PDGF-regulated rab4-dependent recycling of αvβ3 integrin from early endosomes is necessary for cell adhesion and spreading

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Background: It has been postulated that the regulation of integrin vesicular traffic facilitates cell migration by internalizing integrins at the rear of the cell and transporting them forward within vesicles for exocytosis at the leading edge to form new contacts with the extracellular matrix. The rab family of GTPases control key targeting events in the endo/exocytic pathway; therefore, these GTPases may be involved in the regulation of cell-matrix contact assembly.

Results: The endo/exocytic cycle of αvβ3 and α5β1 integrins was studied using mouse 3T3 fibroblast cell lines. In serum-starved cells, internalized integrins were transported through rab4-positive, early endosomes and arrived at the rab11-positive, perinuclear recycling compartment approximately 30 min after endocytosis. From the recycling compartment, integrins were recycled to the plasma membrane in a rab11-dependent fashion. Following treatment with PDGF, αvβ3 integrin, but not α5β1, was rapidly recycled directly back to the plasma membrane from the early endosomes via a rab4-dependent mechanism without the involvement of rab11. This rapid recycling pathway directed αvβ3 to numerous small puncta distributed evenly across the dorsal surface of the cell, and the integrin only became localized into focal complexes at later times following PDGF addition. Interestingly, inhibition of PDGF-stimulated αvβ3 recycling using dominant-negative rab4 mutants compromised cell adhesion and spreading on vitronectin (a ligand for αvβ3), but adhesion to fibronectin (a ligand for α5β1 and αvβ3) was unchanged.

Conclusions: We propose that growth factor-regulated, rab4-dependent recycling of αvβ3 integrin from early endosomes to the plasma membrane is a critical upstream event in the assembly of cell-matrix contacts.

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Received: **9 April 2001**
Revised: **18 June 2001**
Accepted: **7 August 2001**

Published: **18 September 2001**

Current Biology 2001, 11:1392–1402

0960-9822/01/$ – see front matter

Background

In order for cells to spread on or migrate across flat surfaces, integrin-containing focal complexes must form rapidly at the peripheral or leading lamellae. Formation of these focal complexes requires integrin engagement but is also driven by growth factors such as PDGF [1]. Many surface receptors, including integrins, participate in an endo/exocytic cycle [2]. They are internalized, delivered to endosomes, and then recycled to the plasma membrane for reutilization. It has been suggested that this cycle may facilitate focal complex assembly by internalizing integrins at the rear of the cell and transporting them forward within vesicles for exocytosis at the leading lamellae [3].

Previous studies have shown that the integrin endo/exocytic cycle is functionally important. Fabbri et al. [4] have shown that a YXXϕ motif in the cytoplasmic tail of β2 integrin is essential for recycling to the plasma membrane following internalization and that disruption of this motif inhibits β2-dependent cell migration. Furthermore, β1 integrins are internalized via a dynamin-dependent step, and inhibition of β1 integrin internalization by expression of dominant-negative dynamin also reduces cell motility [5]. It is unclear, however, whether cell-signaling pathways coordinate cell motility by regulating the recycling of integrins.

Transferrin receptor (TFN-R) recycling operates in two distinct time domains; a short circuit recycling pathway directly from early endosomes to the plasma membrane [6] and an indirect route involving transit through the perinuclear recycling compartment [7]. The rab family of small GTPases control key targeting events in these recycling pathways. Rab 11 localizes to the perinuclear recycling compartment and has been shown to control recycling from this compartment as well as transport to the trans-Golgi network [8–10]. Rab4, on the other hand, is localized predominantly to early endosomes [6] and, to a lesser extent, to recycling endosomes where it colocalizes with rab11 [11, 12], and it is thought to be involved in recycling from both of these compartments. ARF6, a GTPase known to regulate cell spreading and motility, has also been shown to regulate endosomal recycling via a pathway distinct from those regulated by rab GTPases [13, 14]. Although some studies have shown that β1 integrins colocalize with rab11 and the transferrin receptor [5], it is unclear, however, which rab- or ARF-dependent steps are involved in the endo/exocytic cycle of integrins.

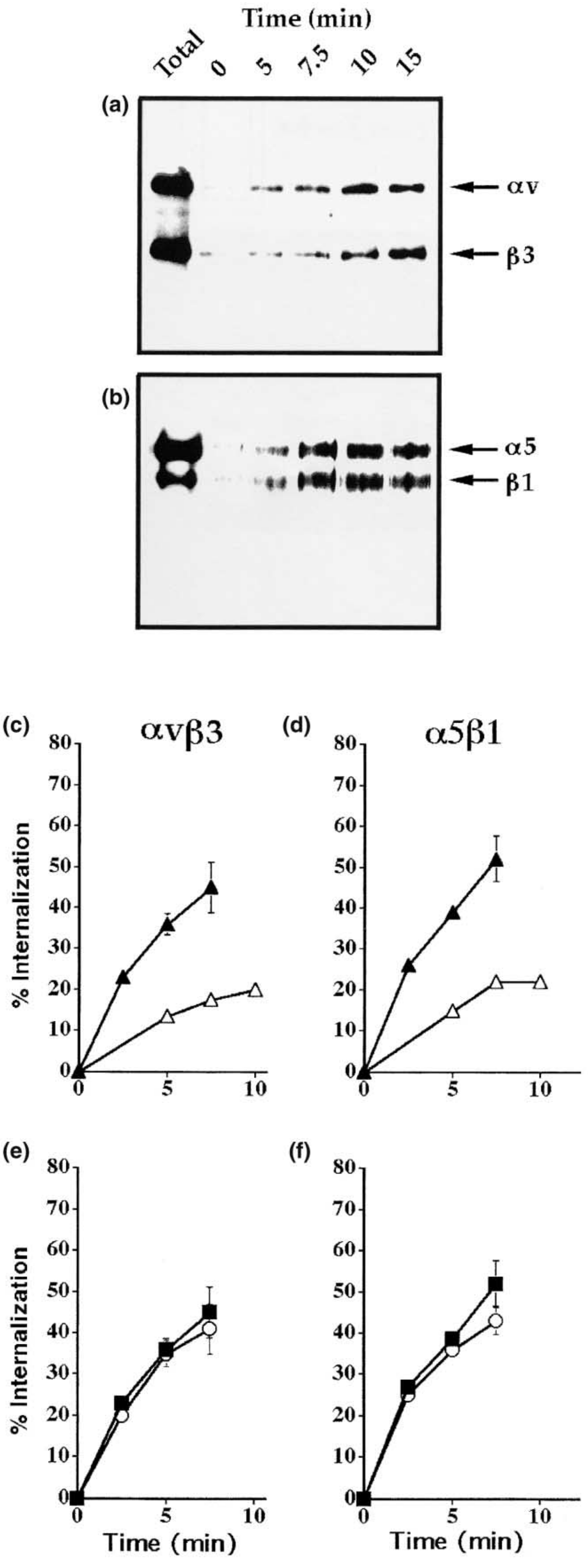
Here, we show that PDGF regulates the recycling rate for αvβ3, but not for α5β1. This process involves rab4 and does not require rab11 or ARF6; thus, it defines a mechanism whereby growth factors can regulate integrins during cell adhesion and spreading.

Results

Internalization of integrins

Preliminary experiments indicated that treatment with PDGF rapidly increased the levels of αvβ3 integrin at the plasma membrane (see the Supplementary material available with this article online). We developed assays for integrin endocytosis and recycling to determine whether PDGF regulation of these processes could account for this change in αvβ3 surface levels. Integrin internalization was determined by surface labeling Swiss 3T3 fibroblasts with NHS-SS-biotin at 4°C, followed by incubation at 37°C for various times. Biotin was removed from proteins remaining on the cell surface by exposure to Sodium 2-mercaptoethanesulphonate (MesNa) at 4°C, and internalized integrin was assessed by immunoprecipitation, followed by Western blotting with streptavidin. Both αvβ3 and α5β1 heterodimers were internalized with similar kinetics, their internal pools reaching a steady level by 10 min (Figure 1a,b). Using capture-ELISA to quantify biotinylated integrins, we found that the addition of the

Figure 1



Internalization of integrins. **(a,b)** Serum-starved Swiss 3T3 fibroblasts were surface labeled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4°C and were warmed to 37°C for the times indicated. Biotin was released from proteins remaining at the cell surface by MesNa treatment at 4°C, the cells were lysed, and integrins were immunoprecipitated with (a) anti-β3 or (b) anti-α5 monoclonal antibodies. Immobilized material was then analyzed by 6% SDS-PAGE, followed by Western blotting with peroxidase-conjugated streptavidin. The positions of α5, αv, β1, and β3 integrin chains are indicated. **(c,d)** Surface-labeled cells were warmed to 37°C in the absence (open triangle) or presence (solid triangle) of 0.6 μM PMQ for the times indicated. Biotin was released from proteins remaining at the cell surface, and biotinylated integrin was determined by capture-ELISA using microtiter wells coated with (c) anti-β3 or (d) anti-α5 integrin monoclonal antibodies. **(e,f)** Surface-labeled cells were warmed to 37°C in the presence of 0.6 μM PMQ and in the absence (open circle) and presence (solid square) of 10 ng/ml PDGF-BB for the times indicated. Internalized integrin was determined as for (c) and (d) using microtiter wells coated with (e) anti-β3 or (f) anti-α5 integrin monoclonal antibodies. (Mean ± SEM from three separate experiments).

receptor recycling inhibitor, primaquine (PMQ) [15], increased the measured internalization rate of αvβ3 and α5β1 integrin (Figure 1c,d), indicating that both integrins recycle back to the plasma membrane very shortly after internalization. Therefore, to measure the effect of PDGF on the *endocytic* rate of integrins, we performed internalization assays in the presence of PMQ. This analysis revealed that PDGF did not affect the endocytic rate for either αvβ3 or α5β1 integrin (Figure 1e,f).

Integrin endosomal compartments

Recycling receptors do not normally accumulate in early endosomes but pass rapidly through them and proceed to the perinuclear recycling compartment where they accumulate before making the last step in recycling to the plasma membrane. Incubation times of 30–60 min at 37°C have been routinely used to load the perinuclear recycling compartment with internalized tracer [16]. Additionally, internalized receptor may be concentrated in the early endosomes by reducing the temperature to slow transport through the endosomal system [17, 18].

To monitor the trafficking of integrin through endosomal compartments, cell-surface αvβ3 was tagged using anti-β3 monoclonal antibody at 4°C. Internalization was initiated by raising the temperature to either 22°C for 15 min or to 37°C for 30 min. Following this, antibody remaining at the cell surface was removed by a low pH wash at 4°C, and αvβ3 integrin and rab proteins were visualized by immunofluorescence. Following the shorter internalization period, αvβ3 integrin became closely colocalized with rab4 in endocytic vesicles distributed relatively evenly about the cytoplasm (Figure 2a–c). During this time, αvβ3 did not reach the perinuclear recycling compartment and showed little colocalization with rab11 (Figure 2d–f). Longer internalization times resulted in the integrin being transported out of the rab4-positive compartment such that, following 30 min at 37°C, αvβ3 was observed to focus in the perinuclear region and colocalize with rab11 (Figure 2g–i). Similar results were obtained for α5β1 integrin (data not shown).

Recycling of integrins

We proceeded to monitor the recycling of integrins from early endosomes and the perinuclear recycling compartment using a pulse-chase approach. Cells were surface-labeled, and internalization was allowed to proceed for 15 min at 22°C to allow integrin to accumulate in rab4-positive early endosomes. Biotin was removed from proteins remaining on the cell surface by exposure to MesNa at 4°C, and internalized integrin was chased from the cells at 37°C for various times in the presence or absence of PDGF. At each time, the cells were reexposed to MesNa to remove biotin from integrins that had recycled back to the cell surface, and the level of biotinylated integrin remaining within the cell was assayed by capture-ELISA. PDGF stimulated the rate of αvβ3 recycling from the early endosomes by approximately 2-fold, such that, in the presence of the growth factor, all internalized integrin had returned to the plasma membrane within 10 min (Figure 3a). A limited amount of α5β1 recycled from this compartment, but this was unaffected by PDGF (Figure 3b).

To monitor recycling of tracer that accumulated in the perinuclear recycling compartment, cells were surface labeled, and internalization was allowed to proceed for 30 min at 37°C. Integrin recycling was then determined as for Figure 3a,b. The recycling rates were similar for both α5β1 and αvβ3 and were unaffected by PDGF (Figure 3c,d), indicating that αvβ3 is subject to PDGF-regulated recycling only when present in the rab4-positive early endosomes and that recycling from endocytic compartments distal to this is refractory to this kind of regulation.

Rab4 dependence of αvβ3 recycling

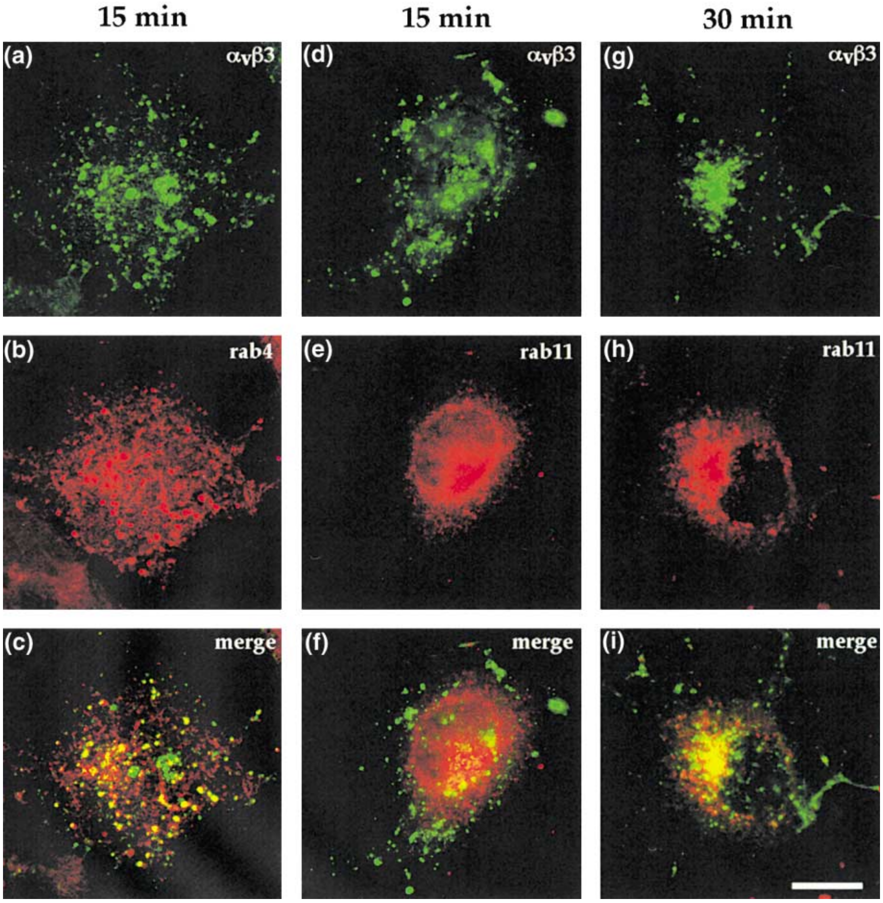
Rab4 is known to regulate the recycling of receptors from early endosomes to the plasma membrane [19]. To investigate the potential involvement of rab proteins in integrin recycling, we employed NIH 3T3 fibroblasts, which are similar to Swiss 3T3s but can be transfected to high efficiency. To measure recycling from transfected cells, we transiently expressed human integrins and detected them using human integrin-specific antibodies. The antibodies used were highly selective for hαvβ3 and hα5β1 and did not crossreact with mouse integrins (Figure 4a). Additionally, when cells were cotransfected with rab4 and human integrins, both receptor and GTPase were expressed in the same cells (Figure 4b,c).

Treatment with PDGF increased recycling of hαvβ3 from early endosomes, and the expression of wild-type rab4 increased both the basal and PDGF-stimulated rates of hαvβ3 recycling (Figure 4d). hα5β1, however, did not recycle directly from these endosomes (Figure 4e).

We employed two dominant-negative mutants to assess the rab4 dependence of αvβ3 recycling; S22Nrab4, which binds GDP poorly but is unable to bind GTP [20], and N121Irab4, which is unable to bind guanine nucleotide [21]. S22Nrab4 significantly reduced, and N121Irab4 completely abolished, PDGF-stimulated recycling of αvβ3 (Figure 4d). These data indicate an absolute requirement for rab4 in this process.

The data shown in Figure 3 suggest that αvβ3 must be present in rab4-positive early endosomes to be amenable to PDGF-regulated recycling. Transport through the recycling compartment is inhibited by dominant-negative mutants of rab11 [8, 9]. Dominant-negative N124Irab11 has no effect on PDGF-induced recycling of αvβ3 (Figure 4d), indicating that the growth factor regulates recycling

Figure 2



Visualization of integrin endosomal compartments. NIH 3T3 fibroblasts were transfected with hαv and hβ3 integrin in combination with **(a–c)** wild-type rab4 or **(d–i)** wild-type rab11. Surface αvβ3 was tagged by incubation with the mouse anti-hβ3 monoclonal antibody for 30 min at 4°C. Surface-bound antibody was allowed to internalize for (a–f) 15 min at 22°C or for (g–i) 30 min at 37°C, and the cells were rapidly cooled to 4°C. Antibody remaining at the cell surface was removed by a low-pH wash, and the cells were fixed and detergent permeabilized. Internalized antibody was visualized using (a,d,g; shown in green) FITC-conjugated anti-mouse, and the cells were counterstained with (b) rabbit anti-rab4 and (e,h) rabbit anti-rab11, followed by detection with Texas Red-conjugated anti-rabbit antibody (shown in red). Yellow indicates colocalization of the two fluorophores. The scale bar represents 16 μm.

directly to the plasma membrane without involving passage of the integrin through the perinuclear recycling compartment.

Experiments employing a 30 min internalization period indicated that both hαvβ3 and hα5β1 integrin recycled at similar rates from the perinuclear recycling compartment, such that, during 30 min of chase, ≈60% of integrin had returned to the plasma membrane (Figure 4f,g), a value similar to that observed in Swiss 3T3s (shown in Figure 3c,d). In complete contrast to PDGF-regulated recycling of hαvβ3 from the early-sorting endosomes, recycling of hαvβ3 and hα5β1 from the perinuclear compartment was unaffected by N121Irab4 but strongly inhibited by dominant-negative N124Irab11 (Figure 4f,g).

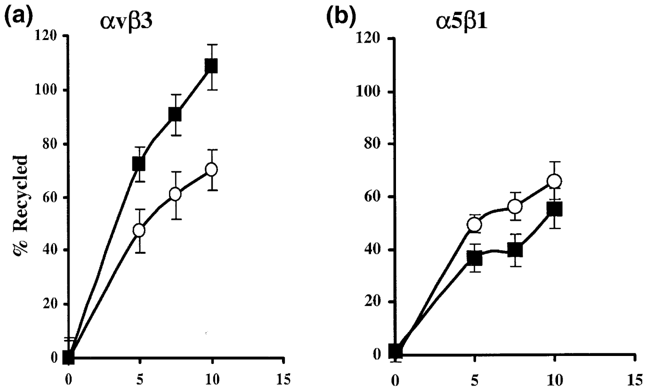
Endosomal recycling can be regulated by the ARF subfamily of GTPases, notably ARF6. We therefore determined the effect of dominant-negative T27NARF6 on PDGF-dependent αvβ3 recycling. Consistent with previous observations that ARF6 regulates a membrane recycling pathway distinct from that controlled by rab4 [13], T27NARF6 did not inhibit αvβ3 recycling (Figure 4d).

Visualization of PDGF-dependent αvβ3 recycling

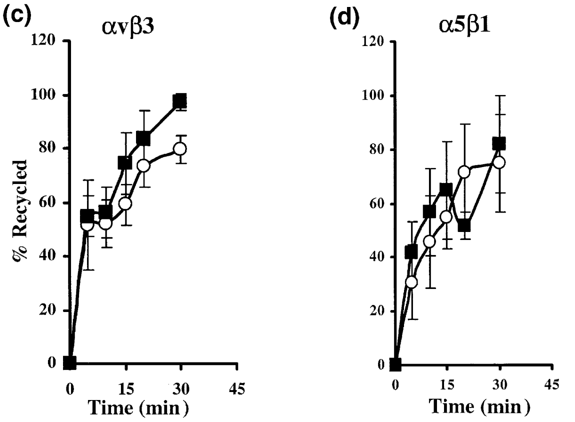
Treatment with PDGF for 10 min increased surface staining for αvβ3 and resulted in its localization to numerous small puncta distributed over the cell surface but sometimes enriched in lamellar ruffles (Figure 5c,d). Examination of optical slices from the confocal image indicate that these puncta were present primarily on the dorsal surface of the cell (data not shown). Following longer treatments with PDGF, αvβ3 was seen to be incorporated into larger complexes in a peripheral distribution that is characteristic of integrin-containing focal complexes (Figure 5e,f) [1].

The time course of the appearance of αvβ3-rich puncta parallels that measured for PDGF-dependent integrin recycling from early endosomes, suggesting that recycling integrin may be targeted to these puncta. Integrin recycling was visualized by tagging αvβ3 with an NHS-SS-biotin-labeled antibody that was allowed to internalize and recycle as shown in Figure 4d. PDGF stimulated the recycling of αvβ3-bound antibody (Figure 5i), and this was targeted to numerous small punctate complexes distributed over the cell surface (Figure 5k).

Figure 3



30 min internalization:



Recycling of α5β1 and αvβ3 integrins. Cells were surface labeled, and internalization was allowed to proceed for **(a,b)** 15 min at 22°C or for **(c,d)** 30 min at 37°C, and biotin was removed from receptors remaining at the cell surface by treatment with MesNa at 4°C. Cells were then rewarmed to 37°C for the times indicated in the absence (open circle) or presence (solid square) 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-ELISA using microtiter wells coated with (a,c) anti-β3 or (b,d) anti-α5 integrin monoclonal antibodies. The proportion of integrin recycled to the plasma membrane is expressed as the percentage of the pool of integrin labeled during the internalization period (values are mean ± SEM from five separate experiments).

Given that PDGF-regulated recycling is dependent on rab4 and that early endosomal recycling of αvβ3 is directed to cell surface puncta, we determined whether rab4 inhibition would affect the appearance of these puncta. S22Nrab4 blocked the assembly of αvβ3-containing puncta (Figure 5g), but membrane ruffling was uncompromised (Figure 5h).

Taken together, these data indicate that recycling of αvβ3 to the plasma membrane via a rab4-dependent pathway directs this integrin to punctate plasma membrane complexes. These puncta subsequently organize into the more familiar integrin-containing focal complexes.

Involvement of rab4 in cell adhesion and spreading

To investigate the possibility that rab4-dependent αvβ3 recycling was necessary for cell adhesion or spreading, cells transfected with wild-type or dominant-negative rabs were adhered to either vitronectin (VN), a good ligand for αvβ3, or fibronectin (FN), a ligand for both α5β1 and αvβ3, in the presence of PDGF. N121Irab4, which abrogated early endosomal recycling of αvβ3, inhibited the adhesion of transfected cells to VN (Figure 6a). Adhesion to FN was unaffected by N121Irab4, indicating that the inhibition was specific for αvβ3-mediated events. S22Nrab4, however, did not affect adhesion to VN (Figure 6a), implying that the partial blockade of αvβ3 recycling with this construct was insufficient to block adhesion.

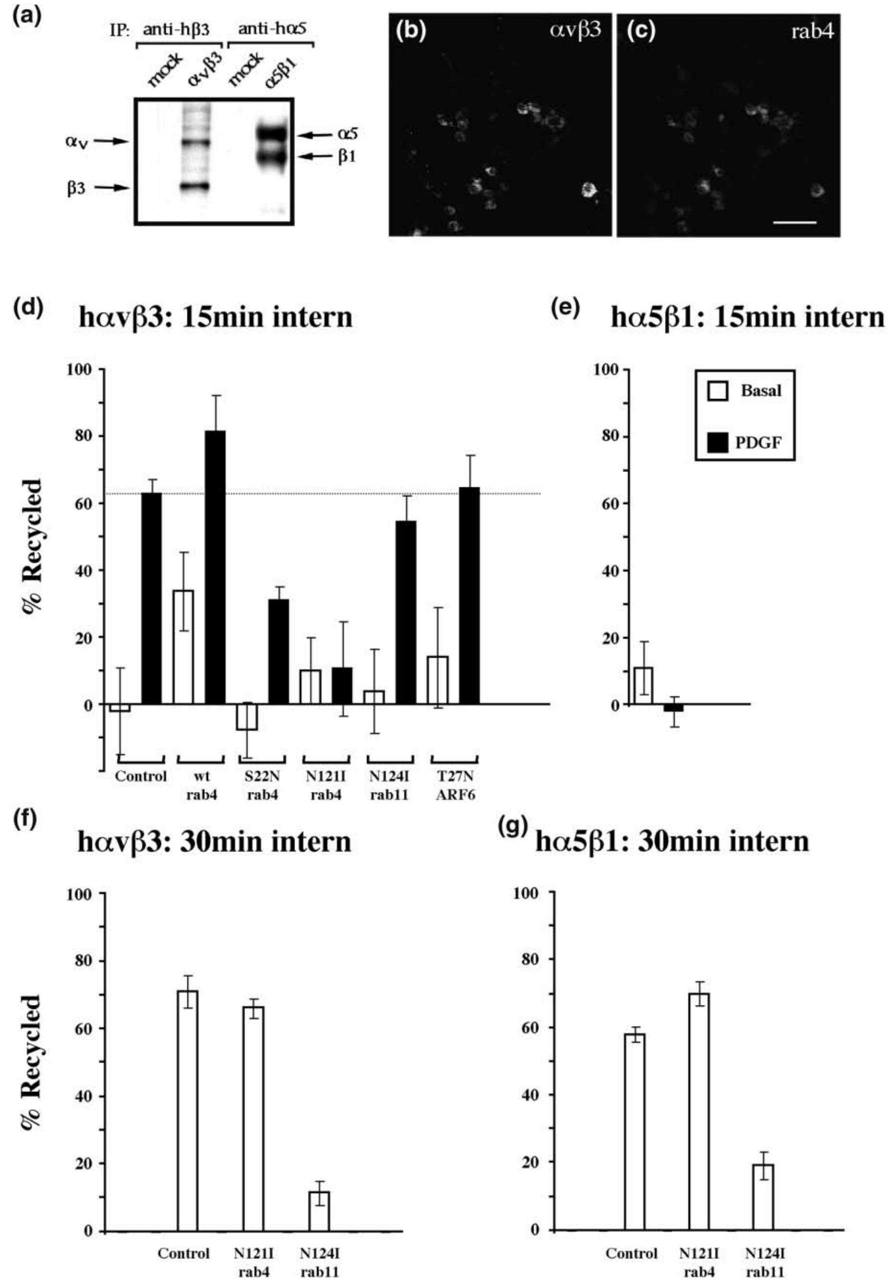
However, partial inhibition of αvβ3 recycling by S22Nrab4 compromised spreading of cells following attachment to VN. Within 1 hr of plating, cells transfected with wild-type rab4 spread better than their untransfected neighbors (Figure 7a,b). In contrast, cells transfected with S22Nrab4 did not spread efficiently on VN and seemed unable to properly organize their actin cytoskeleton (Figure 7c,d). Spreading on FN was similar irrespective of transfection with wild-type rab4 (Figure 7e; indicated by arrow) or S22Nrab4 (Figure 7f; indicated by arrow), consistent with the observation that α5β1 recycling is unaffected by dominant-negative rab4. Quantification of cell area indicated that S22Nrab4 reduced spreading on VN by approximately 50% (Figure 6b), a value consistent with the reduction of αvβ3 recycling by this construct.

In contrast to dominant-negative rab4s, N124Irab11 did not inhibit the adhesion of cells to either matrix protein (Figure 6a) and even increased spreading onto VN (Figure 6b). Therefore, direct recycling of integrin from early endosomes to the plasma membrane, and not that from the perinuclear recycling compartment, is critical for αvβ3 function.

Discussion

We have characterized regulation of the endo/exocytic cycle of αvβ3 integrin by PDGF and found that it plays a role in the functioning of the integrin during cell adhesion and spreading. In the absence of serum, αvβ3 integrin is internalized and passes through the early endosomes to arrive at the perinuclear recycling compartment approximately 30 min after internalization. The integrin is then recycled to the plasma membrane in a rab11-dependent fashion. However, following treatment with PDGF, αvβ3 integrin is recycled directly back to the plasma membrane from early endosomes in a rab4-dependent fashion with-

Figure 4



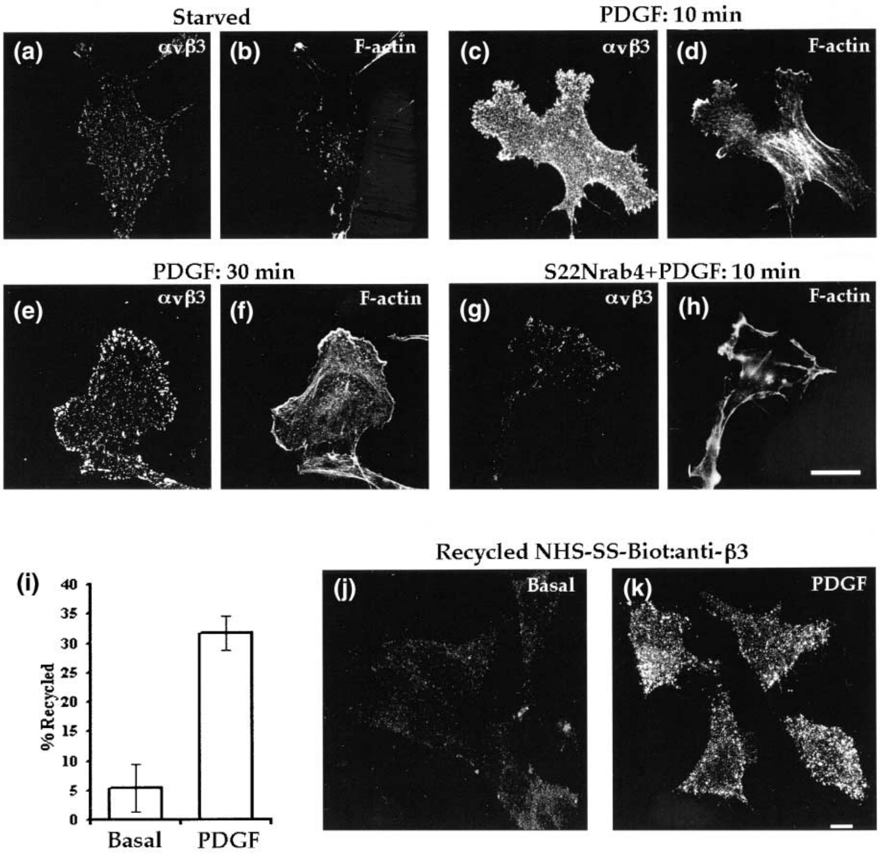
Rab4 dependence of αvβ3 recycling. **(a)** NIH 3T3 fibroblasts were transfected with human αv and β3 (αvβ3) or human α5 and β1 (α5β1) integrins or empty vector controls (mock). Cells were surface labeled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4°C and were lysed. Lysates were immunoprecipitated (I.P.) with anti-human β3 (hβ3) or anti-human α5 (hα5) monoclonal antibodies. Immobilized material was then analyzed by 6% SDS-PAGE, followed by Western blotting with peroxidase-conjugated streptavidin. The migration positions of human αv, β3, α5, and β1 integrin chains are indicated. **(b,c)** Cells were transfected with human αv and β3 integrins in combination with wild-type rab4. Following transfection, the cells were fixed, permeabilized, and costained for (b) human β3 integrin and (c) rab4 as for Figure 2. The scale bar represents 150 μm. **(d–g)** NIH 3T3 fibroblasts were transfected with (d,f) human αvβ3 or (e,g) α5β1 integrins either alone (Control) or in combination with wild-type rab4 (wtrab4), S22Nrab4, N121Irab4, N124Irab11, and T27NARF6 as indicated. Cells were surface labeled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4°C, and internalization was allowed to proceed for (d,e) 15 min at 22°C or for (f,g) 30 min at 37°C. Cells were exposed to MesNa at 4°C, and internalized integrin was chased back to the cell surface at (d,e) 37°C for 10 min in the absence (open bars; Basal) and presence (solid bars) of 10 ng/ml PDGF-BB or for (f,g) 30 min at 37°C in the absence of PDGF. Cells were then reexposed to MesNa, and biotinylated integrin was determined by capture-ELISA using microtiter wells coated with (d,f) anti-human β3 or (e,g) anti-human α5 monoclonal antibodies. Values are mean ± SEM from at least three separate experiments.

out the involvement of rab11 or ARF6. This direct recycling pathway delivers αvβ3 to numerous small puncta distributed evenly across the dorsal surface of the cell, and the integrin only later becomes localized into focal complexes. α5β1 integrin is not regulated in this way, but undergoes rab11-dependent endo/exocytic cycling even in the presence of PDGF. Furthermore, dominant-negative rab4s, but not rab11s, reduce adhesion and spreading on VN, demonstrating that rapid recycling from early endosomes is required for αvβ3 integrin-dependent processes to proceed efficiently.

Regulation of recycling from early endosomes

Glut4 and E-cadherin are examples of proteins recycled to the plasma membrane in response to stimulation. E-cadherin recycles from early endosomes in response to cell-cell contact [22]. Insulin stimulation of Glut4 recycling has been shown to be dependent on rab4 [23], suggesting that this GTPase is a target for regulation of receptor recycling by growth factors. Rab4 is proposed to be necessary for the formation of vesicles involved in recycling proteins from the early endosome to the cell surface. Overexpression of wild-type rab4 reduces the ability of

Figure 5



Visualization of PDGF-dependent recycling of αvβ3 integrin. **(a–h)** NIH 3T3 fibroblasts were transfected with hαvβ3 integrin either (a–f) alone or (g,h) in combination with S22Nrab4. Following transfection, the cells were serum starved for 30 min and then challenged with 10 ng/ml PDGF-BB for (c,d,g,h) 10 min or for (e,f) 30 min, or they were (a,b) allowed to remain quiescent. Cells were fixed in 2% paraformaldehyde, surface αvβ3 was visualized by indirect immunofluorescence, and F-actin was counterstained with Texas Red-conjugated phalloidin. Surface-only integrin staining was obtained by the addition of the primary antibody prior to the detergent-permeabilization step. The scale bar represents 20 μm. **(i)** Anti-β3 antibody was biotinylated with NHS-SS-biotin and bound to the surface of the cells at 4°C. Biotinylated antibody was allowed to internalize for 15 min at 22°C, and the cells were exposed to MesNa at 4°C to remove biotin from antibody remaining on the plasma membrane. Internalized antibody was chased back to the cell surface at 37°C for 10 min in the absence and presence of 10 ng/ml PDGF-BB. Cells were then reexposed to MesNa, and biotinylated antibody was determined by capture-ELISA using microtiter wells coated with anti-hamster monoclonal antibody. **(j,k)** Biotinylated antibody was bound, internalized, and recycled in the (j: Basal) absence and (k) presence of PDGF as for (i). Following this, the cells were fixed, and recycled antibody was visualized using FITC-conjugated streptavidin (surface-only labeling being ensured by the omission of a detergent-permeabilization step). The scale bar represents 16 μm.

the TFN-R to reach acidic endosomes, presumably by rapidly mistargeting internalized receptors directly to the plasma membrane [19]. Consistent with this, we found that overexpression of wild-type rab4 enhanced PDGF-dependent rapid recycling of αvβ3, while dominant-negative rab4 mutants opposed this. The pulse-chase experiments presented in Figures 3 and 4 indicate that, in order to be amenable to PDGF-regulated recycling, αvβ3 must be present in early endosomes at the time of growth factor addition. Following the addition of PDGF, αvβ3 may recycle directly from the early endosomes or, alternatively, may proceed to the plasma membrane via an indirect route through the perinuclear recycling compartment. Previous studies showed that transport through, and exit from, the perinuclear recycling compartment requires the function of rab11 [8, 9]. N124Irab11 has no effect on the ability of PDGF to trigger αvβ3 recycling, indicating that the direct route to the plasma membrane is regulated by the growth factor.

PDGF regulation of rab4 may occur via activation of PI 3-kinases that can regulate guanine nucleotide exchange on rab4 [24]. We have found that inhibition of PI 3-kinase with wortmannin, albeit unable to affect αvβ3 endocytosis, potently inhibited its recycling (data not shown). Additionally, PKB/Akt, a kinase activated downstream of PI 3-kinase, regulates rab fuction in macrophages [25]. We are currently investigating the involvement of PI 3-kinases and PKB/Akt in growth factor-regulated αvβ3 recycling.

αvβ3 recycling and the regulation of cell adhesion and motility

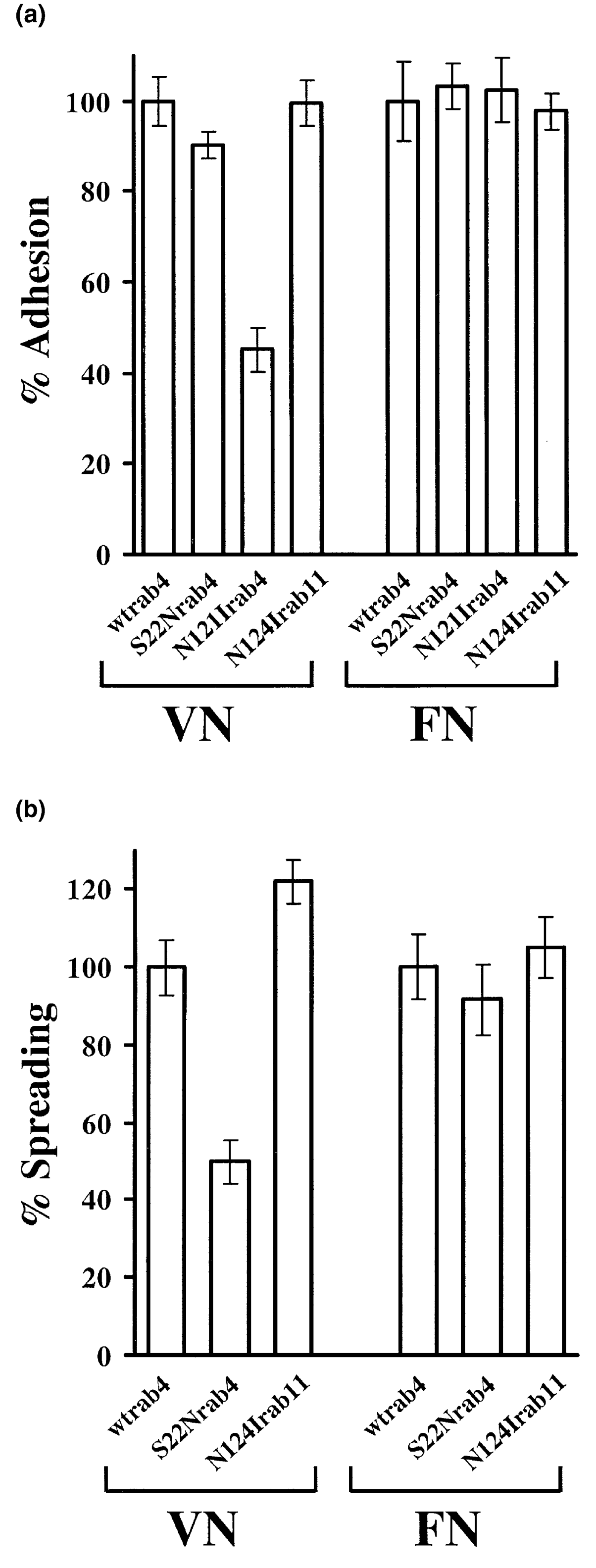
Tagging αvβ3 with antibodies revealed that PDGF-induced recycling of αvβ3 occurs over the dorsal cell surface and is not targeted directly to focal contacts. The early endosomal recycling pathway may, therefore, play a general role in regulating integrin-mediated events. Indeed, inhibition of PDGF-regulated αvβ3 recycling using dominant-negative rab4 mutants impaired cell adhesion and nonpolarized spreading. This suggests that this aspect of regulated vesicular transport may facilitate integrin activation rather than mediate vectorial delivery to the leading edge of migrating cells.

It has been suggested that factors such as PDGF promote cell spreading by modulating the affinity of the integrins for their ligands, a mechanism that has been termed inside-out signaling [26]. The precise and rapid regulation of the affinity of integrins for their ligands is a feature of many members of this family. A classic example of this is the activation of the platelet receptor αIIbβ3 following thrombin stimulation. Upon stimulation, αIIbβ3 undergoes a conformational change rendering it competent to bind ligand, and the total levels of active receptor exposed at the cell surface are increased. The affinity of both α4β1 [27] and the β2 integrins αLβ2 and αMβ2 [28] for their ligands is also regulated by exposure of cells to various stimuli. Although certain signaling pathways, including PI 3-kinase activation, have been implicated in mediating inside-out activation, the mechanism through which this is achieved remains unclear. Here, we have found that the rapid recycling of αvβ3 to the cell surface is dependent on rab4 and that this can be regulated by PDGF, a growth factor that promotes cell spreading and motility. This rapid recycling pathway must be necessary for integrin function, as dominant-negative rab4 mutants block αvβ3-mediated cell adhesion and spreading on vitronectin. Therefore, modulating the flux of integrin to and from the cell surface is a mechanism through which the cell can achieve the inside-out activation of integrins. It will be interesting to determine whether the same mechanism can regulate other integrins.

Conclusions

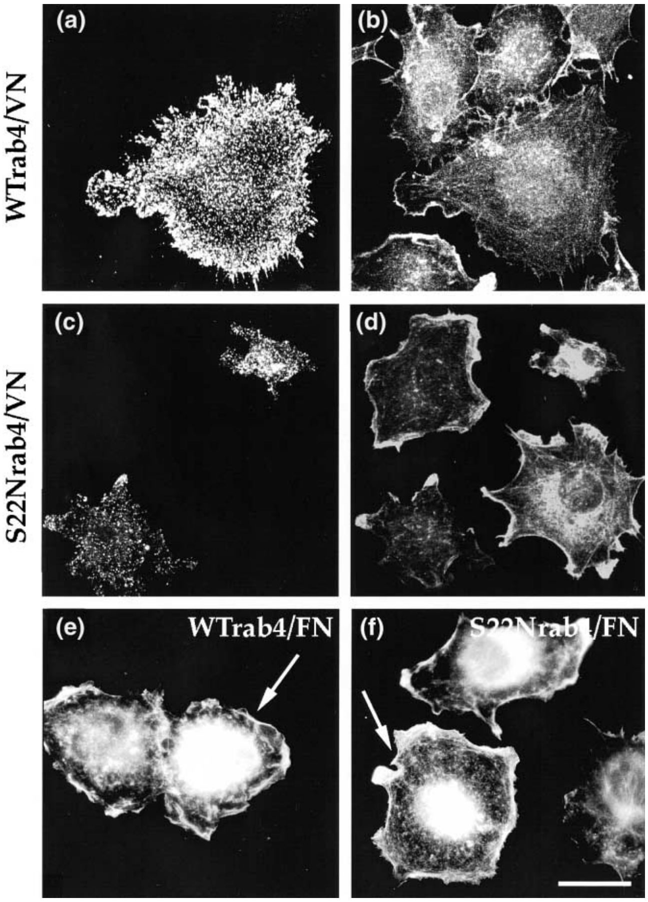
We have shown that PDGF stimulates rab4-dependent recycling of αvβ3 integrin from early endosomes to the cell surface. Inhibition of this process using dominant-negative rab4 mutants impaired cell adhesion and spreading on ligands for αvβ3. These data describe for the first time a mechanism through which growth factors can regu-

Figure 6



The effect of dominant-negative rab4s on cell adhesion and spreading. NIH 3T3 cells were transfected with wild-type rab4 (wtrab4), S22Nrab4, N121Irab4, or N124Irab11 in combination with a β-galactosidase transfection marker. The cells were then briefly trypsinized and allowed to adhere to either vitronectin (VN) or fibronectin (FN) in the presence of 10 ng/ml PDGF-BB for 1 hr. Following this, the unattached cells were washed off with ice-cold PBS, and attached cells were fixed and stained for β-galactosidase expression. **(a)** The number of β-galactosidase-expressing cells adherent to the VN or FN matrices was expressed as a proportion of those adherent to poly-L-lysine in the same experiment. **(b)** Adherent cells were then photographed with a digital camera, and the area of transfected cells was determined by delineation of the cell envelope using “NIH image” software. The data are expressed as a percentage of the cell area of wild-type rab4-expressing cells following spreading on VN. (Values are mean ± SEM).

Figure 7



The effect of S22Nrab4 on cell spreading. NIH 3T3 fibroblasts were transfected with human αv and β3 integrins in combination with **(a,b,e)** wild-type rab4 (wtrab4) or **(c,d,f)** S22Nrab4. The cells were then briefly trypsinized and were allowed to adhere to either (a–d) vitronectin or (e,f) fibronectin in the presence of 10 ng/ml PDGF-BB for 1 hr. Cells were fixed in 2% paraformaldehyde and double stained for (b,d,e,f) F-actin and (a,c) cell-surface human β3 integrin. The arrows in (e) and (f) indicate transfected cells. The scale bar represents 40 μm.

late integrin recycling and, furthermore, that this has functional consequences during cell adhesion and spreading.

Materials and methods

Cell culture and transfection

Swiss and NIH 3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) with 10% fetal calf serum (Globepharm) and 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Life Technologies) at 37°C with 10% CO_2. For transient transfection experiments, NIH 3T3 fibroblasts were grown to 50% confluence, fed with fresh DMEM containing 10% fetal calf serum, and transfected with integrin, rab, and ARF6 constructs (all cDNAs were ligated into pcDNA3; see the Supplementary material for details of constructs) using Fugene 6 (Roche) according to the manufacturer's instructions. The ratio of Fugene 6 to DNA was maintained at 3 μl Fugene 6:1 μg DNA. Integrin recycling and cell adhesion/spreading assays were carried out 24 hr posttransfection.

Internalization and recycling

Internalization and recycling of integrins was measured using a modification of the method described in [29].

Internalization

Cells were serum starved for 30 min, transferred to ice, washed twice in cold PBS, and surface labeled at 4°C with 0.2 mg/ml NHS-SS-biotin (Pierce) in PBS for 30 min. Labeled cells were washed in cold PBS and transferred to DMEM at 37°C with or without 10 ng/ml PDGF-BB (PeproTech) in the presence and absence of 0.6 μM primaquine to allow internalization. At the indicated times, the medium was aspirated, and the dishes were rapidly transferred to ice and washed twice with ice-cold PBS. Biotin was removed from proteins remaining at the cell surface by incubation with a solution containing 20 mM MesNa in 50 mM Tris (pH 8.6) and 100 mM NaCl for 15 min at 4°C [30]. MesNa was quenched by the addition of 20 mM iodoacetamide (IAA) for 10 min, and the cells were lysed in 200 mM NaCl, 75 mM Tris, 15 mM NaF, 1.5 mM Na_3VO_4, 7.5 mM EDTA, 7.5 mM EGTA, 1.5% Triton X-100, 0.75% Igepal CA-630, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 1 mM 4-(2-Aminoethyl)benzynesulphonyl fluoride (AEBSF). Lysates were passed three times through a 27G needle and were clarified by centrifugation at 10,000 × g for 10 min. Supernatants were corrected to equivalent protein concentrations and levels of biotinylated integrin were determined by capture-ELISA, or integrins were isolated by immunoprecipitation and analyzed by SDS-PAGE.

Recycling

Following surface labeling, cells were transferred to serum-free DMEM at 22°C for 15 min or 30 min at 37°C to allow internalization of tracer into early endosomes and the perinuclear recycling compartment, respectively. Cells were returned to ice and washed twice with ice-cold PBS, and biotin was removed from proteins remaining at the cell surface by reduction with MesNa. 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. At the indicated times, cells were returned to ice and biotin was removed from recycled proteins by a second reduction with MesNa. Biotinylated integrins were then determined by capture-ELISA.

Capture-ELISA

Maxisorb 96-well plates (Life Technologies) were coated overnight with 5 μg/ml appropriate anti-integrin antibodies (see the Supplementary material for full antibody information) in 0.05 M Na_2CO_3 (pH 9.6) at 4°C and were blocked in PBS containing 0.05% Tween-20 (PBS-T) with 5% BSA for 1 hr at room temperature. Integrins were captured by overnight incubation of 50 μl cell lysate at 4°C. Unbound material was removed by extensive washing with PBS-T, and wells were incubated with streptavidin-conjugated horseradish peroxidase (Amersham) in PBS-T containing 1% BSA for 1 hr at 4°C. Following further washing, biotinylated integrins were detected by a chromogenic reaction with ortho-phenylenediamine.

Immunoprecipitations

Magnetic beads (Dynal) conjugated to anti-integrin antibodies were incubated with lysates overnight at 4°C with constant rotation. Beads were washed six times with lysis buffer, and immunoisolated material was eluted by boiling for 10 min in nonreducing Laemmli sample buffer. Integrin chains were resolved by 6% nonreducing SDS-PAGE and were visualized by Western blotting with peroxidase-conjugated streptavidin.

Immunofluorescence microscopy

Cells were plated onto glass coverslips and grown to 50%–70% confluency over 3 days and were transfected 24 hr prior to experimentation where appropriate. Cells were serum starved for 30 min and treated with 10 ng/ml PDGF-BB for an additional 10 or 30 min prior to fixation in 2% paraformaldehyde in PBS for 20 min at room temperature. Following fixation, nonspecific binding was blocked for 1 hr in PBS containing 10% FCS (PBS-FCS). The cells were then incubated with anti-β3 monoclonal antibodies at 5 μg/ml for 1 hr at room temperature. Following this, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and then reblocked in PBS-FCS. Detection was by FITC-conjugated secondary antibodies. The actin cytoskeleton was counterstained with Texas Red-conjugated phalloidin in PBS for 10 min at room temperature.

For tracking the internalization of $\alpha v\beta 3$ integrin, NIH 3T3 fibroblasts were transfected with hαv and h$\beta 3$ integrin in combination with wild-type rab4 or wild-type rab11. Following serum starvation, surface $\alpha v\beta 3$ was tagged by incubation with mouse anti-h$\beta 3$ monoclonal antibody for 30 min at 4°C in PBS containing 1% BSA. Surface-bound antibody was allowed to internalize for 15 min at 22°C or for 30 min at 37°C, and the cells were rapidly cooled to 4°C. Antibody remaining at the cell surface was removed by incubation in acid-PBS (corrected to pH 4.0 by the addition of HCl) at 4°C for 6 min. The cells were then fixed in 2% paraformaldehyde and were detergent permeabilized. Internalized antibody was visualized using FITC-conjugated anti-mouse, and the cells were counterstained with rabbit anti-rab4 and rabbit anti-rab11, followed by detection with Texas Red-conjugated anti-rabbit antibody.

Cell adhesion and spreading assays

Tissue culture plates (Nunc, 24-well) were coated with fibronectin (Sigma, F-1141) or vitronectin (Sigma, V-8379) at concentrations of 20 μg/ml overnight at 4°C and were blocked with 2% BSA. Cells were transfected with wild-type or dominant-negative rab4 in conjunction with a β-galactosidase-expressing marker construct (see the Supplementary material). Following transfection, cells were harvested by trypsinization and were collected by centrifugation in the presence of 20 μg/ml soybean trypsin inhibitor. The cell suspensions were then added immediately to ligand-coated wells in serum-free DMEM containing 10 ng/ml PDGF-BB. 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 overnight at 37°C. For cell adhesion assays, the numbers of stained cells per well were counted and expressed as a proportion of total transfected cells for each condition. To obtain an index of cell spreading, the area of cells expressing β-galactosidase was determined by delineation of the cell envelope using "NIH image" software.

Supplementary material

Supplementary material including important data documenting the effect of various growth factors on the surface expression of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins on serum-starved Swiss 3T3 fibroblasts is available at http://images.cellpress.com/supmat/supmat.htm. Additional methodological information on the sources and generation of antibodies and cDNAs is also included.

Acknowledgements

This work was generously supported by grants from the Wellcome Trust (to J.C.N.) and the Dutch Cancer Society (to P.v.d.S.). J.C.N. is holder of a Wellcome Trust Career Development Fellowship. We gratefully acknowledge Fedor Berditchevsky, Margaret Frame, and Dave Critchley for critical reading and helpful comments on the manuscript. We would like to thank Chris D'Lacey and Kul Sikand for their invaluable assistance with the confocal microscopy. Many thanks to Dave Jones and Shamshad Cockcroft for generously providing ARF6 constructs.

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