The integrin β tail is required and sufficient to regulate adhesion signaling to Rac1

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Summary

Rac1 is a small Rho family GTPase that regulates changes in cell morphology associated with cell spreading and migration. Integrin-mediated adhesion is known to activate Rac1 and to regulate the interaction of Rac1 with downstream effectors. Currently, it is not clear how integrins signal Rac1 activation following cell adhesion. Integrin β cytoplasmic domains (β-tails) are known to be required for integrin-mediated cell spreading, and isolated β tails expressed as tac-β tail chimeras can inhibit cell spreading indicating that protein interactions with β tails can regulate this process. Our recent studies demonstrated that the expression of constitutively activated Rac1 can restore cell spreading inhibited by tac β tail chimeras, suggesting a role for Rac1 in the regulation of cell spreading by β tails. Hence, we examined the role of β tails in integrin activation of Rac1. By using recombinant wild-type and mutant integrin heterodimers, we demonstrate that integrin β tails are required for adhesion to increase Rac1-GTP loading. We demonstrate that clustering tac-β tail chimeras, on the surface of cells in suspension, activates Rac1. Thus, β tails are not only required, but also sufficient for integrin-triggered Rac1 activation. Our findings indicate that integrin β-tails are an important link between integrin engagement and Rac1 signaling, and that protein interactions initiated at β tails are sufficient for integrins to regulate Rac1 activity.

Key words: Rac, Integrin, β cytoplasmic domain

Introduction

Integrin-mediated cell adhesion to components of extracellular matrices and basement membranes is a fundamental cellular process required for the maintenance of tissue architecture (Isacke and Horton, 2000; Sheppard, 2000). Cell adhesion is initiated by the binding of integrins to their extracellular matrix ligands in cell attachment, which is followed by changes in cell morphology leading to cell spreading and the formation of additional integrin-matrix contacts (Small et al., 1999). Integrin engagement activates a number of intracellular signaling proteins including tyrosine kinases, serine/threonine kinases, lipid kinases, and small GTP-binding proteins (Giancotti and Ruoslahti, 1999; Geiger et al., 2001; Miranti and Brugge, 2002). These signals regulate the adhesion process itself, and cooperate with signals from other cell surface receptors to regulate various aspects of cell behavior (Giancotti and Ruoslahti, 1999; Geiger et al., 2001; Miranti and Brugge, 2002).

Integrins regulate the activities of RhoA, Rac1 and Cdc42 (Ren et al., 1999; Arthur et al., 2000; Danen et al., 2000; del Pozo et al., 2000; Etienne-Manneville and Hall, 2001; Price et al., 1998). These small GTP-binding proteins in turn regulate cell adhesion and changes in cell morphology by triggering dynamic changes in the actin cytoskeleton (Kjoller and Hall, 1999). Integrin activation of Rac1 and Cdc42 signaling induces the formation of lamellapodia and filopodia, which are necessary for cell spreading (Kjoller and Hall, 1999). The disruption of Cdc42 or Rac1 signaling by the expression of the dominant negative proteins N17Cdc42 or N17 Rac1 inhibits the spreading process (Berrier et al., 2000; Clark et al., 1998; Price et al., 1998). Interestingly, the regulation of RhoA by integrins during cell adhesion is biphasic (Ren et al., 1999). Initially, integrin signaling triggers a transient inhibition of RhoA activity upon cell attachment to promote cell spreading (Arthur and Burridge, 2001; Ren et al., 1999). RhoA is subsequently re-activated (Ren et al., 1999), inducing actinomyosin contractility, the formation of stress fibers, and focal adhesions, which are important steps in the adhesion process following cell spreading (Chrzanowska-Wodnicka and Burridge, 1996).

The mechanisms linking integrins to the regulation of Rho family GTPases are not fully defined. However, integrin-mediated activation of tyrosine kinases may play a central role. For many integrins, intact integrin β tails are required to trigger increases in tyrosine phosphorylation of FAK, Cas and paxillin following cell adhesion (Guan et al., 1991; Schaffner-Reckinger et al., 1998; Wennerberg et al., 1998; Wennerberg et al., 2000), suggesting important roles for integrin β tails in this process. Additionally, clustering isolated integrin β1 or β3 tails induces the tyrosine phosphorylation of these same proteins indicating that interactions with β tails are sufficient to trigger increases in tyrosine phosphorylation of FAK, Cas and paxillin (Akiyama et al., 1994; Bodeau et al., 2001; Tahiliani et al., 1997). Since the activation of tyrosine phosphorylation provides a pathway linking integrin engagement to many downstream signaling events (Cary and

Guan, 1999; Parsons et al., 2000; Pawson and Scott, 1997), clustering integrin β tail-associated proteins may also be sufficient to initiate other signaling events, such as the regulation of Rho family GTPases.

In addition to playing an important role in the activation of tyrosine kinase signaling, integrin β tails also play a central role in regulating cell spreading. Previous studies by others demonstrated that integrin β tails are required for integrins to promote cell spreading (Ylanne et al., 1993). We showed that the expression of tac-β tail chimeras can inhibit cell spreading, indicating that protein interactions mediated by β tails can regulate the spreading process (Bodeau et al., 2001; LaFlamme et al., 1994). More recently, we demonstrated that integrin β tails are required downstream of Rac1 signaling in cell spreading, and that constitutively active Rac1 can restore cell spreading inhibited by tac β tail chimeras, suggesting that β tails may also be involved in Rac1 activation (Berrier et al., 2000). In our current study, we examine the role of integrin β tails in regulating Rac1 activation, and demonstrate that integrin β tails are both required and sufficient for integrins to signal increases in Rac1 GTP-loading.

Materials and Methods

Cells, transient transfections and plasmids

Human foreskin fibroblasts (Vec Technologies) and the A5 and ETC12 CHO stable cell lines, generously provided by Mark Ginsberg, (Ylanne et al., 1993) were cultured and transfected as previously described (Berrier et al., 2000). Plasmids encoding chimeric receptors containing the extracellular and transmembrane domains of the tac subunit of the IL-2 receptor connected to the wild-type β1 and β3 cytoplasmic domains have been described previously (LaFlamme et al., 1992; LaFlamme et al., 1994). Site-directed mutagenesis to generate the wild-type Rac1 construct was performed using myc-tagged Rac1S78 as template DNA in a two-step PCR reaction utilizing internal overlapping primers each amplified with 5′ and 3′ primers that anneal to the flanking vector sequences. The second round of PCR used the products of the first round together with the 5′ and 3′ vector primers. The sequences of the internal overlapping primers are 5′-GCAAACAGATGTGTTCTTAATTTGCTTTT-3′ and 5′-GAAAA-GCAAATTAAGAACACATCTGTTTG-3′. The sequences of the 5′ and 3′ vector primers are 5′-GAAGCTGATCTCC-3′ and 5′-CTGCAGGAATTC-3′. The final PCR product was inserted into pRK5-myc as a BamHI/EcoRI fragment. The pRK5myc-Rac1 plasmid was sequenced to confirm the integrity of the wild-type Rac1 sequence.

Assay for Rac1-GTP loading

In general, cells were harvested with trypsin and allowed to recover for 2.5 hours at 37°C in serum-free medium. Cells were then kept in suspension or either allowed to adhere to fibrinogen (Enzyme Research Laboratories), collagen I (Vitrogen 100, Cohesion), or fibronectin (kindly provided by Jane Sottile). Non-adherent cells were removed from the coated dishes with two washes with cold PBS. The amount of Rac1-GTP was assayed in cell lysates as described previously (Benard et al., 1999). In brief, a fraction of the lysate was incubated with glutathione-agarose beads coated with bacterially expressed GST-PAK [PAK amino acid residues 67-150 (Benard et al., 1999)]. The levels of Rac1 in the GST pull-down assay were analyzed by western blot with monoclonal antibody, clone 102 to Rac1 (BD transduction laboratories) and sheep anti-mouse horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech). The bands were visualized with ECL enhanced chemiluminescence (Amersham Pharmacia Biotech). Quantitation was performed by densitometric scanning of the autoradiogram (Molecular Dynamics Scanner and ImageQuant software, Amersham Pharmacia Biotech). Several different autoradiogram exposures were used to quantitate each experiment. In each case, the levels of Rac1 GTP-loading in cells kept in suspension were normalized to 1. Western blot analysis of one-fiftieth of the cell lysates was performed to correct for the lysate levels of Rac1. Statistical analysis of the data was performed using the Kruskall Wallis one way analysis of variance on rank. Differences between time points were determined by Dunnett's method using SigmaStat software (Jandel Corp.). Significance was set at $P<0.05$.

Clustering the integrin β tail

Transiently transfected fibroblasts were harvested with trypsin and allowed to recover for 2.5 hours at 37°C in serum-free medium. Purified 7G7B6 mouse monoclonal antibody to the tac epitope (Upstate Biotechnology) was used to coat magnetic beads conjugated with goat anti-mouse IgG (Polysciences). Chimeric receptors were clustered on the cell surface by incubating the transfected cells with antibody-coated magnetic beads in serum-free medium (Akiyama et al., 1994; Tahiliani et al., 1997). Prior to the clustering assay, flow cytometry of a fraction of the transfected cells was performed in order to ensure that similar numbers of tac-, tac-β1-, or tac-β3-expressing cells were incubated with the magnetic beads. Tac-expressing cells were magnetically sorted and the levels of Rac1-GTP were assayed in lysates of the positively sorted cells as described previously (Benard et al., 1999). The levels of Rac1-GTP in the control tac-receptor-expressing cells were normalized to 1. Since measuring endogenous Rac1 activity is unmanageable for more than one tac construct per trial due to the large numbers of transiently transfected cells needed for the analysis of Rac1 GTP-loading, we measured the activity of co-transfected Rac1.

Flow cytometry

Cells were harvested, immunostained and fluorescence intensity was analyzed on individual cells using a FACScan flow cytometer (Becton Dickinson) and Cellquest software (Becton Dickinson) as described previously (Mastrangelo et al., 1999). Surface expression of the αIIbβ3 integrin receptor was detected using the FITC-conjugated anti-CD41 monoclonal antibody, clone P2 (Immunotech). The surface expression of the transfected tac and tac-β tail chimeras was detected using a FITC-conjugated anti-CD25 monoclonal antibody (Becton Dickinson). The expression of myc-tagged Rac1 was detected in cells permeabilized with Cytofix/Cytoperm (Pharmingen) using a rabbit polyclonal anti-myc antibody (Santa Cruz Biotechnology) and a goat anti-rabbit phycoerythrin conjugated secondary antibody (Molecular Probes).

Results

The integrin β tail is required for integrins to regulate Rac1 activity

To determine whether the integrin β-tail is required for adhesion to activate Rac1, we utilized two CHO stable cell lines: A5 cells that stably express αIIbβ3 and ETC12 cells that stably express αIIbβ3Δ728 containing a β3-tail truncation (Ylanne et al., 1993). Parental CHO cells do not adhere to fibrinogen; however, expression of αIIbβ3 and αIIbβ3Δ728 confers A5 and ETC12 cells with the ability to adhere to fibrinogen (Ylanne et al., 1993). If the integrin β-tail is important for integrin-mediated adhesion to activate Rac1, then Rac1 GTP-loading in A5 cells will increase after adhesion to fibrinogen, whereas in ETC12 cells Rac1 activity will not

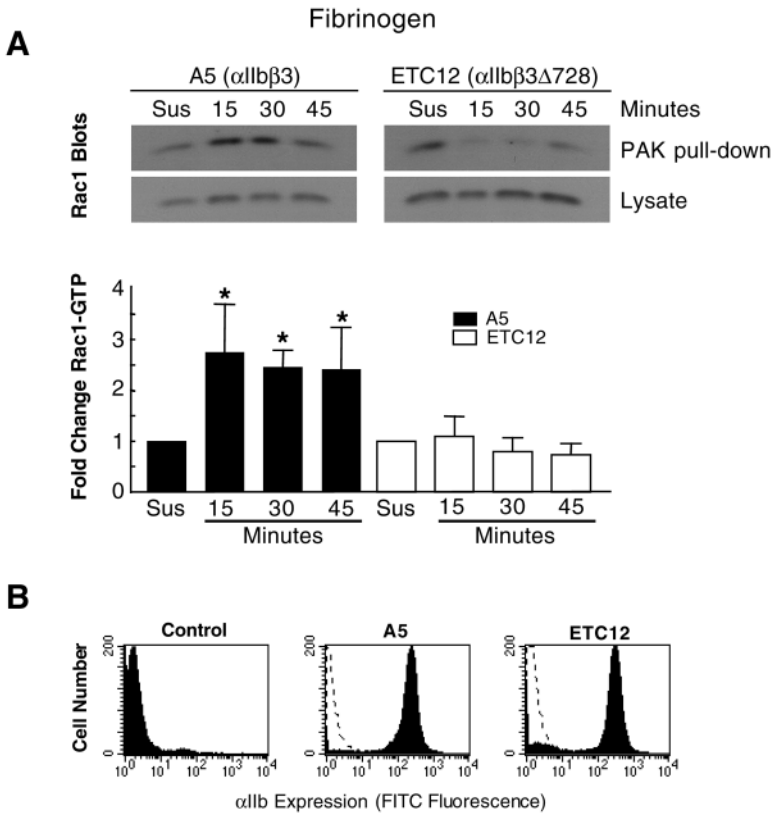

Fig. 1. Comparison of Rac1 activation in A5 cells and ETC12 cells adhered to fibrinogen. (A) A5 cells stably expressing αIIbβ3 and ETC12 cells stably expressing αIIbβ3Δ728 were incubated in suspension for 2.5 hours and then aliquots of these cells were adhered to fibrinogen (15 μg/ml) for 15, 30 or 45 minutes as indicated. The amount of Rac1 GTP-loading was compared in cell lysates from A5 and ETC12 cells. The levels of Rac1 were detected by western blot in the GST-PAK pull-down assay (top panel) and the lysate (bottom panel) are shown. The average fold change in Rac1-GTP±s.e.m. from five trials is shown in the bar graph (A5 solid bars, ETC12 open bars). The level of Rac1-GTP in the suspended cells was normalized to 1. Significant differences are indicated (*). (B) Surface levels of αIIbβ3 and αIIbβ3Δ728 on A5 and ETC12 cells were analyzed by flow cytometry. The x-axis is the FITC fluorescence intensity of A5 or ETC12 cells stained with the directly conjugated P2-FITC monoclonal antibody that recognizes the αIIb subunit (solid line) or with an IgG-FITC control (dotted line). The y-axis is cell counts. In control samples, human fibroblasts were stained with P2-FITC or IgG-FITC.

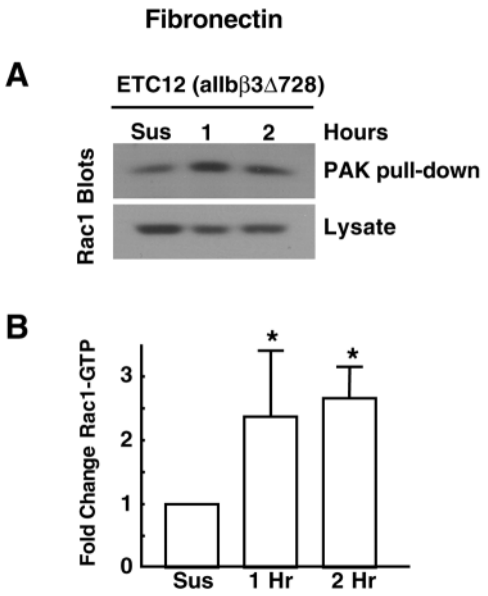

Fig. 2. Analysis of Rac1 activation in ETC12 cells adhered to fibronectin. (A) ETC12 cells were incubated in suspension and then aliquots of these cells were adhered to fibronectin (10 μg/ml) for 1 or 2 hours as indicated and the amount of Rac1 GTP-loading was compared in cell lysates. The levels of Rac1 detected by western blot in the GST-PAK pull-down assay (top panel) and the lysate (bottom panel) are shown. (B) The average fold change in Rac1 GTP-loading±s.e.m. from three trials is shown in the bar graph. The level of Rac1-GTP in the suspended cells was normalized to 1. Significant differences are indicated (*).

change upon adhesion to fibrinogen. To analyze Rac1-GTP loading, we measured the amount of Rac1 bound to a glutathione S-transferase fusion protein containing the p21-binding domain of PAK1 (GST-PBD) (Benard et al., 1999). We assayed the levels of Rac1 activity at the onset of A5 cell spreading on fibrinogen. When Rac1 GTP-loading was assayed in A5 cells, the levels were on average 2.4 fold higher in A5 cells adhered to fibrinogen in comparison to suspended A5 cells (Fig. 1A). In contrast, the levels of Rac1 GTP-loading were similar in the ETC12 cells that were adhered to fibrinogen or kept in suspension (Fig. 1A). The difference in the ability of adhesion to fibrinogen to activate Rac1 in A5 and ETC12 cells is not due to differences in the surface expression of αIIbβ3 and αIIbβ3Δ728 (Fig. 1B) (Ylanne et al., 1993). However, it is possible that adhesion of ETC12 cells to fibrinogen failed to trigger Rac1 activation, because ETC12 cells lack necessary components to activate this pathway. To examine this possibility, we analyzed the ability of endogenous α5β1 to trigger Rac1 activation when ETC12 cells were adhered to fibronectin, a ligand for α5β1. We assayed the levels of Rac1 activity at the onset of cell spreading on fibronectin. The levels of Rac1 GTP-loading were induced on average 2.5-fold by adhesion to fibronectin (Fig. 2), demonstrating that integrin engagement can activate Rac1 in ETC12 cells. Thus, taken together our results demonstrate that integrin β tails are required for adhesion-triggered Rac1 activation. In addition, our studies further indicate that adhesion mediated by integrin receptors containing either the β1 or β3 subunit can signal Rac1 activation in CHO cells.

Clustering an isolated integrin β1 or β3 tail is sufficient to enhance Rac1 activation

To further our understanding of how integrin receptors regulate Rac1 activity, the mechanisms involved in the requirement for integrin β tails were examined. Clustering integrin β tails in normal fibroblasts is sufficient to activate protein tyrosine phosphorylation and may lead to the formation of signaling complexes through recruitment of SH2-domain containing signaling and adaptor proteins (Bodeau et al., 2001; Pawson and Scott, 1997). We reasoned that clustering isolated integrin

β tails could trigger Rac1 activation, if integrin β tail-associated complexes are sufficient to signal to Rac1. Alternatively, it is possible that integrin β tails play only a permissive role, and that additional pathways are required for Rac1 activation. To distinguish between these possibilities, we tested whether clustering tac-β-tail chimeras could trigger increases in Rac1 GTP-loading. Since we have previously clustered β tail chimeras to induce protein tyrosine phosphorylation in transiently transfected primary human fibroblasts, we chose to perform our assay for Rac activation in this cell type. Additionally, cells were co-transfected with myc-tagged, wild-type Rac1 to facilitate detection of Rac1 by western blot in the clustering assay.

Integrin-mediated cell adhesion of several fibroblast cell lines has been shown to regulate Rac1 activity (Danen et al., 2000; del Pozo et al., 2000; Arthur et al., 2000; Price et al., 1998). To confirm that integrin-mediated adhesion of primary human fibroblasts induced Rac1 activation, Rac1 GTP-loading of endogenous Rac1 was assayed following cell adhesion to collagen I. The levels of Rac1 GTP-loading increased on average 2.1-fold in primary fibroblasts adhered to collagen I for 15 minutes when compared to the levels in suspended cells (Fig. 3A,B), demonstrating that integrins can mediate the activation of Rac1 in primary human fibroblasts. We also analyzed the effect of cell adhesion on the activity of transiently expressed recombinant wild-type Rac1. The activity of the myc-tagged wild-type Rac1 was enhanced on average 2.5-fold by adhesion to collagen I (Fig. 3C), confirming that transiently expressed myc-tagged Rac1 can also be regulated by cell adhesion. Furthermore, a comparison of the levels of GTP-loading of wild-type Rac1 or constitutively active L61Rac1-transfected cells revealed that overexpression of wild-type myc-tagged Rac1 does not induce constitutive activation of Rac1 in suspended cells (data not shown).

To test whether integrin β-tails are sufficient to signal Rac1 activation, human fibroblasts were transiently co-transfected with the myc-tagged wild-type Rac1 and either the control tac receptor or chimeric receptors expressing tac with the intracellular domains of integrin β1A (tac-β1) or β3A (tac-β3) (LaFlamme et al., 1994). A fraction of the tac, tac-β1 or tac-β3 transiently transfected cells were each stained for cell surface expression of the tac epitope and flow cytometry was performed to determine the percentage of cells that express tac, tac-β1 or tac-β3 (Fig. 4A). Equal numbers of tac-expressing cells were then incubated with anti-tac antibody-coated magnetic beads to cluster tac on the surface of the tac-, tac-β1- or tac-β3-transfected cells while in suspension. The tac-expressing cells were magnetically sorted. The enrichment of tac-epitope-expressing and Rac1 co-transfected cells by this method was confirmed by flow cytometry experiments (Fig. 4B). Lysates from the positively selected cells were prepared and analysis of Rac1 GTP-loading revealed that clustering of tac-β1 or tac-β3 for 15 minutes resulted in an average of 4.7 or 4.1-fold increase in Rac1 activity, respectively, in comparison to Rac1 activity induced by clustering the control tac receptor (Fig. 4C,D). These experiments indicate that clustering an isolated integrin β1or β3 tail is sufficient to signal Rac1 activation in human fibroblasts.

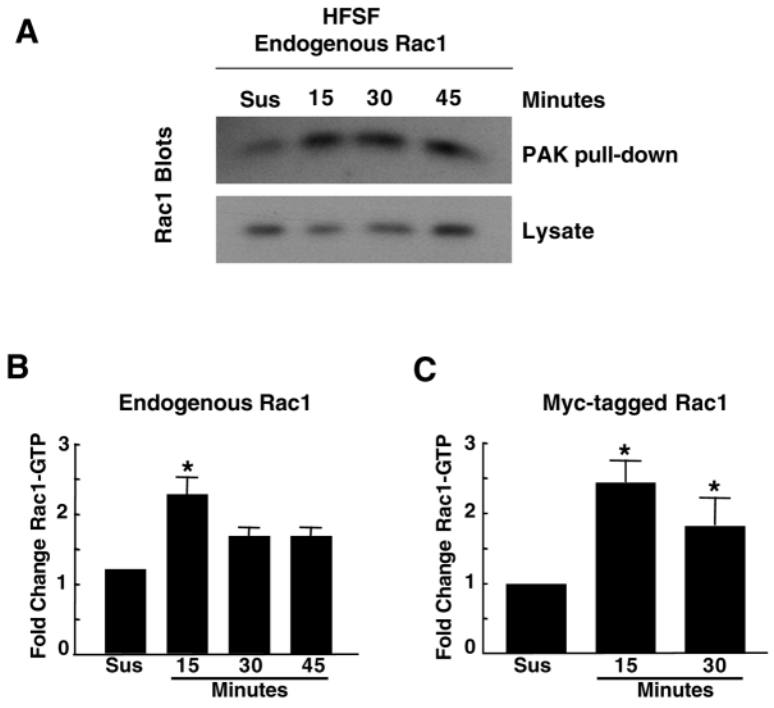

Fig. 3. Adhesion to collagen I increases the GTP-loading of endogenous Rac1 and transfected myc-tagged Rac1 in primary human fibroblasts. (A) Primary human fibroblasts were incubated in suspension for 2.5 hours and then aliquots of these cells were adhered to collagen I (50 μg/ml) for 15, 30 or 45 minutes as indicated. The amount of Rac1 GTP-loading was compared in lysates from suspended and adhered cells. The levels of Rac1 detected by western blot in the GST-PAK pull-down assay (top panel) and the lysate (bottom panel) are shown. (B) The average fold change in endogenous Rac1 GTP-loading±s.e.m. from three trials is shown in the bar graph. The level of Rac1-GTP in suspended cells was normalized to 1. (C) Primary human fibroblasts were transiently transfected with myc-tagged wild-type Rac1 and the amount of Rac1 GTP-loading was compared in lysates from suspended cells and cells adhered to collagen I for 15 and 30 minutes. The average fold change in Rac1-GTP loading±s.e.m. from three trials is shown in the bar graph. The level of Rac1-GTP in the suspended transfected cells was normalized to 1. Significant differences are indicated (*).

Discussion

Rac1 can be regulated by signaling pathways that activate guanine nucleotide exchange factors (GEFs), and/or inhibit GTPase activating proteins (GAPs) or guanine nucleotide dissociation inhibitors (GDIs) (Kjoller and Hall, 1999). Recent studies have demonstrated that integrin engagement during cell adhesion enhances Rac1 GTP-loading and also triggers the membrane-targeting of Rac1, the dissociation of RhoGDI, and the interaction of Rac1 with its downstream effectors (del Pozo et al., 2000; del Pozo et al., 2002). In our study, we demonstrate that integrin β tails are both required and sufficient to signal increases in Rac1 GTP-loading.

Our result that clustering isolated β tails can trigger Rac1 activation indicates that proteins that bind to β tails can initiate the pathway leading to increases in

Rac1 activity. Although we do not know the identity of these β tail binding protein(s), we have recently shown that clustering isolated β tails is sufficient to trigger the tyrosine phosphorylation of p130Cas, paxillin and FAK (Akiyama et al., 1994; Bodeau et al., 2001). The phosphorylation of these proteins provides potential pathways linking β tails to Rac1 activation. The tyrosine phosphorylation of p130Cas is known to trigger the formation of a Cas/Crk complex with p180Dock, which in turn can activate Rac1 (Kiyokawa et al., 1998a; Kiyokawa et al., 1998b). Phosphorylated paxillin can also bind CrkII, suggesting that CrkII may similarly couple paxillin to Rac1 activation (Petit et al., 2000). Also, integrin-triggered tyrosine phosphorylation of FAK can lead to the activation of PI 3-kinase, and PI 3-kinase signaling is known to participate in Rac1 activation (Chen and Guan, 1994; Kjoller and Hall, 1999; Reiske et al., 1999).

Additional studies are needed to define the molecular pathway linking integrin β tails with Rac1 activation. If tyrosine phosphorylation is required for β tails to trigger Rac1 activation, talin is an attractive candidate for a β tail binding protein responsible for this linkage. Talin binds to both the β tail and to FAK (Chen et al., 1995; Liu et al., 2000), and thus could nucleate the assembly of signaling complexes leading to FAK activation, a cascade of tyrosine phosphorylation and the subsequent activation of Rac1. Mutations in regions of the β tail implicated in talin binding inhibit the induction of tyrosine phosphorylation by β tails (Bodeau et al., 2001; Tahiliani et al., 1997), supporting a role for talin in the activation of tyrosine phosphorylation by integrins. However, there are other potential pathways linking β tails with the regulation of Rac1. ILK is also a β tail binding protein that could initiate signaling to Rac1. ILK can bind to paxillin linking integrin β tails to Rac1 through the formation of an ILK/paxillin/p95PKL/PIX complex. However, it is important to note that although PIX is a Rac1 GEF, the effect of the formation of this complex on PIX exchange activity has not yet been determined. ILK can also bind to PINCH and could link integrin β tails with Rac1 through the formation of an ILK/PINCH/Nck2/Dock180 complex, since Dock180 can trigger Rac1 activation (Tu et al., 2001; Turner, 2000; Wu and Dedhar, 2001; Kiyokawa et al., 1998a). However, the role of integrin-mediated adhesion or tyrosine kinase signaling in the formation of these complexes is also not yet clear. Future studies will focus on identifying the components linking β tails with Rac1 and the mechanisms of their action.

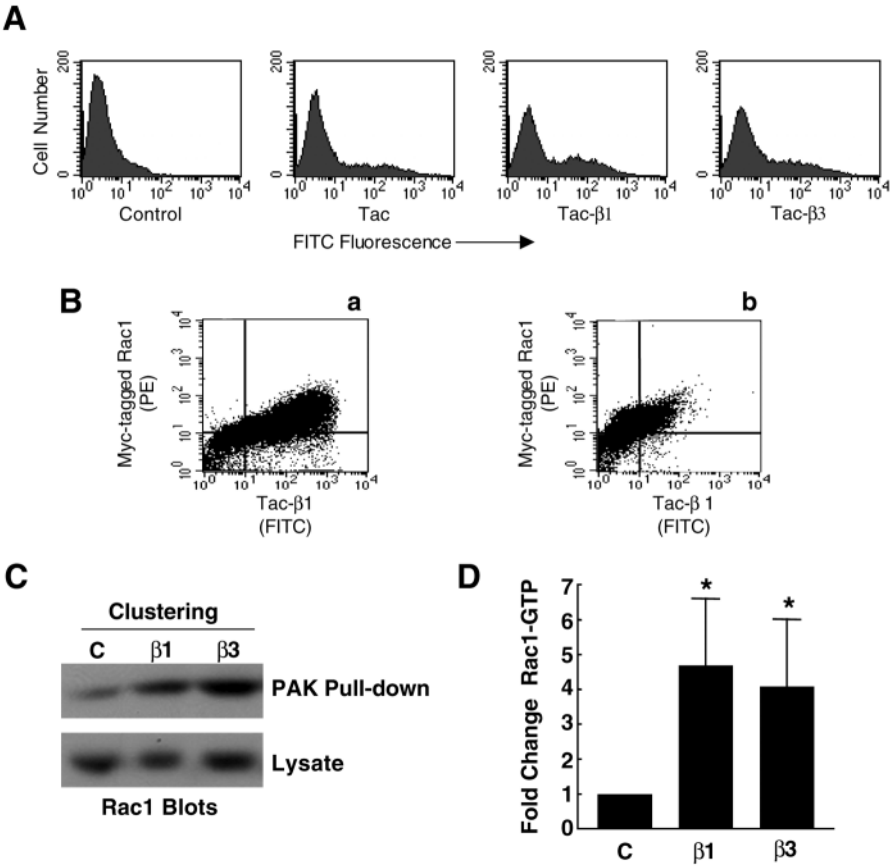

Fig. 4. Clustering integrin β1 or β3 tails is sufficient to activate Rac1 in primary human fibroblasts. (A) Tac-, tac-β1- and tac-β3-transfected primary human fibroblasts were analyzed for surface expression of the tac epitope by flow cytometry. The x-axis is the FITC fluorescence intensity of transfected cells stained with the directly conjugated anti-CD25-FITC monoclonal antibody (Becton Dickinson) that recognizes the tac epitope of the IL-2-receptor. Transfected cells were also stained with IgG-FITC (Becton Dickinson) as a negative control. (B) Primary human fibroblasts co-transfected with tac-β1 and myc-tagged Rac1 were placed in suspension and one fraction of these cells was stained for expression of the tac epitope at the cell surface, permeabilized and then stained for expression of the myc epitope to demonstrate the distribution of co-transfected cells (a). The second fraction was incubated in a clustering assay with anti-tac antibody-coated magnetic beads (b). The remaining cells that were not selected magnetically for Rac activity assays were stained for tac and myc expression to demonstrate the depletion of tac positive and Rac co-transfected cells. Two-color flow cytometry was performed to analyze the distribution of tac and myc expression for the starting and negatively sorted cells. The x-axis is the FITC fluorescence intensity of cells stained with the directly conjugated anti-CD25-FITC monoclonal antibody. The y-axis is the PE fluorescence intensity of cells stained with anti-myc primary antibody and PE conjugated secondary antibody. (C) Normal human fibroblasts were co-transfected with wild-type Rac1 and either the control tac receptor (C), tac-β1 (β1) or tac-β3 (β3). Equal numbers of tac-expressing cells were incubated in clustering assays for 15 minutes. The levels of transfected Rac1 in the lysates of the clustered cells detected by western blot of the GST-PAK pull-down assay (top panel) and the lysate (bottom panel) are shown. (D) The average fold change in Rac1 GTP-loading±s.e.m. from five clustering trials is shown in the bar graph. The fold change in Rac1 GTP-loading was normalized relative to the levels in control tac-transfected cells. Significant differences are indicated (*).

After the completion of this work and while this paper was in preparation, Hirsch and colleagues (Hirsch et al., 2002) published that adhesion-triggered Rac1 activation is inhibited

in cells expressing β1 integrins containing mutant β tails. Our results are in agreement with these findings since we demonstrate that truncation of β tails on the integrins mediating adhesion inhibits the integrin-activation of Rac1. We extend these results by demonstrating that integrin β tails are sufficient to trigger increases in Rac1 GTP-loading. Our results indicating that wild-type αIIbβ3 expressed in CHO cells can activate Rac1, however, differ from those published recently by Miao and colleagues (Miao et al., 2002). These authors conclude that β1 but not β3 integrins can activate Rac1 upon adhesion to fibronectin. We demonstrate that αIIbβ3-mediated adhesion to fibrinogen and α5β1-mediated adhesion to fibronectin can both trigger Rac1 activation, indicating that both β1 and β3 integrins can regulate Rac1. Our experiments and the studies by Miao and colleagues differ in experimental design and this may account for the observed difference in the ability of β3 integrins to activate Rac1. We assayed Rac1 activity after 15 minutes of adhesion in contrast to 4 hours used in the assay described by Miao and colleagues. Also, we assayed Rac1 activation after adhesion to fibrinogen, whereas Miao and colleagues measured Rac1 activity following adhesion to fibronectin. Thus, it is possible that integrin transmembrane signaling to Rac1 may differ depending on whether particular β3 integrins bind to fibronectin or fibrinogen. Clearly, additional studies are needed to fully understand the mechanisms by which integrins regulate Rac1 and how these mechanisms may differ for different integrin heterodimers and different integrin ligand interactions.

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