Kinases that associate with integrins are likely to mediate the assembly/disassembly of cell:matrix junctions during cell migration. Here we show that ERK1 associates with \( \alpha_{v}\beta_{3} \) integrin following the addition of platelet-derived growth factor to serum-starved Swiss NIH 3T3 fibroblasts in an interaction that is mediated by the central region of the \( \beta_{3} \) integrin cytodomain. \( \alpha_{v}\beta_{3}\)-ERK1 association occurred prior to focal complex formation and was seen to initiate in small punctate complexes primarily in the peripheral regions of the plasma membrane. Expression of a dominant negative mutant of ERK1 (but not ERK2) significantly reduced the spreading of cells on vitronectin, whereas cell spreading on fibronectin was unaffected by inhibition of ERK1. In contrast, inhibition of ERK activation by PD98059 had no effect on the platelet-derived growth factor-regulated Rac1-dependent flux of \( \alpha_{v}\beta_{3} \) integrin from early endosomes to the plasma membrane, an event that is also necessary for cells to spread efficiently on vitronectin. We propose that \( \alpha_{v}\beta_{3} \) integrin must recycle to the plasma membrane via the Rab4 pathway and recruit active ERK1 in order to function efficiently.

The integrins have received a great deal of attention in the literature over the last few years, not only for their ability to bind and model the extracellular matrix, but also due to their ability to activate a number of cell signaling cascades that influence a range of biological processes including cell growth, differentiation, migration, and apoptosis (1). The distal stretches of many of these signaling pathways are reasonably well defined. For instance, ligation of integrins is well established to both activate the MEK/ERK signaling axis and to initiate the assembly/disassembly of cell:matrix junctions (12). There are several ways in which these associations may involve these interactions. Prior to engagement with the extracellular matrix, integrins must be rendered competent to bind ligand via a process termed "inside-out" signaling (12). There are several ways in which the ability of ligand-competent integrin may be increased. Growth factors have recently been shown to regulate delivery of \( \alpha_{v}\beta_{3} \) from endosomal compartments to the plasma membrane, and this process is necessary for efficient integrin function (13). Once upon the cell surface, conformational changes in an individual heterodimer may increase its affinity for monovalent ligand (12). This is well documented to be a key event in the activation of integrins prior to focal complexes (14). For example, clusters of \( \alpha_{v}\beta_{3} \) integrin form at early stages during cell spreading and clearly prior to the assembly of focal complexes (15). Several growth factor-activated signaling pathways have been implicated in the activation of integrins prior to focal complex assembly. For instance, the small GTPase, RhoA regulates delivery of \( \alpha_{v}\beta_{3} \) to the plasma membrane (13); phosphorylation of integrin-linked kinase (16) and H-Ras (17) are clearly involved in this process.

Kinases that associate with integrins are likely to mediate the assembly/disassembly of cell:matrix junctions during cell migration. Here we show that ERK1 associates with \( \alpha_{v}\beta_{3} \) integrin following the addition of platelet-derived growth factor to serum-starved Swiss NIH 3T3 fibroblasts in an interaction that is mediated by the central region of the \( \beta_{3} \) integrin cytodomain. \( \alpha_{v}\beta_{3}\)-ERK1 association occurred prior to focal complex formation and was seen to initiate in small punctate complexes primarily in the peripheral regions of the plasma membrane. Expression of a dominant negative mutant of ERK1 (but not ERK2) significantly reduced the spreading of cells on vitronectin, whereas cell spreading on fibronectin was unaffected by inhibition of ERK1. In contrast, inhibition of ERK activation by PD98059 had no effect on the platelet-derived growth factor-regulated Rac1-dependent flux of \( \alpha_{v}\beta_{3} \) integrin from early endosomes to the plasma membrane, an event that is also necessary for cells to spread efficiently on vitronectin. We propose that \( \alpha_{v}\beta_{3} \) integrin must recycle to the plasma membrane via the Rab4 pathway and recruit active ERK1 in order to function efficiently.

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ERK1 Associates with α5β3 Integrin

volved in integrin affinity modulation; and the calcium-dependent protease, calpain, has recently been shown to mediate integrin clustering (15). However, the overall picture is far from complete.

Clearly, any kinase or other signaling protein found to associate with an integrin shortly following cell activation but prior to its incorporation into focal complexes would be potentially of interest as a possible regulator of integrin function. To identify these proteins, we immunoprecipitated integrins from fibroblasts shortly following activation with platelet-derived growth factor (PDGF-BB) and screened them for associated proteins that were rich in phosphoryrosine and phosphothreonine. We report that following PDGF addition, active ERK1 is an abundant component of α5β3 integrin immunoprecipitates. The association of ERK with α5β3 forms in plasma membrane complexes prior to delivery of integrin to focal complexes. Moreover, we find that association of active ERK1 with integrin, ERK, and Rab constructs (integrin and Rab cDNAs were expressed and purified as GST fusion proteins) was appropriate. Antibody and fusion protein-coated beads were incubated with lysates for 2 h at 4°C with constant rotation. Unbound proteins were removed by extensive washing in lysis buffer, and specifically associated proteins were eluted from the beads by boiling for 10 min in Laemmli sample buffer. Proteins were resolved by SDS-PAGE (8% gels under reducing conditions for ERKs, the hexa-His epitope, phosphothreonine, and phosphoseryl; 6% gels under nonreducing conditions for integrins) and analyzed by Western blotting as described previously (20).

**Integrin Recycling Assay**—This was performed as described previously (13). Cells were serum-starved for 30 min, transferred to ice, washed twice in cold PBS, and surface-labeled at 4°C with 0.2 μg/ml NHS-SS-biotin in PBS for 30 min. Labeled cells were transferred to serum-free DMEM at 22°C for 15 min to allow internalization of tracer into early endosomes. Cells were returned to ice and washed twice with ice-cold PBS, and biotin was removed from proteins remaining at the cell surface by incubation with a solution containing 20 mM sodium ortho-mercaptoethanesulfonate (MesNa) in 50 mM Tris pH 8.6, and 100 mM NaCl for 15 min at 4°C. The internalized fraction was then chased from the cells by returning them to 37°C in serum-free DMEM in the absence or presence of 10 ng/ml PDGF-BB for 10 min. Cells were returned to ice, and biotin was removed from recycled proteins by a second reduction with MesNa. MesNa was quenched by the addition of 20 mM iodoacetamide for 15 min at room temperature, and the cells were lysed in 200 mM NaCl, 75 mM Tris, 1.5 mM NaF, 7.5 mM EDTA and 7.5 mM EGTA, 1.5% (v/v) Triton X-100, 0.75% (v/v) Igepal CA-630, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride. Lysates were passed three times through a 27-gauge needle and clarified by centrifugation at 10,000 × g for 10 min at 4°C. For immunoprecipitations, magnetic beads conjugated to sheep anti-mouse IgG were blocked in PBS containing 0.1% (w/v) BSA and then bound to anti-integrin or anti-His6 monoclonal antibodies. For pull-downs, anti-mouse magnetic beads were bound to mouse anti-rabbit IgG, followed by anti-GST or GST-integrin fusion proteins as appropriate. Antibody and fusion protein-coated beads were incubated with lysates for 2 h at 4°C with constant rotation. Unbound proteins were removed by extensive washing in lysis buffer, and specifically associated proteins were eluted from the beads by boiling for 10 min in Laemmli sample buffer. Proteins were resolved by SDS-PAGE (8% gels under reducing conditions for ERKs, the hexa-His epitope, phosphothreonine, and phosphoseryl; 6% gels under nonreducing conditions for integrins) and analyzed by Western blotting as described previously (20).

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal rat anti-mouse α5 integrin (clone 5H10–27 (MF55)), hamster anti-mouse β3 integrin (clone 2C9.G2), and mouse anti-human β3 integrin (clone VI-PL2) were purchased from Pharmingen (San Diego, CA). Monoclonal mouse anti-ERK1/2 (s13–6200) from Novex (San Francisco, CA) was used for immunofluorescence, and polyclonal rabbit anti-ERK1/2 (sc-93) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and monoclonal anti-Thr202/Tyr204 phospho-ERK1/2 from New England Biolabs (Beverly, MA) were used for Western blotting. Peroxidase-conjugated anti-phosphotyrosine (clone 4G10) was from BD Transduction Laboratories (San Diego, CA). Monoclonal mouse anti-phospho-Thr202/Tyr204 (clone 41B3) and PD98059 (513000) were from Calbiochem. Fluorescein isothiocyanate-conjugated goat anti-mouse and Texas Red-conjugated anti-rabbit immunoglobulins were from Southern Biotechnology (Birmingham, AL). Texas Red-conjugated phalloidin was purchased from Molecular Probes (Leiden, The Netherlands). Magnetic sheep anti-mouse IgG Dynabeads (Dynal, Oslo, Norway) and bovine serum albumin (BSA) were from First Link Ltd. NHS-Ss-biotin (21351) and enhanced chemiluminescence reagents (ECL) were from Pierce and Warriner Ltd. (Chester, Cheshire, UK). Cell culture medium and Maxisorb 96 well plates were purchased from Invitrogen, and fetal calf serum (FCS) was from Sera-Q (Tunbridge Wells, Kent, UK). The Fucose 6 transfection reagent was from Roche Diagnostics GmbH (Mannheim, Germany). PDGF-BB (100–14B) was from PreproTech Inc. (Rocky Hill, NJ). Streptavidin-conjugated horseradish peroxidase was from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). All other reagents including a monoclonal antibody against the hexa-His epitope tag (anti-polyHISTIDINE, clone HIS-1) were purchased from Sigma.

**Cell Culture and Transfection**—Swiss and NIH 3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.02 μg/ml amphotericin B at 37°C with 10% CO2. For transient transfection experiments, NIH 3T3 fibroblasts were grown to 50% confluence, transfected with integrin, ERK, and Rab constructs (integrin and Rab cDNAs were ligated into pcdNA3 and are as described in Ref. 13; His-ERK1, His-ERK1K–R, HisERK2, and His-ERK2–K–R were in pCMV5 and are as described in Ref. 18) using Fugene 6 according to the manufacturer's instructions. The ratio of Fugene 6 to DNA was maintained at 3 μl of Fugene 6:1 μg of DNA. Immunoprecipitations, integrin recycling, and cell spreading assays were carried out 24 h post-transfection. Expression and Purification of GST-Integrin Cytodomain Fusion Proteins—PCR-amplified DNA fragments corresponding to aa 728–762, 728–756, 728–748, and 728–741 of the human sequence of β3 integrin and to aa 764–798 of the human sequence of β3 integrin were subcloned into the BamHI-EcoRI site of the pGEX-2TK vector. GST fusion proteins were expressed in Escherichia coli strain BL21 and purified as described previously (19).

Immunoprecipitations and Pull-downs—Cells were grown to 90% confluence, serum-starved for 30 min, and treated with PDGF (10 ng/ml), epidermal growth factor (30 ng/ml), or lysophosphatidic acid (1 μg/ml) where appropriate. Following this, cells were washed twice in ice-cold PBS and lysed in a buffer containing 200 mM NaCl, 75 mM Tris-HCl pH 7.5, 15 mM NaF, 1.6 mM Na2VO4, 7.5 mM EDTA, and 7.5 mM EGTA, 0.5% (v/v) Triton X-100, 0.25% (v/v) Igepal CA-630, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (1.14 μM/liter culture area; giving a final concentration of 0.3% (v/v) nonionic detergent following dilution of the lysis buffer in the PBS wetting the cells) and scraped from the dish with a rubber policeman. Lysates were passed three times through a 27-gauge needle and clarified by centrifugation at 10,000 × g for 10 min at 4°C. For immunoprecipitations, magnetic beads conjugated to sheep anti-mouse IgG were blocked in PBS containing 0.1% (w/v) BSA and then bound to anti-integrin or anti-His6 monoclonal antibodies. For pull-downs, anti-mouse magnetic beads were bound to mouse anti-rabbit IgG, followed by anti-GST or GST-integrin fusion proteins as appropriate. Antibody and fusion protein-coated beads were incubated with lysates for 2 h at 4°C with constant rotation. Unbound proteins were removed by extensive washing in lysis buffer, and specifically associated proteins were eluted from the beads by boiling for 10 min in Laemmli sample buffer. Proteins were resolved by SDS-PAGE (8% gels under reducing conditions for ERKs, the hexa-His epitope, phosphothreonine, and phosphoseryl; 6% gels under nonreducing conditions for integrins) and analyzed by Western blotting as described previously (20).

**Cell Adhesion and Spreading Assays**—24-well tissue culture plates were coated overnight at 4°C with fibronectin (F-1141; Sigma) or...
vitronectin (V-8379; Sigma) at concentrations of 20 μg/ml and then blocked with 2% (w/v) BSA. Cells were transfected with Rab4 or His-ERK constructs in conjunction with a β-galactosidase-expressing marker construct and, 24 h following transfection, were harvested by trypsinization and collected by centrifugation in the presence of 20 μg/ml soyabean trypsin inhibitor. The cell suspensions were added immediately to ligand coated wells in serum-free DMEM containing 10 ng/ml PDGF-BB in the presence and absence of 12 μM PD98059. Cells were allowed to attach for 60 min, and nonadherent cells were removed by washing six times with PBS. Attached cells were fixed for 1 min in 0.2% glutaraldehyde containing 5 mM EGTA, and β-galactosidase-expressing cells were visualized by incubation with 5 mM potassium ferricyanide and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) overnight at 37 °C. To obtain an index of cell spreading, the area of cells expressing β-galactosidase was determined by delineation of the cell envelope using the NIH Image software.

RESULTS

Active ERK1 Is Associated with α5β3 Integrin—Swiss 3T3 fibroblasts were serum-starved for 30 min and then stimulated with 10 ng/ml PDGF for 10 min or allowed to remain quiescent. Cells were immediately cooled to 4 °C and surface-labeled with NHS-SS-Biotin. Attached cells were lysed in a buffer containing 0.5% (v/v) Triton X-100 and 0.25% (v/v) Igepal CA-630. Lysates were immunoprecipitated (IP) with monoclonal antibodies against mouse α5 (mα5) and β3 (mβ3) integrins. Immobilized material was then analyzed by Western blotting with peroxidase-conjugated streptavidin (SA-HRP) (B), anti-phosphotyrosine (PY) (C), anti-ERK1/2 (D–F), and anti-phospho-ERK1/2 (D). The positions of α5, αv, β1, and β3 integrin chains, immunoglobulin heavy chain (IgG HC), and ERK1/2 are indicated.

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To gain insight into key biochemical events that occur during the early stages of focal complex assembly, we investigated the complement of cell-signaling proteins that communoprecipitate with integrins shortly following treatment of cells with growth factor. Therefore, integrin immunoprecipitates were analyzed by Western blotting with antibodies recognizing phosphotyrosine and phosphothreonine. In serum-starved cells, low levels of phosphotyrosine- or phosphothreonine-containing proteins communoprecipitated with either α5β1 or α5β3 integrins (Fig. 1, B and C). However, following the addition of PDGF, a 44-kDa protein (p44) that was rich in both phosphotyrosine and phosphothreonine was particularly abundant in immunopre-
ERK1 Associates with α,β3 Integrin

Fig. 2. ERK1 is associated with human α,β3 integrin expressed in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were transfected with human α,β3 integrin (hαβ3; A–D), α,β3, in combination with His-ERK1 or His-ERK2 (E–F), or empty vector control (A–D; mock) and then serum-starved and challenged with PDGF-BB for 10 min. Following surface labeling, cells were lysed and immunoprecipitated (IP) with monoclonal antibodies against human β3 integrin (A–E; hβ3) or the hexa-His epitope (6XHis) (F). Immobilized material was analyzed by Western blotting with peroxidase-conjugated streptavidin (SA-HRP) (A), anti-human β3 integrin (hβ3) (A and E–F), anti-phosphotyrosine (PY) (B), anti-phosphothreonine (PT) (C), anti-ERK1/2 (D), anti-phospho-ERK1/2 (E), or the hexa-His epitope (6XHis) (E and F). The positions of α3 and β3 integrin chains, immunoglobulin heavy chain (IgG HC), ERK1/2, and HisERK1/2 are indicated.

cipitates of α,β3 integrin (Fig. 1, B and C). p44 was only present at low levels in αβ2 integrin immunoprecipitates, and, moreover, there was no phosphotyrosine signal at 120 or 70 kDa, indicating that pp125FAK and paxillin were not associated with either integrin. We were, however, able to coimmunoprecipitate small quantities of a phosphotyrosine-containing protein of 190 kDa with α,β3, and this is likely to represent association of the PDGF receptor with the integrin (21).

A previous report has documented the presence of the ERKs in newly forming focal complexes (22). Both p44 ERK1 and p42 ERK2 would be expected to be rich in phosphotyrosine and phosphothreonine following cell activation, so we tested for the presence of these kinases in the integrin immunoprecipitates. Western blotting with an antibody that recognizes both p44 ERK1 and p42 ERK2 revealed that p44 comprised ERK1, and this was associated with α,β3 only following PDGF treatment (Fig. 1D). A small quantity of ERK1 was found to be associated with α,β3 integrin; however, this was not increased by the addition of PDGF (Fig. 1D). Interestingly, only relatively small amounts of p42 ERK2 were associated with α,β3, indicating that recruitment of ERK1 was isoform-specific. ERK1 must be phosphorylated on both threonine 202 and tyrosine 204 to be active, and the presence of signals for phosphothreonine and phosphothreonine in p44 implied that it was indeed active ERK1. This was confirmed by Western blotting using a phosphospecific antibody that recognized ERKs only when phosphorylated at both of these positions (Fig. 1D). The phosphotyrosine-containing protein at 60 kDa (p60; Fig. 1B) is likely to represent a Src family kinase. We are currently investigating the identity of p60 and the significance of its association with α,β3 integrin.

We investigated the time course over which the ERK1–α,β3 complex was established. Tyrosine phosphorylation of protein bands corresponding to the PDGF receptor (190 kDa), pp125FAK (125 kDa), and paxillin (70 kDa) was maximal ~4 min after PDGF addition and subsided over the following 12 min (Fig. 1E, upper panel). Recruitment of ERK1 to α,β3 integrin was slower than this and was maximal at 8 min following PDGF addition (Fig. 1E, lower panel). The association persisted for at least 16 min following PDGF addition (Fig. 1E) but abated somewhat over the following hour (data not shown). PDGF, lysophosphatidic acid, and epidermal growth factor were all able to induce substantial increases in tyrosine phosphorylation of a number of cellular proteins (notably of a band corresponding to pp125FAK) (Fig. 1F, upper panel). However, of the growth factors tested in the present study, only PDGF was able to elicit appreciable recruitment of ERK1 to α,β3 (Fig. 1F, lower panel). Increased tyrosine phosphorylation of a 36-kDa
protein (marked p36 in Fig. 1, E and F, upper panels) was PDGF-specific and, moreover, occurred at a rate that paralleled recruitment of ERK1 to αβ3. Preliminary data indicate that p36 is likely to be annexin II (not shown), and the possible role of this protein in the assembly of integrin-containing complexes is discussed later.

To determine whether ERK1 was also able to associate with human αβ3 and to confirm that its presence in mouse αβ3 immunoprecipitates was not an artifactual characteristic of the antibody employed, human integrins were transfected into NIH 3T3 fibroblasts, and αβ3 was immunoprecipitated with a monoclonal antibody that was specific for the human β3 integrin chain (hβ3). Surface labeling indicated that this antibody did not precipitate mouse αβ3 (Fig. 2A), and accordingly we were unable to detect any phosphotyrosine/phosphothreonine-containing proteins or ERKs associated with anti-human β3 monoclonal antibody-coated beads when they were incubated with lysates prepared from mock-transfected cells even following PDGF treatment (Fig. 2, B–D). Following transfection of NIH 3T3 fibroblasts with the human α3 and β3 integrin chains, surface-labeled proteins corresponding to the α3β3 heterodimer were prominent in the immunoprecipitates. These displayed increased surface expression following the addition of PDGF (Fig. 2A), although Western blotting with an antibody recognizing the hβ3 chain revealed that the total quantity of integrin present in the immunoprecipitates was unaffected (Fig. 2A). Moreover, in transfected cells, a profile of phosphotyrosine/phosphothreonine-containing proteins, similar to that found associated with the mouse integrin, were immunoprecipitated with human αβ3 following the addition of PDGF (Fig. 2, B and C), and Western blotting revealed that the 44-kDa band (p44) comprised active ERK1 (Fig. 2D).

To confirm the ability of αβ3 integrin to recruit ERK1 in preference to ERK2 and to further test the specificity of this interaction, we performed immunoprecipitation studies on NIH 3T3 fibroblasts following transient expression of epitope-tagged ERKs. Following the addition of PDGF, recruitment of His-ERK1 to αβ3 immunoprecipitates was seen using an monoclonal anti-His6 antibody to detect the epitope-tagged protein (Fig. 2E). Furthermore, Western blotting with anti-hβ3 indicated that αβ3 integrin was precipitated by immunosolubilation of His-ERK1 using magnetic beads conjugated to anti-His6 (Fig. 2F). In contrast, following transient expression of His-ERK2, this epitope-tagged kinase was not detected in integrin immunoprecipitates (Fig. 2E); nor did β3 integrin co-precipitate with His-ERK2 (Fig. 2F). It is important to note that in these experiments cellular expression of His-ERK1 was somewhat greater than His-ERK2. This discrepancy was only observed when the ERKs were coexpressed with αβ3 integrin (compare Fig. 2, E and F, with the expression levels of His-ERKs in Fig. 9C, where no exogenous αβ3 is expressed) and may indicate that overexpression of αβ3 integrin can support increased cellular levels of ERK1. It is unlikely, however, that the lack of co-immunoprecipitation of His-ERK2 with αβ3 (and vice versa) is due to this discrepancy, since we have performed experiments in which the expression of His-ERK2 was raised to exceed that of His-ERK1 (by increasing the quantity of cDNA employed for the transfection) and obtained similar results.

Recruitment of ERK to the β3 Integrin Cytodomain—A number of studies have indicated that the cytodomains of integrin β subunits are responsible for recruiting signaling kinases and cytoskeletal proteins to the heterodimer (6, 23). To test the involvement of the β3 cytodomain in ERK1 recruitment, we constructed a series of β3 integrin truncation mutants (Fig. 3A). These were aimed at sequentially deleting the membrane-distal NITY motif including Ile757 (Δ757), which has been implicated in the assembly of focal adhesions, the sequence intervening in the membrane-proximal NPXY and the NITY motifs (Δ749), and finally the removal of a large proportion of the integrin cytodomain (Δ728).

The three truncation mutants, Δ757, Δ749, and Δ728, were all expressed at levels similar to that observed for full-length β3, were immunoprecipitated efficiently with the anti-hβ3 monoclonal antibody (Fig. 3B), and formed heterodimers with the α3 subunit (not shown). ERK1 coimmunoprecipitated equally well with both full-length β3 integrin and Δ757, indicating that the NITY motif and Ile757 are not necessary for association with the kinase. However, removal of the 749 EAT-STFTTY758 sequence that intervenes in the NPXY and NITY motifs resulted in a substantial reduction in ERK1 recruitment (Fig. 3B).

To determine whether the β3 cytodomain was sufficient on its own to recruit active ERK, lysates from PDGF-stimulated NIH 3T3 fibroblasts were incubated with GST fusion proteins corresponding to the β3 integrin cytodomain and truncation mutants thereof (Fig. 3C). Interestingly GST-β3 cytodomain was able to associate with both ERK1 and ERK2, whereas

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**Fig. 2.** The β3 integrin cytodomain recruits ERK. A and B, full-length β3 integrin and the Δ757, Δ749 and Δ728 truncation mutants of β3 integrin shown in A were transiently expressed in NIH 3T3 fibroblasts together with the α3 integrin subunit. Transfected cells were serum-starved and challenged with 10 ng/ml PDGF-BB for 10 min. Following this, the monolayers were lysed and immunoprecipitated with monoclonal antibodies against human β3 integrin. Immobilized material was analyzed by Western blotting with anti-human β3 integrin (B, upper panel) and anti-ERK1/2 (B, lower panels). C and D, lysates from PDGF stimulated NIH 3T3 fibroblasts were incubated with magnetic beads conjugated to GST or the GST-integrin cytodomain fusion proteins indicated in C. Immobilized material was analyzed by Western blotting for ERK1/2 (D, upper panels), and the loading of the GST fusion proteins was confirmed by Western blotting for GST (D, lower panels).
GST-β₁ was less effective in this regard (Fig. 3D) and, consistent with the immunoprecipitation studies shown in Fig. 3B, association of ERKs was lost upon removal of the EATST-FTN sequence between the NPXY and NITY motifs (Fig. 3D). These results indicate that the cytodomain of the integrin subunit is both necessary and sufficient to recruit an ERK-containing complex to integrin and that the central region of the cytodomain is involved in establishment of this association.

Recruitment of αvβ₃ and ERK to Plasma Membrane Complexes—We have previously shown that αvβ₃ integrin is incorporated into punctate plasma membrane complexes immediately following Rab4-dependent recycling, and these are subsequently redistributed into peripheral focal complexes (13). Given that ERK1 association with αvβ₃ was established within 10 min of PDGF addition, we wished to determine whether the kinase was also recruited to plasma membrane complexes. Cells were serum-starved for 30 min and treated with PDGF for 10 min, and surface αvβ₃ and ERKs visualized by confocal immunofluorescence microscopy. In serum-starved cells, immunoreactive ERKs were seen to focus in the perinuclear region, perhaps suggesting sequestration of the kinase upon an endomembrane compartment or even at the microtubule organizing center (Fig. 4C). Upon the addition of PDGF, ERKs rapidly redistributed such that they could now be seen in the nucleus and also dispersed into a punctate array across the cell surface (Fig. 4G). This resembled the distribution assumed by αvβ₃ (Fig. 4E), and examination of the higher magnification confocal micrograph shown in Fig. 5 revealed a close colocalization of ERKs and αvβ₃ integrin in these small punctate structures, which were particularly enriched toward the cell periphery (Fig. 5E). These complexes did not contain caveolin 1, so they are distinct from caveolin-containing membrane islands; nor did they contain paxillin or other markers of focal adhesions and complexes (data not shown). αvβ₁ integrin was present in large deposits and a fibrillar distribution reminis-
MEK in order to be recruited to \( \alpha_3 \beta_3 \) integrin.

It is important to note that the concentration of PD98059 employed for these experiments (12 \( \mu \)M) was sufficient to negate association of ERK1 with integrin, had it only partial effects on the PDGF-induced recruitment of ERKs to the nucleus (Fig. 6F).

**Association of ERK1 with \( \alpha_3 \beta_3 \) Is Not Necessary for Integrin Recycling**—We have previously shown that PDGF increases recycling of \( \alpha_3 \beta_3 \) from early endosomes to the plasma membrane via a Rab4-dependent mechanism (13). To investigate the possibility that recruitment of ERK1 to \( \alpha_3 \beta_3 \) is necessary for recycling, we studied the effect of PD98059 on \( \alpha_3 \beta_3 \) recycling. Recycling of \( \alpha_3 \beta_3 \) from early endosomes to the plasma membrane was assayed using the enzyme-linked immunosorbent assay-based method we have described previously (13), and PD98059 had no effect on the ability of PDGF to drive \( \alpha_3 \beta_3 \) recycling from early endosomes (Fig. 7). These data are consistent with the images presented in Fig. 6, E–G, where PD98059 suppressed the colocalization of \( \alpha_3 \beta_3 \) with ERK but did not affect surface expression of the integrin. It is interesting to note, however, that the integrin-containing complexes were smaller and more numerous in the presence of PD98059 (Fig. 6E), indicating that ERK may act to cluster \( \alpha_3 \beta_3 \).

Recycling of \( \alpha_3 \beta_3 \) Is Not a Prerequisite for Association of ERK1 with \( \alpha_3 \beta_3 \)—Having shown that delivery of \( \alpha_3 \beta_3 \) to the plasma membrane was independent of ERK1 recruitment to the integrin, we wished to determine whether the recycling of \( \alpha_3 \beta_3 \) integrin was a prerequisite for its association with ERK1. Recycling of \( \alpha_3 \beta_3 \) from early endosomes to the plasma membrane was powerfully stimulated by PDGF, and, consistent with our previous studies (13), this component of integrin vesicular transport was completely ablated by expression of the dominant negative Rab4 construct, N121Irab4 (Fig. 8B). However, association of ERK1 with \( \alpha_3 \beta_3 \) was unaffected by expression of N1211rab4 (Fig. 8A), despite the blockade of integrin recycling effected by this dominant negative construct.

Recycling of \( \alpha_3 \beta_3 \) and Active ERK1 Are Required for Cell Spreading on Vitronectin—To investigate the role of ERK1 in \( \alpha_3 \beta_3 \) integrin function, cells were allowed to spread on vitronectin, a good ligand for \( \alpha_3 \beta_3 \). We have previously shown that PDGF increases \( \alpha_3 \beta_3 \) function.

Fig. 5. \( \alpha_3 \beta_3 \) integrin and ERK localize to punctate plasma membrane complexes. Swiss 3T3 fibroblasts were serum-starved for 30 min, challenged with 10 ng/ml PDGF-BB for 10 min, and then fixed in 2% paraformaldehyde. Surface \( \alpha_3 \beta_3 \) integrin was visualized by indirect immunofluorescence (A and B; green). Following this, cells were detergent-permeabilized and counterstained for cellular ERK1/2 (C and D; red). Colocalization of the two fluorophores is shown in yellow (E and F). Images are presented as a single confocal optical slice centered ~0.5 \( \mu \)m above the plane of the substratum. Bars, 10 \( \mu \)m (A, C, and E; left panels) and 2.5 \( \mu \)m (B, D, and F; right panels).

Following a longer (30-min) exposure to PDGF, ERKs were still visible in the nucleus, but the surface complexes were no longer prominent, and the kinase was incorporated into a fine array of focal complexes in the peripheral lamellae that paralleled the distribution of \( \alpha_3 \beta_3 \) integrin (Fig. 4, I–L). Taken together, these immunofluorescence and biochemical data suggest that shortly following growth factor addition, ERK1 and \( \alpha_3 \beta_3 \) form a physical association within small punctate complexes in the plasma membrane. These then subsequently redistribute to form focal complexes in the peripheral lamellae.

**Association of ERK1 with \( \alpha_3 \beta_3 \) Integrin Requires the Activity of MEK**—ERK1 is activated by phosphorylation on threonine 202 and tyrosine 204 by the dual specificity kinase, MEK1/2. We investigated whether treatment of cells with the MEK inhibitor, PD98059 (24), affected the association of \( \alpha_3 \beta_3 \) with ERK1 and the recruitment of ERK1 to \( \alpha_3 \beta_3 \)-containing complexes. Serum-starved cells were treated with 12 \( \mu \)M PD98059 for 10 min, following which they were challenged with PDGF and lysed for immunoprecipitation or fixed for immunofluorescence. This concentration of PD98059 completely ablated association of ERK1 with immunoprecipitates of \( \alpha_3 \beta_3 \) (Fig. 6A), and, in accordance with this, markedly reduced the appearance of ERK in integrin-rich plasma membrane complexes (Fig 6, B–G). These data show that ERK1 must be phosphorylated by
clonal antibodies against the mouse H9252 precipitated (lysed, and allowed to remain quiescent. Cells were challenged with 10 ng/ml PDGF-BB or of ERK with H9251 labeled during the internalization period (values are mean plasma membrane is expressed as a percentage of the pool of integrin

ion (18). Both of these dominant negative ERK mutants were

developed. To test this, we employed dominant negative mutants of ERK1 and ERK2 (ERK1K–R and ERK2K–R, respectively), which have previously been shown to oppose ERK-induced c-fos expression and cell transformation in an isoform-specific fashion (18). Both of these dominant negative ERK mutants were expressed at similar levels in NIH 3T3 fibroblasts (Fig. 9C), but ERK1K–R inhibited spreading onto vitronectin by ~60%, whereas ERK2K–R was ineffective in this regard (Fig. 9B). Additionally, ERK1K–R did not compromise spreading on fibronectin, indicating that ERK1 activity is particularly focused toward the function of α3β3 and does not impinge on the function of other fibronectin-binding integrins such as α3β1.

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FIG. 6. PD98059 inhibits association of ERK with α3β3 integrin. A, serum-starved Swiss 3T3 fibroblasts were incubated for 10 min in the absence or presence of 12 μM PD98059 and then challenged with 10 ng/ml PDGF-BB or allowed to remain quiescent. Cells were lysed, and α3β3 integrin was immunoprecipitated (IP) from the lysates with monoclonal antibodies against the mouse β3 integrin chain. Immobilized material was then analyzed by Western blotting with an antibody against ERK1/2. The migration positions of the ERKs1/2 are indicated. B–G, serum-starved Swiss 3T3 fibroblasts were incubated for 10 min in the absence (B–D) or presence (E–F) of 12 μM PD98059, challenged with 10 ng/ml PDGF-BB, and then fixed in 2% paraformaldehyde. Surface α3β3 integrin was visualized by indirect immunofluorescence (B and E, green). Following this, cells were detergent-permeabilized and counterstained for cellular ERK (C and F, red). Colocalization of the two fluorophores is shown in yellow (D and G). Bar, 10 μm.

FIG. 7. PDGF-stimulated recycling of α3β3 does not require the activity of MEK. Serum-starved Swiss 3T3 fibroblasts were surface-labeled with 0.2 mg/ml NHS-SS-Biotin for 30 min at 4 °C, and internalization was allowed to proceed for 15 min at 22 °C in the presence and absence of 12 μM PD98059. Biotin was removed from receptors remaining at the cell surface by treatment with MesNa at 4 °C, and cells were rewarmed to 37 °C for 10 min in the absence or presence of 10 ng/ml PDGF-BB to allow recycling to the plasma membrane, followed by a second reduction with MesNa. Cells were lysed, and integrin biotinylation was determined by capture enzyme-linked immunosorbent assay using microtiter wells coated with anti-mouse β3 integrin monoclonal antibodies. The proportion of integrin recycled to the plasma membrane is expressed as a percentage of the pool of integrin labeled during the internalization period (values are mean ± S.E. from three separate experiments).

DISCUSSION

Here we show that following addition of PDGF to serum-starved fibroblasts, a 44-kDa protein that is rich in phosphotyrosine and phosphothreonine coimmunoprecipitates with α3β3 integrin. Western blotting with phosphospecific antibodies revealed that this protein was active ERK1. Experiments in which the C-terminal region of the β3 integrin subunit was truncated, and pull-downs with GST-β3 fusion proteins show that the cytodomain of the integrin is both necessary and sufficient to recruit ERK and moreover that the 749EATST-FTN59 sequence interposing the NPXY and NITY motifs may be critical to this. Immediately following PDGF addition, ERK was seen to colocalize with α3β3 in numerous small complexes at the plasma membrane, and only later did these redistribute to focal complexes in the peripheral lamellae. The association of ERK1 with α3β3 was particularly sensitive to treatment of the cells with the MEK inhibitor, PD98059; however, this compound had no effect on the Rab4-dependent flux of integrin from early endosomes to the plasma membrane. Correspondingly, inhibition of Rab4 had no effect on recruitment of ERK1 to α3β3 integrin, indicating that integrin recycling and the recruitment of active ERK1 are not interdependent. Expression
of a dominant negative mutant of ERK1 (but not ERK2) significantly reduced the spreading of cells onto vitronectin, whereas cell spreading on fibronectin was unaffected by inhibition of ERK1, consistent with a special role for this isoform of ERK in the regulation of \( \alpha_\beta_3 \) (but not \( \alpha_\beta_2 \)) integrin function. PD98059 also reduced cell spreading on vitronectin, to the same extent as did dominant negative Rab4, and the effects of Rab4 and MEK inhibition were not additive. Taken together, these data indicate that \( \alpha_\beta_3 \) must recycle to the plasma membrane via the Rab4 pathway and recruit ERK1 in order to function efficiently.

**Role of Integrins in ERK Translocation**—In resting cells, ERK is retained in the cytoplasm in tight association with the microtubule cytoskeleton (25), and it is likely, therefore, that the perinuclear accumulation of ERK that we observe in serum-starved fibroblasts indicates association with the microtubule organizing center. Upon stimulation, ERK translocates from the cytoplasm to the nucleus, where it influences gene expression by phosphorylating transcription factors. This enhances expression of a number of early response genes, such as c-fos (26), and ultimately leads to the induction of cyclin D1 and progression through the G_1 phase of the cell cycle (27). The engagement of integrin is known to profoundly enhance ERK activation in response to growth factor addition, and this provides a rationale for the much studied phenomenon of anchorage-dependent growth (28). Enhancement of ERK signaling is thought to be mediated by a diverse array of integrin-activated signaling pathways, most of which also lead to reorganization of the actin cytoskeleton. Indeed, a recent study has shown that integrin-mediated adhesion is necessary for efficient nuclear translocation of ERK via a mechanism that clearly requires an intact actin cytoskeleton (29). It is possible that association of ERK with the focal adhesion machinery may facilitate delivery of the kinase to the nucleus. Two aspects of our data, however, argue against this. First, ERK1 recruitment to \( \alpha_\beta_3 \) is only fully established ~8 min following PDGF addition. However, the translocation of ERK to the nucleus is, if anything, faster than this, arguing against a sequence of events whereby ERK is obliged to associate with \( \alpha_\beta_3 \) and passage through focal complexes in order to reach the nucleus. Second, the concentration of PD98059 employed in the present study was found to completely ablate association of ERK1 with \( \alpha_\beta_3 \) but had no effect on nuclear accumulation of ERK. This implies that different pools of cytoplasmic ERK are destined for transport to the nucleus and the plasma membrane following growth factor addition, the activation of the former being less sensitive to treatment of cells with PD98059 than the latter.

Our data indicate that overexpression of \( \alpha_\beta_3 \) integrin favors increased cellular expression levels of His-ERK1. This suggests that \( \alpha_\beta_3 \) may play a role in the stabilization of the...
ERK1 protein, most likely by incorporation of the kinase into an integrin complex. The coordinated synthesis and degradation of many signaling proteins is likely to be integral to the establishment of anchorage-dependent growth, and it is possible that the ability of \( \alpha_\beta_3 \) integrin to support increased cellular levels of ERK1 may contribute to this.

A Role for ERK at the Plasma Membrane—A number of recent studies have shown that ERK has an important role in the cytoplasm and that this is likely to be distinct from its activity in the nucleus. The sea star oocyte homologue of ERK1 directly phosphorylates myosin light chain kinase (30), and more recently activation of ERKs with a constitutively active MEK has been shown to enhance cell migration via phosphorylation of myosin light chain kinase (31). A more recent study has demonstrated that active ERK is recruited to focal adhesions and controls their assembly by virtue of its ability to phosphorylate and activate myosin light chain kinase (22). Thus, if phospho-ERK levels are lowered using U0126 (a more potent MEK inhibitor than PD98059), the assembly of focal complexes is inhibited, and consequently the ability of cells to spread on the extracellular matrix is compromised. We are able to confirm that ERK is indeed targeted to focal complexes and furthermore show that this is likely to be achieved by its association with an extracellular matrix receptor, \( \alpha_\beta_3 \) integrin.

Recruitment of ERK is clearly mediated by the cytodomain of the \( \beta_3 \) integrin subunit. The extreme C-terminal region of \( \beta_3 \) is known to associate with both endonexin (32) and Syk (6), and a previous study has shown that Ile\textsuperscript{757} is critical for targeting \( \alpha_\alpha_\beta_3 \) to focal adhesions (33). However, removal of the C-terminal NITY motif, including Ile\textsuperscript{757}, has no effect on ERK recruitment to \( \alpha_\beta_3 \), or to GST-\( \beta_3 \) cytodomain fusion proteins. On the other hand, our data indicate that the \( ^{74} \text{KDA}^{757} \) sequence (immediately N-terminal to the NITY motif) is required for association with ERK. It has been known for some time that this portion of the \( \beta_3 \) cytodomain is critical for integrin function. A serine to proline substitution in this region has been shown to reduce \( \beta_3 \) integrin function in cell attachment, spreading, and the initiation of tyrosine phosphorylation of pp125\( \text{FAK} \), and paxillin (35). A recent report has documented a direct association of ERK with the cytodomain of \( \beta_3 \) integrin (36). Synthetic peptides containing the central region of the \( \beta_3 \) cytodomain were shown by these workers to bind directly to ERK. It is interesting to note that a TSTF motif in this region is conserved between \( \beta_3 \) and \( \beta_8 \) and is not present in other \( \beta \)-integrin cytodomains. Further experiments will be aimed at testing the ability of the TSTF motif in \( \beta_3 \) to associate directly with ERKs.

Our data indicate a special relationship between ERK1 and \( \alpha_\beta_3 \), which is not shared by ERK2. First, ERK1 (and not ERK2) is recruited to immunoprecipitates of \( \alpha_\beta_3 \), and second His-ERK1-K\textsuperscript{R} opposes \( \alpha_\beta_3 \)-mediated cell spreading, whereas His-ERK2-K\textsuperscript{R} is ineffective in this regard. From this, it would be tempting to speculate that this is due to an integrin-binding site that is present in ERK1 but not ERK2. However, it is clear that GST-\( \beta_3 \) cytodomain fusion proteins have the capacity to bind equally well to both ERK1 and ERK2. This may indicate that selectivity for ERK1 requires the presence of the \( \alpha_\beta_3 \) heterodimer or alternatively may be influenced by accessory factors that are unable to function in the pull-down assays.

Our data indicate that the association of \( \alpha_\beta_3 \) with ERK1 occurs rapidly following the addition of PDGF and that the resulting complex localizes to punctate clusters in the plasma membrane prior to its incorporation into focal complexes. Hitherto, many studies have focused on the role of integrins in focal adhesions and complexes, and it is generally accepted that integrins are brought into close proximity with the various signaling molecules that mediate focal adhesion signaling, as a consequence of the activity of the Rho subfamily GTPases, such as RhoA and Rac (37). However, it is now becoming clear that certain pathways promote the association of integrins with other signaling components upstream of focal complex assembly. A recent study has highlighted a novel integrin complex, referred to as an integrin cluster, that forms upstream of Rac activation and focal complex assembly (15). These workers reported that integrin clusters differ from focal complexes in both their distribution and molecular composition. The \( \alpha_\beta_3 \)-ERK-containing complexes described in this study also differ in their composition from focal complexes. For instance, they do not stain for established focal adhesion markers, such as vinculin and paxillin; nor do any of these proteins coimmunoprecipitate with \( \alpha_\beta_3 \) following the addition of PDGF. It is interesting to speculate what kind of cellular structure these \( \alpha_\beta_3 \)-ERK-rich complexes may be. Labeling experiments with \( ^{125}\text{I} \)orthophosphate have indicated that, even following extensive washing with nonionic detergent (0.5% (v/v) Triton X-100 and 0.25% (v/v) Igepal), labeled phospholipids are tightly associated with \( \alpha_\beta_3 \) immunoprecipitates. Moreover, the quantity of coimmunoprecipitating phospholipid increased dramatically in response to the addition of PDGF, and this was opposed by PD98059 (data not shown). Reorganization of lipid rafts and other plasma membrane lipid subdomains has been reported to occur following activation of a number of signaling pathways (41). It is possible, therefore, that active ERK1 can act at the plasma membrane to induce clustering of integrin into large detergent-resistant raftlike membrane microdomains. Preliminary data indicate that p36 (Fig. 1, E and F) is likely to be annexin II (not shown). This Ca\textsuperscript{2+}-, phospholipid-, and actin-binding protein is known to be a substrate for the PDGF receptor and Src tyrosine kinases (38), and more recently it has been shown to localize to plasma membrane lipid rafts (39) and participate in the reorganization of these domains during cell attachment (40). It will be interesting to determine whether annexin II is recruited to \( \alpha_\beta_3 \)-rich membrane complexes and to investigate the possibility that it has a role in recruiting ERK1 to the plasma membrane.

Integrins have been reported to associate with many other types of transmembrane and other proteins, which in principle may coalesce to form an extensive network (42). Indeed, \( \alpha_\beta_3 \) integrin is known to associate with CD47, or integrin-associated protein, in a plasma membrane lipid raft (43). Also, the integrin-associated tetraspannin, CD81, has been shown to localize to such a membrane domain (42), and it is interesting in this regard that we observe CD81 to colocalize with \( \alpha_\beta_3 \) in puncta following PDGF treatment (data not shown).

Following expression of dominant negative Rab4, \( \alpha_\beta_3 \) would be expected to accumulate in early endosomes. This construct, however, does not reduce the recruitment of ERK1 to \( \alpha_\beta_3 \) immunoprecipitates and indicates that the association of active kinase to the integrin may be established either on the surface of endosomes or at the plasma membrane. Indeed, large integrin-tetraspannin complexes have been detected on intracellular vesicles (10), and this implies that associations made between integrins and other signaling molecules and membrane proteins may persist while the integrin engages in endocytic cycling. There is mounting evidence that the incorporation of membrane proteins into raftlike domains is a key sorting event in the secretory pathway (44). However, it is unlikely that recruitment of active ERK to \( \alpha_\beta_3 \) at the endosome is necessary to direct its recycling, since the rate of delivery of early endo-
somal α₃β₃ to the plasma membrane was clearly unaffected by PD98059.

The observation that dominant negative ERK1 compromises cell spreading on vitronectin, but not on fibronectin, implicates the activity of this ERK in the activation of α₃β₃ integrin. Many integrins, including α₅β₁, can assume different states with respect to ligand binding and engagement, and there are many examples of the transition between these states being controlled by signaling pathways within the cell, a phenomenon termed inside-out signaling (12). The affinity of an individual integrin heterodimer for its ligand may be increased, and changes in the lateral mobility and clustering of integrins can also affect the avidity of integrin binding to multivalent ligands. Regulation of either the affinity or avidity of an integrin will have profound influence on the ability of cells to spread on the extracellular matrix. The ability of the platelet integrin α₅β₁ to bind fibronogen has been shown to be inhibited by dominant negative mutants of Raf-1 or MEK1 (45). This implicates the MEK/ERK signaling axis in inside-out signaling mediated by dominant negative mutants of Raf-1 or MEK1 (45). This implicates the MEK/ERK signaling axis in inside-out signaling mediated by dominant negative mutants of Raf-1 or MEK1 (45). This implicates the MEK/ERK signaling axis in inside-out signaling mediated by dominant negative mutants of Raf-1 or MEK1 (45).

In summary, our studies have identified a novel association of active ERK1 kinase with α₃β₃ integrin that is established in advance of the incorporation of the integrin into focal complexes. Formation of this complex was not necessary for the trafficking of α₃β₃ through the Rab4-dependent recycling pathway; nor was the activity of Rab4 required for association of α₅β₁ with ERK. However, the activity of ERK1 and Rab4 are clearly required for cells to spread on vitronectin. We suggest that recruitment of ERK1 to α₅β₁ and the Rab4-dependent recycling pathway are parallel growth factor-activated events that are necessary for integrin function.

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