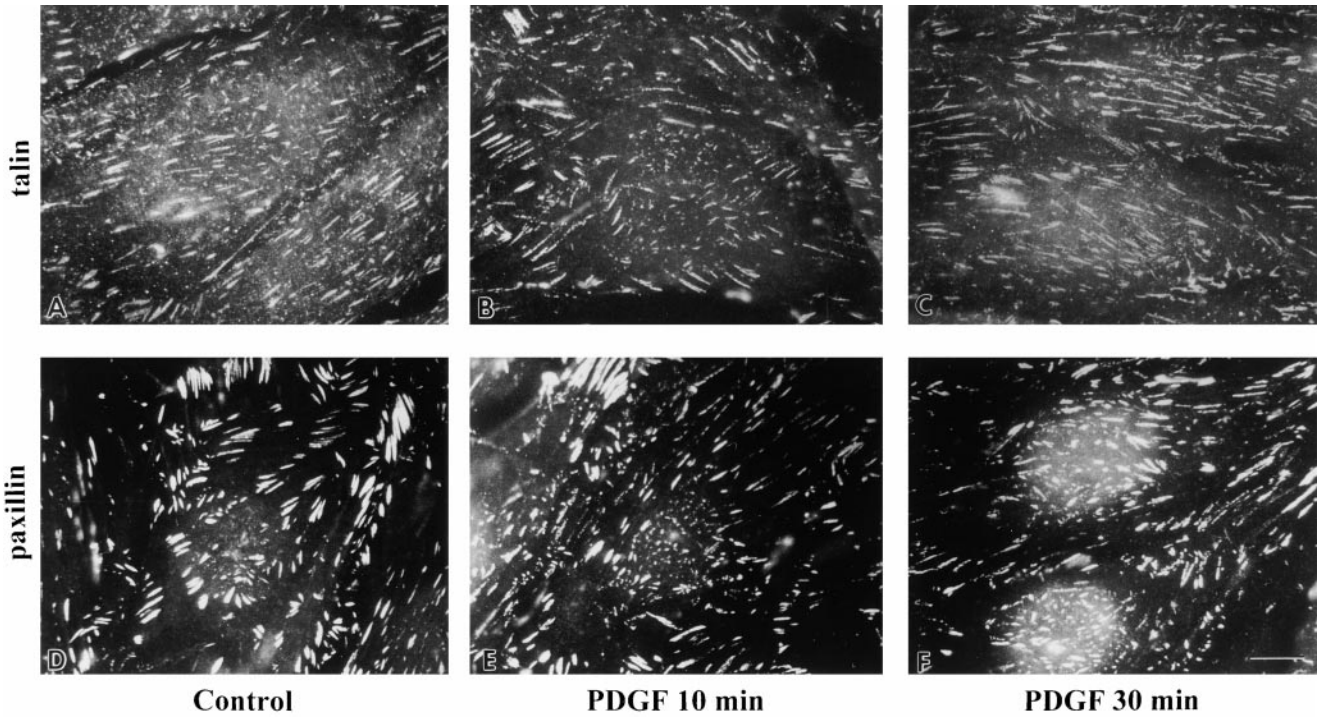


Figure 5. Talin- and paxillin-staining focal adhesion plaques remain in PDGF-treated REFs. Cells were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, rinsed and incubated in DME for 1 h, treated as indicated, and processed for fluorescence microscopy as described in Materials and Methods. Cells were stained with antitalin (A–C) or antipaxillin (D–F). (A and D) Control; (B and E) PDGF 10 min; (C and F) PDGF 30 min; (E) PDGF 10 min; (F) PDGF 30 min. The concentration of PDGF was 30 ng/ml. Results are representative of two to three separate experiments.

lated focal adhesion protein proposed to play an important role in the formation of the plaque (Burridge and Chrzanowska-Wodnicka, 1996). In control cells, paxillin staining was localized to focal adhesions (Fig. 5 D). Similar to talin, paxillin-staining focal adhesions were also observed in cells treated with PDGF for 10 and 30 min (Fig. 5, E and F). These results demonstrate that PDGF stimulation of PI 3-kinase does not induce complete loss of the focal adhesion complex, but rather modulates a restructuring of the plaque.

Since talin, which binds directly to the β_3 integrin, and paxillin were maintained in plaque structures, we proposed that the $\alpha_v\beta_3$ integrins also remained in an active and clustered state. In an attempt to test this hypothesis directly, we stained REFs with various antibodies recognizing this receptor. However, we could not find an antibody that would recognize $\alpha_v\beta_3$ integrin in fixed rat cells. As an alternative approach, we examined the effect of PI 3-kinase activation on the localization of the $\alpha_5\beta_1$ integrin in REFs plated on purified fibronectin. Although a similar loss of stress fibers and α-actinin–staining focal adhesions was observed in the cells plated on fibronectin, the $\alpha_5\beta_1$ integrin remained localized to the focal adhesion plaques in PDGF-treated cells (Fig. 6). In addition, the loss of vinculin-staining focal adhesions and maintenance of talin- and paxillin-staining plaques was observed in PDGF-treated REFs plated on fibronectin (data not shown). Focal adhesion restructuring was also observed in PDGF-treated REFs plated on purified vitronectin (data not shown).

Focal adhesion kinase (FAK) is an important mediator of integrin signaling involved in the recruitment and tyrosine phosphorylation of focal adhesion proteins after integrin activation. The localization of FAK in PDGF-treated REFs was also examined by immunofluorescence microscopy. Staining of focal adhesion plaques with anti-FAK was observed in control and PDGF (for 10 and 30 min)-treated cells (data not shown). However, the intensity of the FAK-staining plaques in PDGF-treated cells appeared to be decreased compared with control cells, suggesting there was some relocalization of FAK. Similar results were observed using antiphosphotyrosine (data not shown). At this point, the significance of these observations remain unclear.

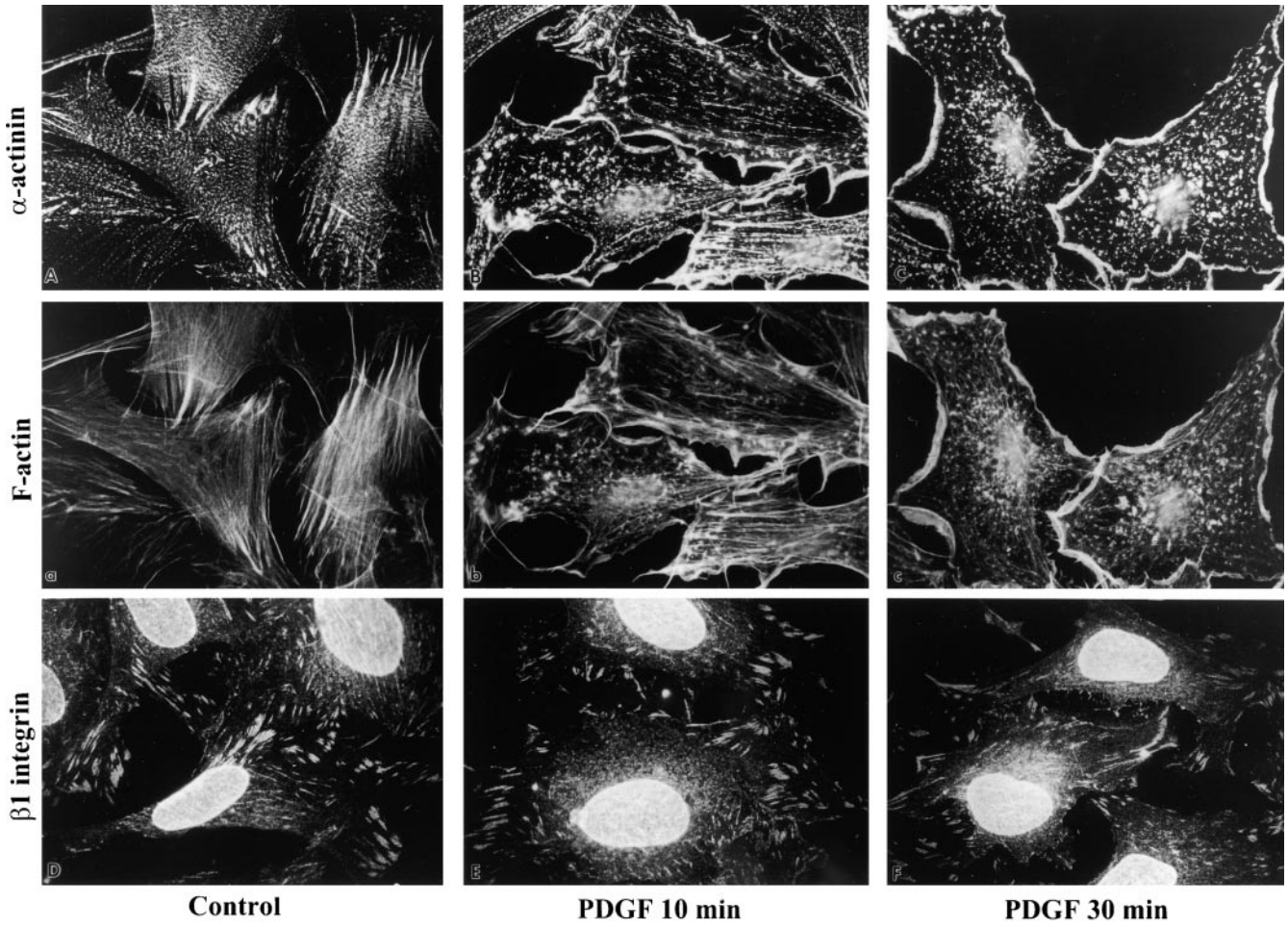


Figure 6. $\alpha_5\beta_1$ integrin–staining focal adhesion plaques remain in PDGF-treated REFs plated on fibronectin. Cells were plated on fibronectin-coated coverslips for 4 h in serum-free DME, treated as indicated, and processed for fluorescence microscopy as described in Materials and Methods. Cells were double labeled with anti–α-actinin (A–C) and phalloidin (a–c) or stained with anti–$\alpha_5\beta_1$ integrin (D–F). (A, a, and D) Control; (B, b, and E) PDGF 10 min; (C, c, and F) PDGF 30 min. The concentration of PDGF was 30 ng/ml. Results are representative of two to three separate experiments.

PtdIns (3,4,5)-P_3, a Major Product of PI 3-kinase Phosphorylation, Is Sufficient to Induce the Restructuring of Focal Adhesions

Signaling through PI 3-kinase–dependent pathways has been demonstrated to be mediated by the lipid products PtdIns (3,4,5)-P_3 and PtdIns (3,4)-P_2 (Toker and Cantley, 1997), and more recently, by the protein kinase activity of PI 3-kinase (Bondeva et al., 1998). To further understand the role PI 3-kinase plays in the restructuring of focal adhesions, the lipid products of PI 3-kinase were examined for the ability to independently induce the restructuring of focal adhesions as observed with PDGF. Several independent studies have demonstrated that purified lipids sonicated to form micelles or vesicles can be added to cells in culture (Toker et al., 1995; Derman et al., 1997; Franke et al., 1997; Heraud et al., 1998; Rizzo et al., 1999). The micelles or vesicles fuse with the cell membrane, and the exogenous lipids are taken up into the cell and are biologically active. In these studies, micelles containing purified phospholipids or vesicles containing the purified phospholipid and phosphatidylserine were added to the cells in culture as described in Materials and Methods.

Treatment of the cells with 25 μM PtdIns (3,4,5)-P_3 for 30 min induced the disassembly of focal adhesions (Fig. 7), redistribution of α-actinin (Fig. 8, A–D), reorganization of the actin cytoskeleton (Fig. 8, a–d), and redistribution of vinculin (Fig. 9). Numerous cells (26.6–40.5%) were observed with IRM images, α-actinin, rhodamine-phalloidin, and vinculin staining nearly identical to that observed in cells treated with PDGF for 10 min. These morphological changes were also observed in cells treated for shorter times (10 min) and lower concentrations (5 μM) of PtdIns (3,4,5)-P_3, however, fewer cells were affected. Although PtdIns (3,4,5)-P_3 induced reorganization of the actin stress fibers into a filamentous actin network, membrane ruffling was not observed (Fig. 8 c). As seen in PDGF-treated cells, PtdIns (3,4,5)-P_3 did not significantly affect talin- or paxillin-staining focal adhesions (data not shown). Cells were also treated with 25-μM concentrations of PtdIns (4,5)-P_2 (Figs. 7 and 9, C, and Fig. 8, D and d), PtdIns (3,4)-P_2, PtdIns (3)-P, and phosphatidylserine (data not shown). None of these phospholipids induced significant changes in the IRM images or immunostaining. Similar results were observed when the cells were treated with mixed lipid vesicles containing a 25-μM concentration of the specific phospholipid and 100 μM phosphatidylserine (data not shown).

As an alternative approach, cells were treated with di-C_{12}-PIP_3/AM, the heptakis (acetoxymethyl) ester of dilauroylphosphatidylinositol (3,4,5)-P_3 (Jiang et al., 1998). The AM esters of this uncharged and membrane-permeant derivative are readily hydrolyzed by intracellular esterases generating biologically active PtdIns (3,4,5)-P_3 inside the cell (Jiang et al., 1998). The loss of α-actinin–staining focal adhesions and stress fibers was observed in 27.5 and 62.5% of the cells treated with 50 and 100 μM di-C_{12}-PIP_3/AM, respectively (Fig. 10). The restructuring of focal adhesions and stress fibers was not observed in cells treated with vehicle alone. These results suggest that PtdIns (3,4,5)-P_3 is sufficient to induce restructuring of focal adhesions in REFs.

PtdIns (3,4,5)-P_3 Binds α-Actinin in PDGF-treated Cells

During restructuring, loss of α-actinin from focal adhesion plaques was observed. In contrast, the distribution of talin and paxillin was not affected. These results suggest that α-actinin is specifically involved in the restructuring of focal adhesions induced by PDGF, and is a potential target for the second messenger action of PtdIns (3,4,5)-P_3. α-Acti-

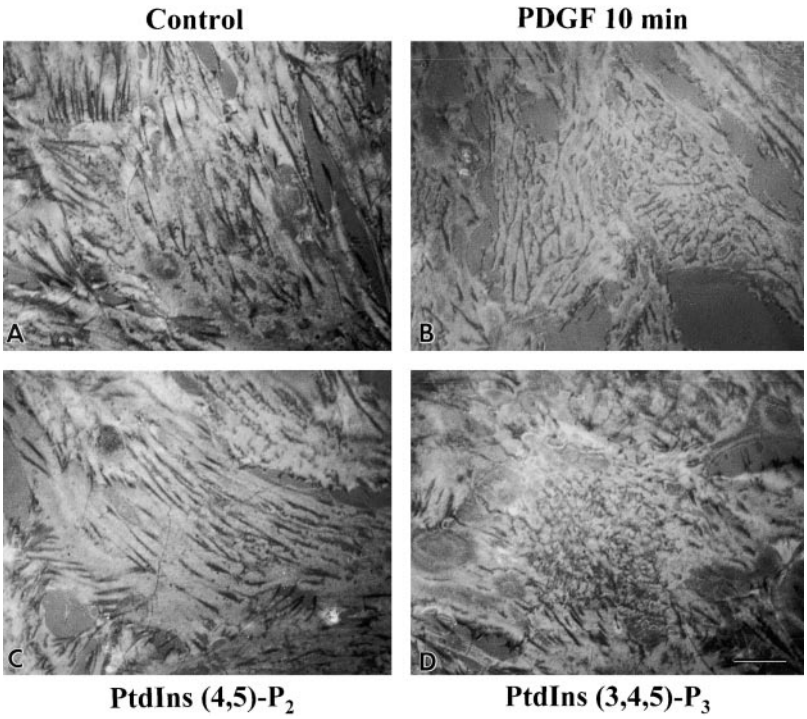


Figure 7. PtdIns (3,4,5)-P_3 induces focal adhesion disassembly. REFs were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, rinsed and incubated in DME for 1 h, treated as indicated, fixed with 3% glutaraldehyde, and examined by IRM as described in Materials and Methods. (A) Control; (B) 30 ng/ml PDGF 10 min; (C) 25 μM PtdIns (4,5)-P_2; (D) 25 μM PtdIns (3,4,5)-P_3. Results are representative of two separate experiments.

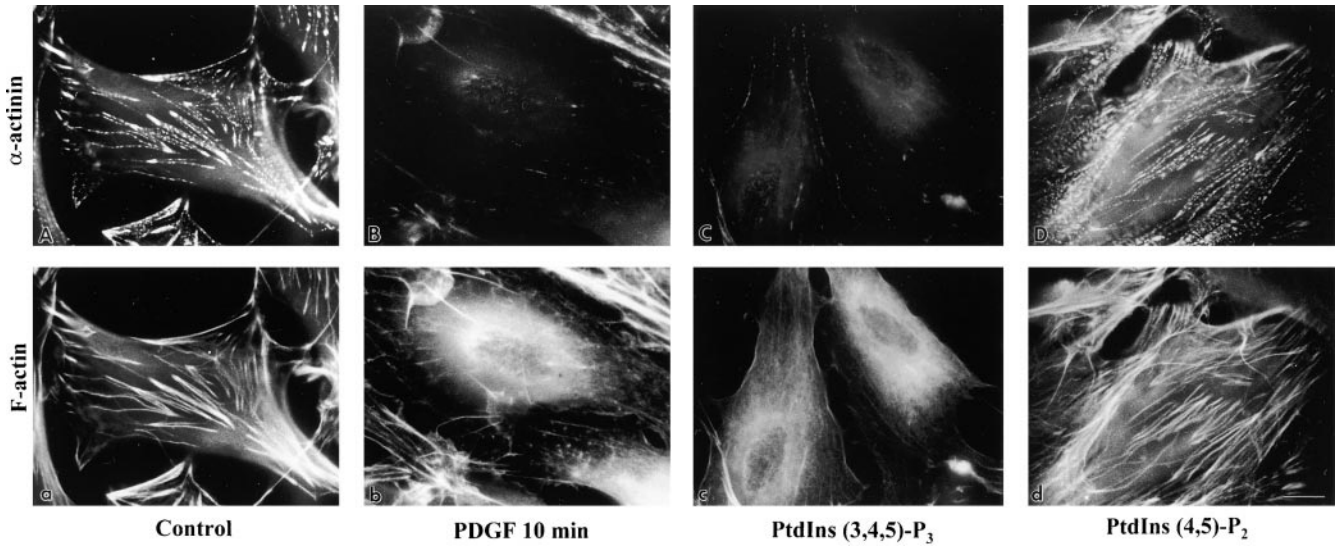


Figure 8. PtdIns (3,4,5)-P_3 induces redistribution of α-actinin. REFs were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, rinsed and incubated in DME for 1 h, treated as indicated, and processed for fluorescence microscopy as described in Materials and Methods. Cells were double labeled with anti–α-actinin (A–D) and phalloidin (a–d). (A) Control; (B) 30 ng/ml PDGF 10 min; (C) 25 μM PtdIns (3,4,5)-P_3 30 min; (D) 25 μM PtdIns (4,5)-P_2 30 min. Results are representative of five separate experiments.

nin previously has been demonstrated to bind acidic phospholipids (Burn et al., 1985; Fukami et al., 1992); however, the binding of α-actinin to PtdIns (3,4,5)-P_3 has not been examined. A P-1-aminopropyl inositol(1,3,4,5)P_4 affinity resin was used to test whether α-actinin can bind to PtdIns (3,4,5)-P_3. The structure of the active group of the inositol(1,3,4,5)P_4 resin is analogous to the inositol phosphate head group of PtdIns (3,4,5)-P_3 (Theibert et al., 1997). Therefore, inositol(1,3,4,5)P_4 affinity chromatography provides a means of screening for PtdIns (3,4,5)-P_3–binding proteins (Hammonds-Odie et al., 1996; Theibert et al., 1997; Blader et al., 1999). Purified α-actinin and α-actinin from BAE and REF cell lysates were found to bind specifically to the affinity resin (Fig. 11 A), indicating that α-actinin may bind PtdIns (3,4,5)-P_3 in vivo. Consistent with previous studies (Hammonds-Odie et al., 1996; Theibert et al., 1997), Ponceau S staining for the total protein showed that a majority of the protein bands from the cell lysates did not bind to the affinity resin, demonstrating a high level of specificity (data not shown).

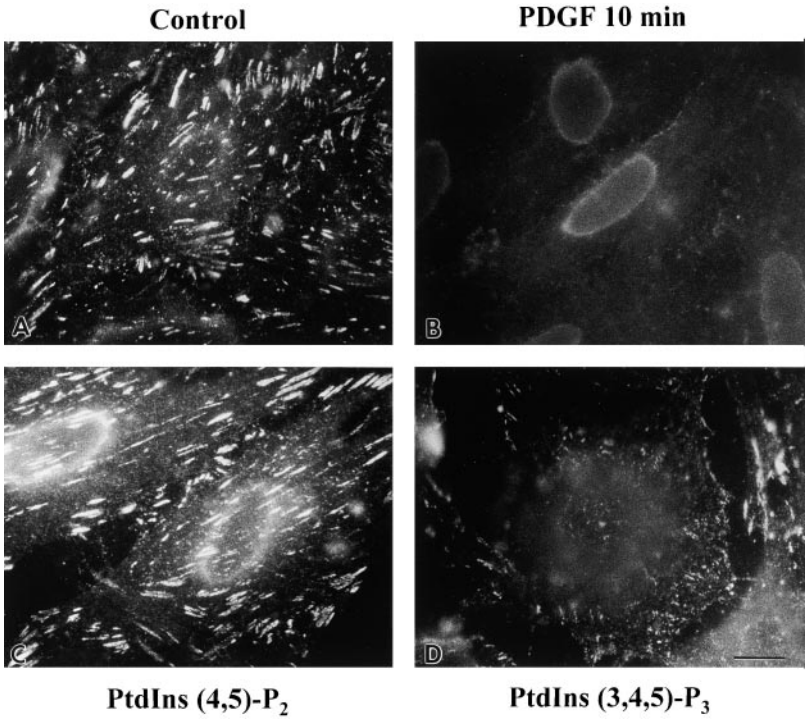


Figure 9. PtdIns (3,4,5)-P_3 induces redistribution of vinculin. REFs were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, rinsed and incubated in DME for 1 h, treated as indicated, and processed for fluorescence microscopy as described in Materials and Methods. (A) Control; (B) 30 ng/ml PDGF 10 min; (C) 25 μM PtdIns (4,5)-P_2 30 min; (D) 25 μM PtdIns (3,4,5)-P_3 30 min. Results are representative of two separate experiments.

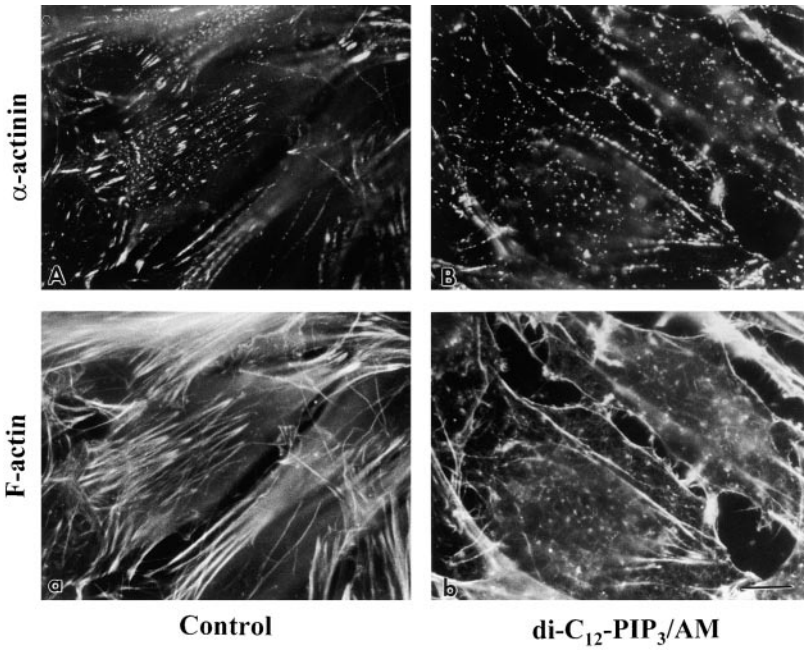


Figure 10. di-C_{12}-PI (3,4,5)-P_3/AM induces redistribution of α-actinin. REFs were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, rinsed and incubated in DME for 1 h, treated as indicated, and processed for fluorescence microscopy as described in Materials and Methods. Cells were double labeled with anti–α-actinin (A and B) and phalloidin (a and b). (A and a) Control; (B and b) 100 μM di-C_{12}-PI (3,4,5)-P_3/AM 30 min. Results are representative of two separate experiments.

To examine the interaction of α-actinin with phosphoinositides, α-actinin was immunoprecipitated from $^{32}P_i$-labeled REFs stimulated with PDGF. In untreated cells, α-actinin was found to bind PtdIns-P_2 and to a lesser degree PtdIns-P (Fig. 11 B), whereas no binding to PtdIns (3,4,5)-P_3 was detected (Fig. 11 C). When the cells were treated with PDGF for 10 min, α-actinin clearly binds to PtdIns (3,4,5)-P_3 with no change detected in the binding to PtdIns-P_2 and PtdIns-P. After 30 min of PDGF treatment, decreased binding of α-actinin to PtdIns (3,4,5)-P_3 was observed, whereas binding to PtdIns-P and PtdIns-P_2 was increased. These results demonstrate that α-actinin binds to PtdIns (3,4,5)-P_3 both in vitro and in vivo, and suggest that α-actinin function is regulated by phosphoinositide binding. PtdIns (3,4,5)-P_3 binding to α-actinin correlated with focal adhesion restructuring. PtdIns-P and PtdIns-P_2 binding to α-actinin appeared unchanged during the initial restructuring of the focal adhesion plaque, but increased during membrane ruffling. Consistent with the results published by Heraud et al. (1998), binding of PtdIns-P and PtdIns-P_2 to talin immunoprecipitated from $^{32}P_i$-labeled REFs was also observed, however, no binding to PtdIns (3,4,5)-P_3 was detected (data not shown).

PtdIns (3,4,5)-P_3 Disrupts the Binding of α-Actinin to the Integrin β Subunit

α-Actinin has been demonstrated to link actin stress fibers to focal adhesions by binding to the cytoplasmic tail of the β_1 and β_3 integrins (Pavalko et al., 1991; Otey et al., 1993; Sampath et al., 1998). Adhesion of cells plated in serum, of which vitronectin is the primary extracellular matrix substrate, occurs primarily through $\alpha_v\beta_3$ integrins (Fath et al., 1989). To examine the effect of PI 3-kinase activation on the interaction of α-actinin with the β_3 integrin, the β_3 integrin was immunoprecipitated from control and PDGF-treated cells. In control cells, coprecipitation of α-actinin was observed with β_3 integrin (Fig. 12 A). However, in cells treated with PDGF for 10 min, a decrease in the amount of α-actinin coprecipitating with β_3 integrin was observed. After 30 min of treatment, coprecipitation of α-actinin with β_3 integrin returned to control levels. When cells were preincubated with 100 nM wortmannin (Fig. 12 A), PDGF did not induce a decrease in α-actinin coprecipitation with β_3 integrin at 10 min, but rather an increase. These results suggest that PI 3-kinase activation is required for the loss of α-actinin association with β_3 integrin in PDGF-treated cells. The increased association of α-actinin with β_3 integrin, which was detected after 10 min of PDGF treatment in cells preincubated with wortmannin was unexpected; however, these results were also observed in the presence of 25 μM LY294002 (data not shown). These results are difficult to interpret, but might suggest that in the absence of PI 3-kinase activation, other signaling pathways stimulated by PDGF actually induce the association of α-actinin with β_3 integrin.

The above results indicate that there is a correlation between the binding of α-actinin to PtdIns (3,4,5)-P_3 and the loss of α-actinin association with β_3 integrin after 10 min of PDGF treatment. These data suggest that PtdIns (3,4,5)-P_3 binding to α-actinin disrupts its interaction with β_3 integrin. To further investigate this hypothesis, we tested whether the addition of exogenous PtdIns (3,4,5)-P_3 to the anti–β_3 integrin immunoprecipitates could disrupt the interaction between α-actinin and β_3 integrin. PtdIns (3,4,5)-P_3 almost completely (~75%) disrupted the interaction between α-actinin and β_3 integrin (Fig. 12 B). PtdIns (3,4)-P_2 induced a slight disruption (~25%), whereas PtdIns (4,5)-P_2, PtdIns (3)-P, and phosphatidylserine did not affect the interaction of α-actinin and β_3 integrin in the anti–β_3 integrin immunoprecipitates. These results suggest that PtdIns (3,4,5)-P_3 disrupts the interaction of α-actinin with β_3 integrin.

α-Actinin has been demonstrated to bind to the highly homologous cytoplasmic tail of the β_1 or β_3 integrin subunits (Pavalko et al., 1991; Otey et al., 1993; Sampath et al., 1998). To confirm that PtdIns (3,4,5)-P_3 was regulating

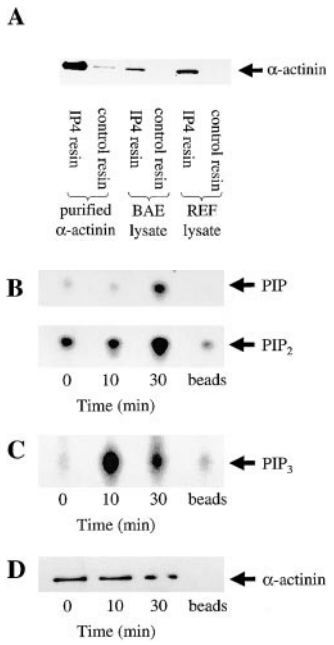


Figure 11. PtdIns (3,4,5)-P_3 binds α-actinin in PDGF-treated cells. (A) 20 μg of purified α-actinin, 500 μg REF cell lysate, or 500 μg BAE cell lysate, was incubated with Affigel-aminopropyl-inositol (1,3,4,5)P_4, washed, and the bound protein was eluted and immunoblotted using anti–α-actinin (1:10,000) as described in Materials and Methods. (B–D) REFs were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, rinsed and incubated for 1 h in phosphate-free DME containing 0.25 mCi/ml $^{32}P_i$ followed by stimulation with 30 ng/ml PDGF for the times indicated. Cells were lysed, α-actinin was immunoprecipitated, and the associated phosphoinositides were extracted or the protein was eluted as described in Materials and Methods. (B) Autoradiograph of the TLC plate exposed for 17 h, optimal time for the visualization of PtdIns-P and PtdIns-P_2. (C) Autoradiograph of the same TLC plate exposed for 48 h, the optimal time for the visualization of PtdIns (3,4,5)-P_3. (D) Immunoblot showing that equal amounts of α-actinin were immunoprecipitated from each sample. Results are representative of four separate experiments.

the interaction of α-actinin with its binding site within the β subunit of the integrin, we developed a purified system to assay the binding of α-actinin to the cytoplasmic tail of the β_1 integrin. Using a GST fusion protein containing the cytoplasmic tail of the β_1 integrin (Sampath et al., 1998), binding was assayed by incubating the purified proteins followed by immunoprecipitation with anti–α-actinin. Binding of the GST-β_1 tail to α-actinin could be detected and quantitated by immunoblotting for GST. Using this assay, we reproduced the results of Sampath et al., showing that the GST-β_1 tail bound specifically to α-actinin (Fig. 13 A). When exogenous phosphoinositides were added to the complexes containing the GST-β_1 tail bound to α-actinin, only PtdIns (3,4,5)-P_3 disrupted the interaction between the two proteins (Fig. 13 B). These data provide strong evidence that PI 3-kinase activation and the production of PtdIns (3,4,5)-P_3 are important components of the signaling mechanism regulating PDGF-induced restructuring of focal adhesions.

Discussion

Cell adhesion involves receptor-mediated cell-surface interactions with the extracellular matrix (Burridge and Chrzanowska-Wodnicka, 1996; Gumbiner, 1996). These interactions play a central role in the organization of the cytoskeleton, thereby regulating cell shape and function. Focal adhesions are specialized structures linking the extracellular matrix to the actin microfilaments through integrin and syndecan transmembrane receptors. The structure of the focal adhesion plaque consists of an elaborate network of interconnecting proteins anchoring the microfilaments to the membrane at the contact site. As the points of closest apposition linking the cytoskeleton to the extracellular matrix, focal adhesions are ideally positioned for regulating the adhesive strength of the cell. It may help to think of the cell as having three grades of adhesiveness: (1) weak adherence, meaning that the cell is attached but not spread; (2) intermediate adherence, characterized by a spread cell that lacks stress fibers and focal adhesions; and (3) strong adherence, indicating a spread cell with stress fibers linked to the extracellular matrix through focal adhesion plaques. Cellular interactions with the extracellular matrix are generally considered to promote adhesion. However, extracellular factors also exist that regulate cell function by stimulating reversal of one or more stages of

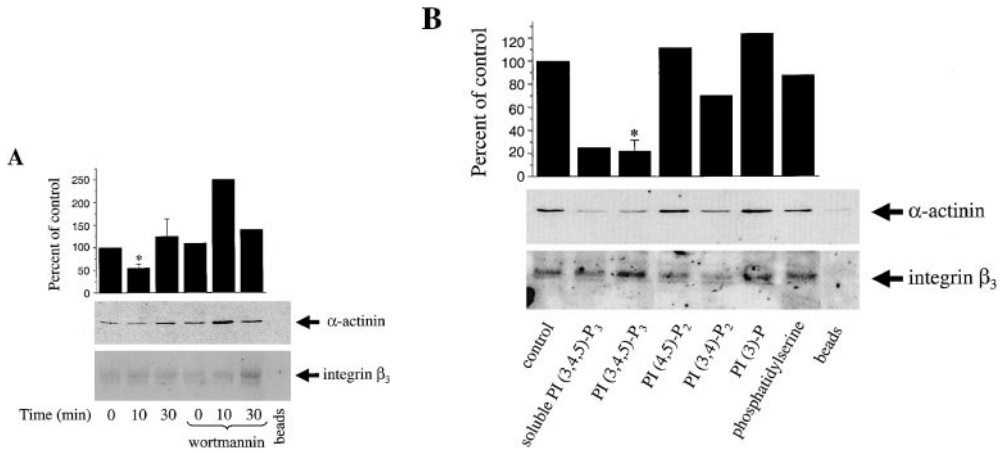


Figure 12. PtdIns (3,4,5)-P_3 disrupts the binding of α-actinin to β_3 integrin. (A) REFs were grown to 80% confluence, serum-deprived for 18 h in 0.2% FBS-DME, and rinsed and incubated in DME for 1 h. Cells were treated with 30 ng/ml PDGF for the indicated times in the absence or presence of 100 nM wortmannin (10-min preincubation), lysates were prepared for immunoprecipitation using anti–β_3 integrin (IgG), and the precipitated protein was examined by immunoblotting with anti–α-actinin (1:5,000) or anti–β_3 integrin (IgM, 1:250) as described in Materials and Materials and Methods. (B) β_3 integrin immunoprecipitates were washed twice and incubated with 20 μg of the indicated phospholipid. Immunoprecipitates were washed, and the protein was eluted and immunoblotted using anti–α-actinin (1:5,000) or anti–β_3 integrin (IgM, 1:250) as described in Materials and Methods. In this experiment, two different forms of PtdIns (3,4,5)-P_3, a water-soluble form and a water-insoluble form sonicated to form micelles, were used. Anti–α-actinin immunoblots were analyzed by scanning densitometry and quantitated using NIH Image. Error bars represent SEM (n = 2–3). Asterisk indicates $P < 0.05$.

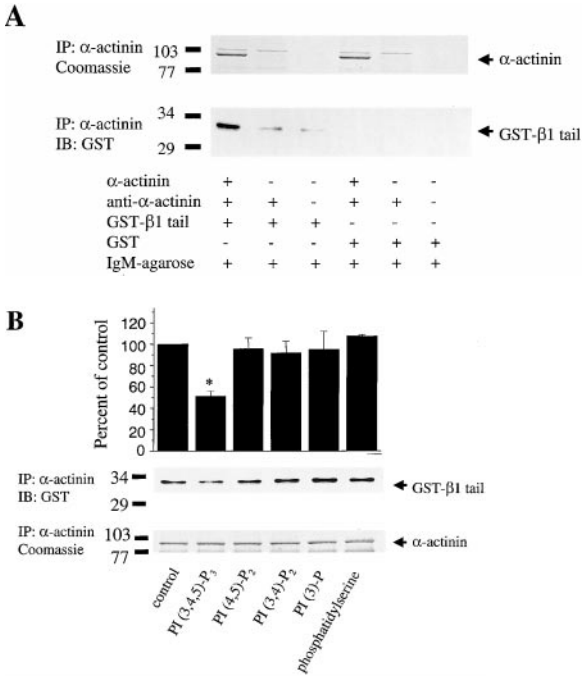


Figure 13. PtdIns (3,4,5)-P_3 disrupts the binding of α-actinin to the cytoplasmic tail of the β_1 integrin. (A) 200 nM of α-actinin and 200 nM GST-β_1 cytoplasmic tail were incubated for 1 h, the complex was immunoprecipitated with anti–α-actinin, and the precipitated protein was examined by Coomassie staining or immunoblotting with anti-GST (1:1,000) as described in Materials and Methods. (B) α-Actinin immunoprecipitates were washed twice and incubated with 20 μg of the indicated phospholipid. Immunoprecipitates were washed, and the protein was eluted and examined by Coomassie staining or immunoblotting with anti-GST (1:1,000) as described in Materials and Methods. Anti-GST immunoblots were analyzed by scanning densitometry and quantitated using NIH Image. Error bars represent SEM ($n = 4$). Asterisk indicates $P < 0.001$.

the adhesion process. A shift from strong adherence to intermediate adherence, places the cell in a malleable state favorable for dynamic processes such as cytokinesis, differentiation, and motility.

Modification of the link between the cytoskeleton and the extracellular matrix is a prominent structural target for the regulation of cellular adhesive strength. Previously, we have demonstrated that thrombospondin 1 stimulates a PI 3-kinase–dependent disassembly of focal adhesions in bovine aortic endothelial cells (Murphy-Ullrich and Hook, 1989; Murphy-Ullrich et al., 1993). In this paper, we have focused on the mechanisms by which PI 3-kinase regulates the disassembly of focal adhesions. Data from studies using PDGF to stimulate the activation of PI 3-kinase in REFs, direct addition of phosphoinositides to REFs, and biochemical approaches suggest all of the following. First, PDGF induces a PI 3-kinase–dependent loss of focal adhesions as measured by IRM (Fig. 2), indicating a significant decrease in the structural integrity of the link between the actin cytoskeleton and the extracellular matrix. Second, examination of the localization of focal adhesion proteins indicates that the activation of PI 3-kinase by PDGF actually induces a restructuring of the focal adhesion plaque rather than a complete dispersal of the complex (Figs. 3–6). Third, PtdIns (3,4,5)-P_3, a lipid product of PI 3-kinase, is sufficient to induce the restructuring of the focal adhesion plaque and reorganization of the actin stress fibers into a filamentous network (Figs. 7–10). Finally, PtdIns (3,4,5)-P_3 binds to α-actinin during the restructuring of the focal adhesion plaque and reorganization of the actin cytoskeleton (Fig. 11), disrupting the interaction of α-actinin with the integrin β subunit (Figs. 12 and 13). These results implicate α-actinin as a specific target for PtdIns (3,4,5)-P_3 involved in the restructuring of focal adhesion plaques and reorganization of the actin cytoskeleton.

In this study, we show that the activation of PI 3-kinase by PDGF and the lipid product PtdIns (3,4,5)-P_3 induce loss of the adhesive link between the actin stress fibers and the extracellular matrix. Yet, the focal adhesion protein complex was not completely dissipated. Rather, PI 3-kinase and PtdIns (3,4,5)-P_3 induced a restructuring of the plaque characterized by the loss of α-actinin and vinculin and the reorganization of the actin stress fibers into a filamentous network. In contrast, the integrin receptor and the focal adhesion proteins talin and paxillin remained localized to the focal adhesion plaques. The effects of PI 3-kinase and PtdIns (3,4,5)-P_3 on the restructuring of focal adhesion plaques indicate that α-actinin and vinculin play an important role in the stabilization and maintenance of actin stress fibers and their link to the extracellular matrix. The presence of α-actinin– and vinculin-containing focal adhesions is associated with decreased cell motility (Rodriguez Fernandez et al., 1992, 1993; Gluck and Ben-Ze'ev, 1994). We propose that the presence of α-actinin and vinculin in focal adhesion plaques is important for the modulation of cellular adhesive strength and motility. Therefore, understanding the mechanisms regulating α-actinin and vinculin activity may provide potential targets for the control of cell motility.

As a prominent structural component of focal adhesions and actin-bundling protein, α-actinin is ideally positioned to regulate the transition of the cell from a strong adhesive to an intermediate adhesive state. The activation of PI 3-kinase, as well as the addition of PtdIns (3,4,5)-P_3, induced a redistribution of α-actinin from focal adhesions and the actin stress fibers to the Triton X-100–soluble fraction. The loss of α-actinin association correlates with the unbundling of the actin stress fibers and reorganization to a filamentous actin network. Wachsstock et al. (1993) demonstrated that mixtures of α-actinin and actin filaments can form in vitro networks or bundles depending on their concentration. At low concentrations, α-actinin and actin filaments form networks indistinguishable from that of actin alone. At high concentrations, α-actinin and actin filament mixtures form bundles with the threshold for bundling dependent on the affinity of α-actinin for the actin filaments. We propose that upon binding of PtdIns (3,4,5)-P_3 to α-actinin, the affinity of α-actinin for actin filaments is altered, resulting in the unbundling of the stress fibers and reorganization to a filamentous network. Studies are currently underway to test this hypothesis.

It has been known for some time that phosphoinositides play an important role in the regulation of the actin cytoskeleton (Janmey, 1994). The activity of many actin-binding proteins are modulated by interactions with vari-

ous phosphoinositides. Both α-actinin and vinculin have been demonstrated to bind different phosphoinositides and PtdIns (4,5)-P_2, in particular, appears to play an important role in their activity during the formation of focal adhesions (Fukami et al., 1992; Johnson and Craig, 1995; Craig and Johnson, 1996; Gilmore and Burridge, 1996). Talin also appears to bind PtdIns (4)-P and PtdIns (4,5)-P_2, although the functional significance of this binding is not clear (Heraud et al., 1998). In this study, we not only demonstrated that α-actinin is regulated by PtdIns (3,4,5)-P_3, but also found evidence of binding to PtdIns-P and PtdIns-P_2. The exact identity of these phosphoinositides has not been determined, however, based on the quantities present, it is likely to be PtdIns (4)-P and PtdIns (4,5)-P_2. Interestingly, binding of these phosphoinositides to α-actinin was detected under basal conditions and remained unchanged after 10 min of treatment with PDGF during focal adhesion restructuring. However, after 30 min of PDGF treatment, the levels of these phosphoinositides increased significantly, correlating with the formation of membrane ruffles. It is not known whether α-actinin binds more than one phosphoinositide at a time, or if there are different populations of α-actinin that bind the different phosphoinositides. We favor the latter hypothesis and suggest that the localized concentration of phosphoinositides determines binding and regulates α-actinin function. Competition between phosphoinositides for binding and the regulation of protein structure and function has been demonstrated to exist for Vav (Han et al., 1998), profilin (Lu et al., 1996), and neurogranin (Lu and Chen, 1997). It will be interesting to see if the same scenario exists for α-actinin and other focal adhesion proteins. In any case, it is now clear that phosphoinositides are present in focal adhesions and play an important role in regulating the structure of the plaque.

A strong link has been established between PI 3-kinase and cell motility (Carpenter and Cantley, 1996). However, much of the work has been focused on Rac and its ability to induce membrane ruffling downstream of PI 3-kinase (Wennstrom et al., 1994a,b; Hawkins et al., 1995; Rodriguez-Viciana et al., 1997; Reiske et al., 1999). In this manuscript, we have presented evidence demonstrating that the regulation of focal adhesion structure is another important and direct target for the lipid products of PI 3-kinase. Several studies have demonstrated that PI 3-kinase is localized to focal adhesions (Geiger et al., 1992; Miyamoto et al., 1995; Plopper et al., 1995; Gillham et al., 1999), further implicating a role for PI 3-kinase in the regulation of plaques. Strong evidence indicates that the strength of adhesion between the cell and the extracellular matrix plays an important role in the regulation of cell motility with an intermediate level of adhesive strength correlating with maximal speed (DiMilla et al., 1991, 1993; Huttenlocher et al., 1996; Palecek et al., 1997). We propose that transition of the cell from a strong adhesive state to an intermediate adhesive state is important for the induction and maintenance of cell motility. Therefore, modification of the link between the actin cytoskeleton and the extracellular matrix is a potential target for the regulation of cell motility.

Recent attention has been focused on PI 3-kinase and its role in tumor growth and metastasis (Cantley et al., 1991; Keely et al., 1997; Shaw et al., 1997). Not only does the activity of PI 3-kinase appear to be upregulated, but PTEN, a phosphatase which dephosphorylates PtdIns (3,4,5)-P_3, is also mutated in some tumor cells resulting in increased levels of PtdIns (3,4,5)-P_3 (Cantley and Neel, 1999; Maehama and Dixon, 1999). The relationship of PI 3-kinase activity and PtdIns (3,4,5)-P_3 to cancer progression emphasizes the importance of understanding the specific mechanisms by which PtdIns (3,4,5)-P_3 regulates cellular function.

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