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

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Supplementary material

Surface expression of integrins
To measure cell-surface expression of α5β1 and αvβ3 integrins, we employed a membrane-impermeant biotinylation reagent, followed by a capture-ELISA assay, to determine the degree of integrin labeling. Briefly, cells were surface labeled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4°C and were lysed. Integrins were captured by overnight incubation in microtiter wells coated with anti-α5 and anti-β3 integrin monoclonal antibodies. Biotinylated integrin was then detected using peroxidase-conjugated streptavidin, followed by a chromogenic reaction with o-phenylenediamine. The assay was established to respond linearly to a range of lyase concentrations (data not shown), and samples were diluted appropriately to lie within this range. Using this approach, we determined surface expression of α5β1 and αvβ3 integrins following treatment of serum-starved Swiss 3T3 fibroblasts with serum factors known to modify the actin cytoskeleton and/or stimulate cell migration. LPA treatment, which produces focal adhesions and stress fibers via activation of rho (S1), induced minimal change in αvβ3 surface expression (Figure S1a). However, greater changes were driven by PDGF and EGF, which induce membrane ruffles via the activation of rac (S2). Notably, PDGF increased αvβ3 surface levels 2-fold. Minimal changes in α5β1 integrin surface expression were observed following the addition of PDGF, EGF, or LPA (Figure S1B), or even 10% serum (data not shown). Inhibition of receptor recycling by a 10-min exposure to primaquine (PMQ) (S3) reduced the surface levels of α5β1 and αvβ3, indicating that, even following serum starvation, these integrins participate in endo/exocytic cycling. To confirm the integrity of immunoisolated material, lysates from serum-starved and PDGF-stimulated cells were immunoprecipitated with anti-α5 and anti-β3 monoclonal antibodies. Immunoprecipitates were analyzed using 8% SDS-PAGE under nonreducing conditions, followed by Western blotting and detection with peroxidase-conjugated streptavidin and enhanced chemiluminescence. Consistent with the capture-ELISA data, the surface expression of αvβ3 was increased by PDGF treatment, while that for α5β1 remained unchanged (Figure S1c).

Antibody information
Monoclonal anti-mouse α5 integrin (clone 5H10-27 [MFR5]), hamster anti-mouse β3 integrin (clone 2C9.G2), mouse anti-human β3 integrin (clone VI-PL2), and mouse anti-human α5 integrin (clone VC5) were purchased from Pharmingen. FITC-conjugated goat anti-mouse and antirat immunoglobulins were purchased from Southern Biotechnology. A rabbit antibody against rab4 was described previously in [S4], and rabbit anti-rab11 (71-5300) was purchased from Zymed.

Generation of cDNAs
The β3 and αv clones were generated by RT-PCR from DX3 RNA prepared using the Promega RNAgent total RNA isolation system. The β3 cDNA was identical to the published sequence, M35999, and was cloned as a HindIII-XbaI fragment into pcDNA-3 (Invitrogen) using restriction sites incorporated into the PCR primer proximal to the initiation codon and distal to the termination codon, respectively. The αv cDNA was identical to the published sequence, M14648, and was cloned as a BamH1-EcoR1 fragment into pcDNA3 again using restriction sites incorporated at the 5’ and 3’ ends of the cDNA. The β1 and α5 integrins were cloned by RT-PCR from K562 RNA prepared using the Promega RNAgent total RNA isolation system. The β1 cDNA was identical to the published sequence, X07979, except that two silent changes were introduced to create a HindIII site, tca to agc (Ser263) and atg to tgt (Leu264). The β1 cDNA was cloned into pcDNA3 as a BamH1-Not1 fragment. The α5 sequence was identical to the published sequence, X06256, except that two silent changes were introduced to create a BamH1 site, agc to tcg (Ser170) and gac to tag (Asp171), and two silent changes were introduced to create an Xba1 site, tcc to tct (Ser611) and tgt to cta (Leu612). The α5 cDNA was cloned into pCDNA3 as a Not1 fragment. Rab11 cDNA was generated by RT-PCR from HeLa cell mRNA and was ligated in the BamH1 site of pcDNA3. Rab11 mutants were made by overlap-extension PCR. Rab4 expression constructs were generated by excising cDNAs with EcoRI from yeast two-hybrid plasmids [S5] and ligating them in pcDNA3. For adhesion and cell spreading assays, the β-galactosidase-expressing construct was pPGKβgeoP [S6].

Supplementary references
The effect of various agents on surface expression of \( \alpha_5 \beta_1 \) and \( \alpha_v \beta_3 \) integrins. Serum-starved Swiss 3T3 fibroblasts were treated with 10 ng/ml PDGF-BB, 1 \( \mu \)g/ml LPA, 30 ng/ml EGF, or 0.6 \( \mu \)M PMQ for 10 min. Cells were surface labeled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4°C and lysed in a buffer containing Triton X-100 and NP-40. Integrins were captured by overnight incubation in microtiter wells coated with (a) anti-\( \beta_3 \) and (b) anti-\( \alpha_5 \) integrin monoclonals. Biotinylated integrin was then detected using peroxidase-conjugated streptavidin, followed by a chromogenic reaction with o-phenylenediamine. Values are expressed as the percentage of control. (Mean ± SEM from five separate experiments. The asterisk indicates that \( p < 0.0001 \), compared with serum-starved control cells). To confirm the integrity of immunoisolated material and antibody specificity, lysates from serum-starved and PDGF-BB-stimulated cells were immunoprecipitated (I.P.) with anti-\( \beta_3 \) and anti-\( \alpha_5 \) monoclonals. (c) Immobilized material was then analyzed by 6% SDS-PAGE under nonreducing conditions, followed by Western blotting and detection with peroxidase-conjugated streptavidin and enhanced chemiluminescence. The positions of \( \alpha_5 \), \( \alpha_v \), \( \beta_1 \), and \( \beta_3 \) integrin chains are indicated.