Integrin-Specific Activation of Rac Controls Progression through the G₁ Phase of the Cell Cycle

Amel Mettouchi,¹,§ Sharon Klein,¹ Wenjun Guo,¹,² Miguel Lopez-Lago,¹ Emmanuel Lemichez,² John K. Westwick,³ and Filippo G. Giancotti¹,§
¹Cellular Biochemistry and Biophysics Program
Memorial Sloan-Kettering Cancer Center
²Sloan-Kettering Division
Graduate School of Medical Sciences
Cornell University
³Laboratory of Plant Molecular Biology
The Rockefeller University
New York, New York 10021
Signal Pharmaceuticals
San Diego, California 92121

Introduction

Exposure to mitogens results in cell proliferation or growth arrest and differentiation, depending on the repertoire of integrins on the cell and the composition of the matrix to which it adheres. It is plausible that a subset of integrins induces one or more signaling events necessary for cell cycle progression, whereas other integrins are unable to do so (Giancotti and Ruoslahti, 1999). However, the nature of these integrin-specific signals and the mechanism by which they are coupled to the cell cycle machinery are incompletely understood.

Focal Adhesion Kinase (FAK) is a crucial mediator of integrin signaling. Upon activation and autophosphorylation at Tyr 397, FAK associates with the SH2 domain of either Src or the p85 subunit of phosphoinositide 3-oh kinase (PI-3K). Src phosphorylates a number of focal adhesion components, including paxillin, tensin, and p130CAS. These interactions link FAK to signaling pathways that promote cell migration and stimulate mitogen-activated protein kinases, such as JNK and in certain cell types ERK (Parsons and Parsons, 1997; Schlaepfer and Hunter, 1998; Cary et al., 1999). Because FAK appears to be activated by most integrins, models based solely on FAK are unlikely to fully explain the integrin-specific control of the cell cycle.

In addition to FAK, some integrins, including α5β1, α5β3, and αvβ3, also activate the Src-family kinase Fyn and, through it, the adaptor protein Shc (Wary et al., 1996; Wary et al., 1998). Caveolin-1 appears to function in this pathway as a membrane adaptor, which couples the integrin α subunit to Fyn and possibly other Src-family kinases carrying a palmitoyl lipid group (Wary et al., 1998; Wei et al., 1999; E. Marcantonio, personal communication). Fyn phosphorylates Shc, which combines with the Grb2/SOS complex, causing activation of the Ras-ERK cascade (Wary et al., 1996; Barberis et al., 2000; Lai and Pawson, 2000).

The signals from integrins and growth factor-receptors converge at multiple levels to promote cell proliferation. In many nonimmortalized cells, the integrins which activate Shc signaling to ERK cooperate with growth factor receptors to promote cell proliferation, whereas other integrins are unable to do so (Wary et al., 1996; Mainiero et al., 1997; Pozzi et al., 1998). In addition to activating Shc, the α5β1 and αvβ3 integrins can form a physical complex with growth factor receptors and contribute to their ability to activate Ras-ERK signaling (Giancotti and Ruoslahti, 1999). Accordingly, Shcα-/- fibroblasts display defective activation of ERK in response to both α5β1-mediated adhesion to fibronectin and growth factor stimulation (Lai and Pawson, 2000). These results raise the possibility that a simultaneous stimulation of Ras by Shc-linked integrins and growth factor receptors may be needed to activate ERK to the extent required for cell cycle progression. In addition, matrix adhesion promotes efficient coupling of Ras to both Raf and MEK in mitogen-stimulated cells (King et al., 1997; Lin et al., 1997), possibly through activation of PAK (Howe and Juliano, 2000). Independently of the mechanism proposed, the activation of ERK is a central feature of all current models of anchorage-dependent cell growth.

Rho-family proteins are critical regulators of oncogenic Ras signaling (Zohn et al., 1998). In particular, the Rho-family protein Rac is required for Ras-mediated transformation and anchorage-independent growth of NIH-3T3 cells (Qiu et al., 1997). The activation of Rac requires interaction of Ras with its immediate downstream effector PI-3K (Rodriguez-Viciana et al., 1997). The catalytic product of PI-3K, phosphatidylinositol-3,4,5-trisphosphate (PIP3), binds to the plekstrin-homology (PH) domain of SOS and increases the guanine nucleotide exchange activity of the adjacent Dbl-homology (DH) domain toward Rac (Nimnuan et al., 1998; Das et al., 2000). In addition to activating JNK, Rac induces lamellipodia and regulates progression through the G₁ phase of the cell cycle (Hall, 1998). Studies with effector-
loop mutants of Rac have indicated that these two latter functions of Rac are related, suggesting that they may be coordinately regulated perhaps in response to integrin activation (Joneson et al., 1996; Lamarche et al., 1996; Westwick et al., 1997). However, the mechanisms by which Rac is activated and controls cell proliferation are unclear.

Understanding the anchorage dependence of normal cells, and by inference the anchorage independence of neoplastic cells, poses three crucial questions. First, what are the integrin-specific signals which control the cell cycle? Second, how are they activated? Third, how do these signals control the cell cycle machinery? Because immortalization and transformation involve the loss of critical cell cycle regulators, we have examined these questions in nonimmortalized cells derived from primary cultures.

Results

Human Umbilical Vein Endothelial cells (HUVECs) express similar levels of α5β1 and α2β1 (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/8/1/115/DC1) and adhere equally well to fibronectin and to laminin-1 or collagen I (Languino et al., 1989; data not shown). Adhesion to fibronectin enables HUVECs to progress through G1, and enter in S phase in response to growth factors, whereas adhesion to laminin results in growth arrest under the same conditions. In M199 medium supplemented with bFGF and insulin, cells plated on laminin-1 exit the cell cycle and undergo apoptosis (Wary et al., 1996). By contrast, in Serum-Free Medium (SFM) supplemented with bFGF, insulin, and EGF (complete SFM), these cells undergo growth arrest but remain viable (not shown). Because the activation of apoptotic pathways could complicate the interpretation of the results, we have examined the mechanism of laminin-1 induced growth arrest in cells plated on fibronectin or laminin-1 in SFM supplemented with bFGF, insulin, and EGF.

Integrin-Specific Control of Cyclin D1

Progression through G1 is regulated by the sequential activation of the two cyclin-dependent kinases (CDKs) Cyclin D/CDK4, 6 and Cyclin E/CDK2, which phosphorylate Rb and thus induce E2F-dependent transcription. Cyclin A is an E2F target gene and activation of Cyclin A/CDK2 promotes entry in S phase (Sherr and Roberts, 1999). We compared the activation of these CDKs in HUVECs synchronized in G0 and plated on fibronectin or laminin-1 (Figure 1A). On fibronectin, CDK-4 was activated approximately 9 hr after exposure to mitogens, and CDK-6 about 2 hr later. Cyclin E/CDK-2 became active approximately 13 hr after release from G0 and Cyclin A/CDK-2 after an additional 8 hr. Notably, both G, CDKs and Cyclin A/CDK-2 were not activated on laminin-1.

The cyclin D-dependent kinases are thought to control the activation of Cyclin E/CDK-2 by two major mechanisms. First, they sequester the CDK-2 inhibitor p27. Second, they phosphorylate the C terminus of Rb and derepress E2F-dependent transcription of Cyclin E (Sherr and Roberts, 1999). To examine whether the deficient activation of Cyclin E/CDK-2 on laminin-1 could be ascribed to defective biosynthesis of Cyclin E, increased binding of p27, or both, we examined the levels of Cyclin E and p27 in HUVECs plated on either fibronectin or laminin-1. The total levels of p27 were high in suspended cells and decreased initially in a similar fashion upon attachment to fibronectin or laminin-1 (Figure 1B). In accordance with the observation that p27 is targeted for degradation by the ubiquitin-proteasome upon phosphorylation by Cyclin E/CDK-2 (Sherr and Roberts, 1999), the levels of p27 decreased dramatically 13 hr after plating on fibronectin. In contrast, the levels of p27 remained elevated for at least 24 hr on laminin-1 (Figure 1B). Cyclin E was induced to lower levels on laminin-1 than on fibronectin. In addition, the amount of p27 associated with Cyclin E was significantly higher on laminin-1 than on fibronectin (Figure 1B). Although we cannot exclude additional mechanisms, these results suggest that the defective activation of Cyclin E/CDK-2, and thereby Cyclin A/CDK-2, on laminin-1 is secondary to the lack of activation of D-type CDKs.

HUVECs express Cyclin D1 and very low levels of Cyclin D3, but no other D-type cyclin (data not shown). Figure 1C shows that Cyclin D1 accumulates to significant levels in cells plated on fibronectin but not laminin-1. These results indicate that the HUVECs undergo cell cycle arrest on laminin-1 because they fail to express Cyclin D1.

Cyclin D1 Is Regulated at the Posttranscriptional Level, Independently of ERK

Because Cyclin D is regulated at the transcriptional level by the Ras-ERK pathway (Lavioe et al., 1996), we asked whether the growth arrest of endothelial cells on laminin-1 was a consequence of defective Ras-ERK signaling. HUVECs were synchronized in G0 and plated for various times on fibronectin or laminin-1 in the presence of different concentrations of bFGF. In the absence of mitogen, adhesion to fibronectin caused a significantly higher activation of ERK than adhesion to laminin-1 (Figure 2A). However, as the concentration of mitogen was increased, the activation of ERK became more sustained, and the difference in ERK activation between cells on fibronectin and laminin-1 smaller (Figure 2A).

Upon treatment with bFGF, insulin, and EGF at the concentrations of our experimental protocol, ERK was activated to similar levels (Figure 2A) (Wary et al., 1996). However, as the concentration of mitogen was increased, the activation of ERK became more sustained, and the difference in ERK activation between cells on fibronectin and laminin-1 smaller (Figure 2A).

The HUVECs spread poorly and displayed a disorganized actin cytoskeleton on laminin-1, especially at early time points. Notably, activated ERK entered the nucleus of these cells efficiently despite the poor cytoskeletal organization (Figure 2B). This suggests that cell spreading is not necessarily a prerequisite for ERK activation. We have previously noted that the fraction of HUVECs which do not undergo apoptosis upon plating on laminin-1 in M199 supplemented with bFGF and insulin display a flat morphology but do not progress through G1, (Wary et al., 1996). Together, these observations imply that cell spreading and exposure to growth factors are
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Figure 1. Integrin-Specific Control of Cyclin D1 Biosynthesis

A) G0-synchronized HUVECs were detached (S) and plated on fibronectin (Fn) or laminin-1 (Lm) in complete SFM for the indicated times. CDK4 and CDK6, Cyclin E, and Cyclin A were immunoprecipitated and subjected to in vitro kinase assay using GST-Rb792-928 (GST-Rb) or Histone H1 (H1) as substrates.

B) HUVECs were treated as in A and subjected to immunoblotting with anti-p27 (top). G0-synchronized HUVECs were plated on Fn or Lm for 15 hr and subjected to immunoprecipitation with normal rabbit serum (C) or antibodies to Cyclin E (E) followed by immunoblotting with antibodies to Cyclin E and p27 (bottom). As assessed by densitometry, the amount of Cyclin E did not increase on Lm but increased approximately by 2-fold on Fn as compared to suspension. Conversely, the amount of p27 associated with Cyclin E decreased by 68% on Lm and by 97% on Fn.

C) Steady-state levels of Cyclin D1 were examined by immunoblotting total lysates of HUVECs treated as in (A, top). Steady state levels of Cyclin D1 mRNA were examined by Northern blotting using GAPDH as a loading control. Radioactivity in individual bands was measured by Phosphorimager. Values were normalized for loading variations and expressed as fold induction over the amount of Cyclin D1 mRNA present in suspended cells (middle). G0-synchronized HUVECs were plated on Fn or Lm with growth factors and incubated for 7 hr. They were then pulse-labeled with 35S-methionine-cysteine for 60 min and lysed after the indicated chase times. The radioactivity in Cyclin D1 was estimated by Phosphorimager and expressed in arbitrary units. These experiments were repeated three or more times with similar results.

not sufficient to promote cell cycle progression: this process requires a postligand binding event elicited by a specific integrin and distinct from spreading.

We next examined why the expression of Cyclin D1 was defective on laminin-1. Northern blotting analysis indicated that the abundance of Cyclin D1 mRNA increased to a very similar extent in cells plated on fibronectin or laminin-1 (Figure 1C). Pulse-chase analysis of cells metabolically labeled with 35S-Methionine/Cysteine revealed that the biosynthesis of Cyclin D1 protein was severely deficient in HUVECs adhering to laminin-1, but the half-life of the protein was not reduced (Figure 1C).

These findings suggest that adhesion to fibronectin induces translation of Cyclin D1 mRNA independently of ERK signaling, whereas adhesion to laminin-1 does not.

Activated Rac Rescues Cyclin D1 Biosynthesis and Cell Cycle Progression on Laminin-1

To identify the signaling pathways involved in integrin-specific control of the cell cycle, we used a complementation approach. Overexpression of activated Ras (V12) and Rac (L61) promoted transit through G1 and entry in S phase in cells on laminin-1 very effectively (Figure 3A). In fact, the percentage of Ras-V12 and Rac-L61
**Figure 2. Saturating Concentrations of Growth Factors Activate ERK to a Similar Extent in Cells Plated on Fibronectin or Laminin-1**

(A) G0-synchronized HUVECs were detached (S) and replated on Fn or Lm. They were then incubated without growth factors, with the indicated concentrations of bFGF, or with 10 μg/ml insulin, 20 ng/ml bFGF, and 10 ng/ml EGF (complete SFM) for the indicated times and subjected to immunoblotting with anti-phospho-ERK. The blots were reprobed with antibodies to total ERK-2 to verify equal loading (not shown).

(B) G0-synchronized HUVECs were fixed or detached and replated on Fn or Lm for 20 min in complete SFM and then fixed. Double immunofluorescent staining was with anti-phospho-ERK antibodies followed by FITC-labeled secondary antibodies (green) and TRITC-conjugated Phalloidin (red). The experiment was conducted in triplicate and the graph shows the percentage (± SD) of HUVECs with nuclear activated ERK.

overexpressing cells entering into S phase was larger than that of control transfecants entering into S on fibronectin. By contrast, activated PI-3K (p110*) and MEK1 (2E) did not rescue entry in S phase on laminin-1. The effect of Rac-L61 was dose-dependent, and 10 μg of plasmid were sufficient to fully rescue entry into S phase on laminin-1. At this dose, activated Cdc42 (V12) and Rho (L63) promoted entry in S phase to a very limited extent; even Ras-V12 was relatively ineffective. Control experiments indicated that all signaling proteins were overexpressed in a comparable and dose-dependent manner. These results suggest that activated Rac rescues endothelial cells from cell cycle arrest on laminin-1 effectively and specifically. The inability of activated MEK to rescue is consistent with the observation that ERK signaling is not defective in HUVECs plated on laminin-1 in complete SFM.

We next examined the mechanism by which activated Rac rescues HUVECs from cell cycle arrest on laminin-1. Activated Ras and Rac were able to restore biosynthesis of Cyclin D1 and activation of Cyclin E/CDK-2 on laminin-1, further supporting the hypothesis that adhesion to laminin-1 does not induce efficient translation of Cyclin D1 mRNA. These results suggest that activated Rac rescues endothelial cell proliferation on laminin-1 by complementing the specific biochemical defect observed in these cells.

Finally, we examined the effect of dominant negative Rac on cells plated on fibronectin. This construct suppressed both accumulation of Cyclin D1 in the nucleus and entry into S phase in these cells (Figure 3C), suggesting that Rac is required for biosynthesis of Cyclin D1 and progression through G1 on fibronectin.

The α5β1, but Not α2β1, Integrin Promotes Activation of Rac

We monitored the activation of Rac in HUVECs plated on fibronectin or laminin-1 in presence of mitogens. Pull-down assays with a GST-fusion protein comprising the CRIB domain of PAK-1 revealed that Rac was activated to a significant extent in cells adhering to fibronectin, but not laminin-1 (Figure 4A). Rac activation required both adhesion to fibronectin and exposure to mitogens,
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Figure 3. Activated Rac Rescues Cell Cycle Progression on Laminin-1, and Dominant Negative Rac Blocks It on Fibronectin

(A) HUVECs were transfected with a vector encoding EGFP-F in combination with the indicated amounts of plasmids encoding activated signaling proteins. After synchronization in G\textsubscript{0}, the cells were plated on Lm in complete SFM for 24 hr and subjected to immunofluorescent staining to estimate the percentage of EGFP-F-expressing cells that had incorporated BrdU. The results represent the average and standard deviations of values obtained from four independent experiments and are expressed as percentages of rescue (see Experimental Procedures). The level of expression of recombinant proteins was verified by immunoblotting (not shown).

(B) HUVECs were transfected with 40 μg of plasmids encoding the indicated activated signaling proteins or empty vector (-). After synchronization in G\textsubscript{0}, the cells were plated on Lm in complete SFM and subjected to anti-Cyclin-D1 immunoblotting (left) or Cyclin E/CDK-2 immunocomplex assay (right).

(C) HUVECs were transfected with a vector encoding EGFP-F alone or in combination with 20 μg of plasmid encoding dominant negative Rac (Rac-N17), synchronized in G\textsubscript{0} and plated on Fn or Lm in complete SFM. Nuclear accumulation of Cyclin D1 and BrdU incorporation were examined by immunofluorescent staining.

As plating in the absence of mitogens resulted only in weak activation of Rac (data not shown). By contrast, Cdc42 and Rho were activated to a small and similar extent on both fibronectin and laminin-1 (see Supplemental Figure S2).

To confirm the integrin-specificity of Rac activation and exclude the possibility that the deficient activation of Rac in cells plated on laminin-1 was a consequence of inefficient ligation of α\textsubscript{2}β\textsubscript{1} or spreading, HUVECs were plated on dishes coated with Mabs reacting with α\textsubscript{5}β\textsubscript{1} or α\textsubscript{2}β\textsubscript{1}. The cells spread to a small and similar extent on the two substrates (not shown). However, ligation of α\textsubscript{5}β\textsubscript{1} caused significant activation of Rac, whereas ligation of α\textsubscript{2}β\textsubscript{1} did not (Figure 4A). Additional control experiments indicated that HUVECs’ adhesion to fibronectin and laminin-1 promotes similar activation of p70\textsuperscript{S6} kinase as well as phosphorylation of 4EBP-1 (M.L-L., A.M., and F.G.G., unpublished data). Finally, transcript profiling indicated that adhesion to laminin-1 is sufficient to induce the expression of several genes in HUVECs (S.K. and F.G.G., unpublished data). These results suggest that α\textsubscript{2}β\textsubscript{1} is intrinsically unable to promote activation of Rac, but it can activate other signaling pathways and affect gene expression. Thus, matrix adhesion regulates Rac in an integrin-specific manner. Together with the results of complementation experiments, these data indicate that endothelial cells undergo growth arrest on laminin-1 because of defective Rac signaling.

Integrin-Specific Activation of Rac Is Mediated by SOS and Requires Both Shc and FAK

Because SOS plays a crucial role in integrin and growth factor receptor signaling (Pawson, 1995) and its ability to activate Rac depends on PI-3K signaling (Nimnal et al., 1998), we examined the role of SOS and PI-3K in integrin-specific activation of Rac. A dominant negative
Figure 4. Rac Is Activated on Fibronectin, but Not Laminin-1, and This Process Requires Shc, FAK, PI-3K, Sos, and Ras

(A) G0-synchronized HUVECs were plated on Fn or Lm (left) or incubated in suspension with anti-α2 or anti-α5 Mab and then plated on anti-mouse IgG coated dishes (right) in complete SFM for the indicated times. Equal amounts of proteins were subjected to GST-PAK pull-down. Rac bound to GST-PAK (top) and Rac in total lysates (bottom) were detected by immunoblotting with anti-Rac.

(B) HUVECs were transfected with a plasmid encoding HA-tagged Rac alone (-) or with the indicated concentrations of activated SOS (myr-SOS) or dominant negative Sos (myr-SOS-Dbl-), PI-3K (p85ΔISH2), Shc (Shc 2YF), FAK (CD2-FAK-Y397F), Ras (Ras-N17), and Crk (Crk-SH3). G0-synchronized cells were detached and plated on Fn for 90 min with growth factors. The activation of tagged-Rac was examined by GST-PAK pull-down followed by immunoblotting with anti-HA. Values of activation were expressed as percentage of activation relative to the control value obtained on Fn with cells expressing HA-Rac only. Proper expression of recombinant proteins was verified by immunoblotting. The graph presents the averages and standard deviations of four independent experiments. Inset shows one experiment with dominant negative SOS.

(C) HUVECs were either left untransfected (-) or transfected with 10 μg of the indicated plasmids, synchronized in G0, and replated on Lm in complete SFM for 90 min. Rac activation was examined by GST-PAK pull-down (left panel). HUVECs were transfected with a vector encoding EGFP-F in combination with plasmids encoding the indicated activated signaling proteins. G0-synchronized cells were plated on Lm in complete SFM for 24 hr and subjected to anti-BrdU staining. The results represent the average and standard deviations of values obtained from three independent experiments and are expressed as percentages of rescue (see Experimental Procedures) (middle panel). HUVECs were transfected with the indicated plasmids, synchronized in G0, and then either kept in suspension or plated on either Fn or Lm in complete SFM for 90 min. Total lysates were probed by immunoblotting with antibodies to phospho-ERK, total ERK-2, phospho-Akt, and total Akt (right panel).
form of SOS, carrying a series of point mutations inactivating the DH domain (myr-SOS-Dbl^~^~), suppressed activation of Rac in HUVECs plated on fibronectin in complete SFM. By contrast, a membrane-targeted form of SOS with an intact DH domain did not inhibit activation of Rac on fibronectin (Figure 4B). Rather, upon high-level overexpression it promoted this process (not shown). Together with the observation that myr-SOS-Dbl^~^~ does not interfere with Ras-ERK signaling (data not shown), these findings point to a specific requirement for the DH domain of SOS in integrin-specific activation of Rac. The activation of Rac on fibronectin was also inhibited by dominant negative PI-3K (p85^-iSH2) (Figure 4B) and the specific inhibitor LY294002 (not shown). These results suggest that the integrin-specific regulation of Rac requires both PI-3K and the DH domain of SOS.

Our prior results imply that the integrins able to recruit Shc, such as α5β1, cooperate with growth factor receptors to promote effective recruitment of Grb2/SOS to the plasma membrane, thereby regulating Ras-ERK signaling (Wary et al., 1996). We thus asked if Shc signaling was required for activation of Rac. As shown in Figure 4B, a dominant negative form of Shc carrying phenylalanine permutations at both tyrosines involved in Grb2 binding (Tyr 239 and Tyr 317; Shc-Y2F) completely suppressed the activation of Rac. The inhibition of Rac activation by Shc-Y2F was unexpected because the growth factors used in this study bind to receptors, which can recruit Grb2/SOS, PI-3K, and Nck independently of Shc, and there is evidence potentially linking all these target-effectors to Rac (Pawson, 1995). Together, the integrin specificity of Rac activation and its inhibition by Shc-Y2F suggest that in normal cells Shc functions at a specific subcellular location to integrate the signals emanating from specific integrins and growth factor receptors and thus activate Rac.

Because activated FAK combines with the SH2 domain of the p85 subunit of PI-3K and thereby recruits the enzyme in proximity of integrins (Chen et al., 1996), we asked whether FAK participated in the activation of Rac. Introduction of a membrane-targeted dominant negative form of FAK (CD2-FAK-Y397F) blocked the activation of Rac (Figure 4B) but not ERK (data not shown). Finally, the activation of Rac was also suppressed by dominant negative Ras (Ras-N17) (Figure 4B). This latter result probably reflects the ability of Ras to contribute to the activation of PI-3K and, through it, Rac (Rodriguez-Viciana et al., 1997), although we cannot exclude that the binding of dominant negative Ras to SOS prevents SOS interaction with Rac. Taken together, these findings suggest that signals from Shc, FAK, and PI-3K converge on SOS to activate Rac.

We finally asked if PI-3K and SOS could rescue endothelial cell proliferation on laminin-1 by activating endogenous Rac. When expressed separately, activated PI-3K and membrane-targeted SOS did not promote significant activation of Rac or entry in S phase on laminin-1. However, coexpression of the two signaling molecules induced activation of Rac and fully rescued cell cycle progression on laminin-1 (Figure 4C). This effect required that the DH domain of SOS be intact. Control experiments indicated that activated PI-3K was sufficient to induce phosphorylation of both Akt and ERK, whereas myr-SOS was only able to promote phosphorylation of ERK. These results are consistent with the hypothesis that SOS and PI-3K function upstream of Rac in integrin-specific control of cell proliferation.

**FAK-PI-3K Signaling Is Defective on Laminin-1**

Because both FAK and PI-3K were required for activation of Rac, we compared the efficiency of FAK-PI-3K signaling in HUVECs plated on fibronectin or laminin-1 in complete SFM. Immunoblotting experiments revealed that the major autophosphorylation site in FAK, Tyr 397, was phosphorylated at a slower rate and to a lower level on laminin-1 than on fibronectin (Figure 5A). Because Tyr 397 in FAK mediates recruitment of PI-3K (Chen et al., 1996), we examined the activity of the fraction of PI-3K associated with FAK in HUVECs adhering to fibronectin or laminin-1. Adhesion to fibronectin caused a 4-fold increase in the activity of FAK-associated PI-3K, whereas adhesion to laminin-1 exerted a very modest effect (Figure 5B). Similar results were obtained in the presence or absence of mitogens (not shown). These results indicate that ligation of α2β1 does not induce effective FAK-PI3K signaling.

**Inefficient Assembly of Focal Complexes and Focal Adhesions on Laminin-1**

We consistently observed that HUVECs adhering to fibronectin spread by extending lamellipodia along the entire cell periphery and rapidly acquired a typical polygonal shape. By contrast, HUVECs adhering to laminin-1 extended lamellipodia only from defined areas of the cell periphery and thus acquired a more elongated, often stellate morphology (not shown).

Adhesion to fibronectin promoted the formation of small peripheral focal complexes as well as more centrally located large focal adhesions, both of which contained the integrin α5 subunit, vinculin (not shown), phosphotyrosine, and paxillin (Figure 6A). In addition, it resulted in the organization of prominent actin stress fibers in the majority of cells (Figure 6A). By contrast, adhesion to laminin-1 promoted formation of focal complexes limited to the edges of lamellipodia. More importantly, cells plated on laminin-1 had only very thin and short focal adhesions, which stained for the integrin α2 subunit, vinculin (not shown), paxillin and phosphotyrosine faintly, and did not display prominent actin stress fibers (Figure 6A). Even cells plated on laminin for a prolonged time did not display mature focal adhesions. These results suggest that, in contrast to α5β1, the α2β1 integrin does not promote formation of focal adhesions and stress fibers efficiently.

We next tested the effect of activated Rho and Rac on the adhesive structures and cytoskeleton of HUVECs adhering to laminin. Introduction of activated Rho did not restore formation of focal adhesions and stress fibers on laminin-1 (not shown), consistent with the observation that Rho activation is not defective on laminin. Because α2β1 from HUVECs binds efficiently to laminin-1 in vitro (Languino et al., 1989; Kirchhofer et al., 1990), its inability to nucleate focal adhesions and stress fibers in vivo may be due to defective interaction with cytoskeletal proteins rather than poor activation of Rho. Transfection of activated Rac did not rescue formation of focal adhesions and stress fibers or promote significant...
spreading of HUVECs adhering to laminin-1 (see Supplemental Figure S3). Yet, it efficiently rescued these cells from growth arrest. This observation suggests that integrin clustering at focal adhesions may be a prerequisite for activation of Rac, rather than vice versa.

Inefficient Phosphorylation of Caveolin-1 and FAK on Laminin-1
Caveolin-1 plays a crucial role in integrin-mediated Shc signaling (Wary et al., 1998). We have recently observed that caveolin-1 is phosphorylated at Tyr 14 in response to integrin ligation (G. Fiucci and F.G.G., unpublished data). To monitor Shc signaling, HUVECs were stained with antibodies reacting specifically with caveolin-1 phosphorylated at Tyr 14. As shown in Figure 6B, adhesion to fibronectin resulted in the accumulation of phosphorylated caveolin-1 at focal complexes and focal adhesions, whereas cells plated on laminin were stained faintly by anti-phospho-caveolin antibodies. Thus, the fraction of caveolin-1 that is phosphorylated on Tyrosine in response to matrix adhesion, and is presumably involved in integrin signaling, localizes to focal complexes and focal adhesions (see also Volonté et al., 2001). We have previously detected a fraction of caveolin-1 at extracellular matrix contacts, but not at focal complexes and focal adhesions (Wary et al., 1998). It is likely that the epitopes recognized by the two antibodies to caveolin-1 used previously are masked at focal complexes and focal adhesions. These results suggest that caveolin-1 colocalizes with integrins at focal complexes, focal adhesions, and extracellular matrix contacts but becomes phosphorylated only at focal complexes and focal adhesions (Figure 6B and data not shown).

To monitor FAK signaling in situ, we stained HUVECs plated on fibronectin or laminin-1 with antibodies reacting with FAK phosphorylated at Tyr 397. As shown in Figure 6B, adhesion to fibronectin resulted in the accumulation of phosphorylated FAK at focal complexes and focal adhesions, whereas cells plated on laminin stained very poorly with anti-phospho-FAK antibodies. Because localization at focal adhesions is required for efficient activation of FAK (Shen and Schaller, 1999), the decreased rate of activation of FAK observed in HUVECs adhering to laminin-1 (see also Figure 5) may be a consequence of the inefficient assembly of focal adhesions on this matrix protein. We conclude that adhesion to laminin-1 does not promote efficient assembly of focal complexes and focal adhesions and efficient signaling through both caveolin-1/Shc and FAK.

Discussion
We provide evidence that in endothelial cells the α5β1 integrin and growth factor receptors cooperate to recruit SOS and increase its guanine nucleotide exchange activity toward Rac. Upon activation, Rac promotes biosynthesis of Cyclin D1, thus initiating the cell cycle events necessary for progression through G1 and entry into S phase. Although adhesion mediated by α5β1 allows efficient activation of ERK in response to mitogens, it does not mediate activation of Rac and progression through G1. These results suggest that Rac couples
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Figure 6. Reduced Assembly of Focal Adhesions and Signaling through Both Shc and FAK on Laminin-1

(A) G0-synchronized HUVECs were detached and replated on Fn or Lm in complete SFM for 4 hr, with the exception of the cell in the upper left panel, which was plated on Fn for 1 hr. The cells were stained with antibodies to paxillin (Pax) or phosphotyrosine (P-Tyr) followed by FITC-conjugated anti-mouse IgGs or with TRITC-conjugated Phalloidin (Actin).

(B) The FAK and Shc signaling pathways are activated at focal complexes and focal adhesions on fibronectin but not laminin-1. G0-synchronized HUVECs were plated on Fn or Lm for 2 hr in complete SFM and doubly stained with antibodies to phospho-caveolin-1 (P-Cav) followed by FITC-conjugated anti-mouse IgGs and antibodies to phospho-FAK (P-FAK) followed by TRITC-conjugated anti-rabbit-IgGs. The structures labeled by both antibodies correspond to focal complexes and focal adhesions as demonstrated by staining with anti-paxillin (not shown).

Signals generated by specific integrins and by growth factor receptors to the cell cycle machinery.

Normal fibroblasts placed in suspension undergo growth arrest prior to the activation of Cyclin E/CDK2 (Guadagno and Assoian, 1991). The Cyclin D mRNA is not synthesized efficiently, presumably as a consequence of defective ERK signaling (Roovers et al., 1999). Therefore, D-type CDKs are not assembled and increased amounts of p27 block Cyclin E/CDK-2 (Zhu et al., 1996). In endothelial cells adhering to laminin, saturating concentrations of growth factors cause efficient activation of ERK and transcription of Cyclin D1. However, Rac is not activated and these cells fail to synthesize Cyclin D1 protein and undergo growth arrest. Introduction of activated Rac, but not MEK, PI-3K, or Akt rescues biosynthesis of Cyclin D1 and progression through G1. These findings suggest that Rac plays a primary role in integrin-specific control of the cell cycle by controlling the translation of Cyclin D1 mRNA.

Our results do not exclude that ERK may also play a physiologically relevant role. In fact, cells in vivo are likely to be exposed to a wide range of concentrations of growth factors. It is possible, and indeed likely, that the ability of specific integrins to activate ERK and thereby regulate Cyclin D transcription becomes important when the amount of growth factors is limiting. In addition, it is known that Rac signaling can promote transcription of Cyclin D1 (Westwick et al., 1997). We speculate that this contribution of Rac may also become important when the amount of growth factors is limiting and ERK is poorly activated. In light of our current results, we propose that ERK and Rac signaling also cooperate to promote Serum Response Element-dependent transcription of Fos (see Figure 7A in Wary et al., 1996). This dual regulation of cell cycle genes, through ERK and Rac, may ensure that the control of cell cycle by the matrix is both stringent and integrin-specific.

What is the mechanism by which growth factor receptors and specific integrins jointly activate Rac? This process requires the DH domain of SOS as well as PI-3K. Structural and functional studies indicate that the PH domain of SOS exerts an allosteric inhibition on the adjacent DH domain. Upon interaction of the PH domain with PIP-3 in the plasma membrane, SOS is thought to undergo a conformational transition that exposes the DH domain and allows it to activate Rac (Soisson et al.,...
We propose that α5β1 and other Shc-linked integrins cooperate with growth factor receptors to recruit the Grb2/SOS complex at sites of integrin-mediated adhesion, where FAK-P13K signaling increases the local concentration of PIP-3 and activates the exchange activity of SOS toward Rac. This model accounts for the ability of both dominant negative Shc and FAK to suppress activation of Rac (Figure 4). The mechanism by which PI-3K is activated upon recruitment by FAK remains to be examined, but the ability of dominant negative Ras to inhibit activation of Rac suggests an involvement of Ras. Several observations suggest that the growth factor and adhesion signals necessary for activation of Rac may be integrated at focal complexes and focal adhesions. First, a fraction of growth factor receptors physically associate with certain integrins, and this interaction presumably occurs at these sites (Giancotti and Ruoslahti, 1999). Second, the fraction of caveolin-1 that is phosphorylated at Tyr 14 in response to integrin signaling—and presumably functions upstream of palmitoylated Src kinases in the recruitment of Shc—is localized at focal adhesions. In addition, overexpression of Shc opposes the disassembly of focal adhesions caused by PTEN (Gu et al., 1999) and ShcA−− fibroblasts display defects in the organization of focal adhesions and stress fibers (Lai and Pawson, 2000). Both results imply a function of Shc at focal adhesions. Third, optimal activation of FAK and thus recruitment of PI-3K require localization of FAK at focal adhesions (Shen and Schaller, 1999). Fourth, activated Rac and PI-3K have been localized at focal complexes and focal adhesions (Manser et al., 1997; Gillham et al., 1999). Finally, endothelial cells plated on laminin-1 in the presence of mitogens do not form mature focal adhesions and fail to activate Rac. Introduction of activated Rac in these cells does not promote assembly of focal adhesions, suggesting that integrin clustering may be a prerequisite for activation of Rac, rather than vice versa.

Because the activation of FAK is mediated by β1, β3, and several other β subunit cytoplasmic domains, it is widely assumed that most integrins can activate FAK. However, optimal activation of FAK requires localization at focal adhesions (Shen and Schaller, 1999). Integrins may differ in their ability to nucleate focal adhesions because this process is regulated by the α subunit cytoplasmic tail (Yamada and Miyamoto, 1995). We have observed that α5β1 promotes nucleation of focal adhesions and efficient signaling through both Shc and FAK,
Integrin Activation of Rac Controls G1 Transit

whereas α2β1 does not nucleate mature focal adhesions and activate both pathways inefficiently, at least in endothelial cells. It is possible that the integrin α subunit tail regulates both Shc and FAK signaling by regulating assembly of focal adhesions.

In addition to the one described here, there are other mechanisms by which integrins may control Rac. In hematopoietic cells, integrins activate the tyrosine kinase Syk and PI-3K, which cooperate to augment the guanine-nucleotide exchange activity of Vav-1 toward Rac (Miranti et al., 1998). In fibroblasts, p130Cas and paxillin recruit the adaptor Crk and, through it, DOCK180, which displays guanine nucleotide exchange activity toward Rac in vitro and promotes spreading in a Rac-dependent manner in cells (Kiyokawa et al., 1998). Notably, a dominant negative version of Crk, which interferes with the recruitment of DOCK180 by both p130Cas and paxillin, exerts no apparent effect on the activation of Rac caused by mitogens in endothelial cells plated on fibronectin (Figure 4), suggesting that DOCK180 is not involved in this process. In addition, whereas matrix adhesion is sufficient for activation of Rac in fibroblasts (del Pozo et al., 2000), it is not in endothelial cells. These observations suggest that the mechanisms, and therefore consequences, of Rac activation differ depending on the cell type and stage of development.

Several lines of evidence suggest a correlation between cell spreading, which in part reflects cytoskeleton tensile forces, and cell division (Chen et al., 1997; Huang et al., 1998). In addition to spreading, Rac induces clustering of integrins (D’Souza-Schorey et al., 1998). This effect may further enhance activation of Rac, spreading, and clustering of integrins. Based on our current results, we speculate that the failure of cells to proliferate when spreading is prevented may reflect inhibition of Rac signaling. It is also known that interaction with a natural fibronectin matrix, but not with fibronectin coated onto a substrate, facilitates Rho activation and cell proliferation (Bourdoulous et al., 1998; Danen et al., 2000). In addition, all Rho family proteins, including Cdc42, are required for cell proliferation (Olson et al., 1995). The coordinate regulation of the cytoskeleton and the cell cycle by Rho proteins may ensure that cells proliferate only upon attaching to a matrix with appropriate geometry and physical properties.

The mechanism of cell cycle control identified here is likely to be important for angiogenesis. During the invasive/proliferative phase of angiogenesis, endothelial cells undergo multiple interactions with a fibronectin-rich interstitial matrix, whereas during the maturation phase they assemble a laminin-rich basement membrane and form a capillary (Risau, 1997). Our current results imply that α2β1 and αvβ3, which have been both implicated in angiogenesis (Elceiri and Cheresh, 1999; Hynes et al., 1999) and share similar signaling properties (Wary et al., 1996), promote endothelial cell proliferation during the invasive/proliferative phase. The α2β1 integrin may instead facilitate growth arrest and differentiation during the maturation phase. Accordingly, laminin and collagen, which bind to α2β1, promote formation of capillary-like structures by endothelial cells in vitro (Montesano et al., 1983; Kubota et al., 1988).

In conclusion, our results indicate that SOS and its downstream target-effector Rac integrate signals from specific integrins and growth factor receptors to promote biosynthesis of Cyclin D1 and progression through the G1 phase of the cell cycle. These findings identify a mechanism by which Rac is activated and controls the cell cycle machinery, thus potentially explaining why Rac is important for both normal cell proliferation and neoplastic transformation.

Experimental Procedures

Cells, Transfections, and Constructs

HUVECs were obtained from Clonetics or Vec Technologies. For transfection, 3 x 104 cells were reseeded in 300 μl SFM with the indicated amounts of vectors and carrier DNA to a total of 60 μg and electropropated at 300 V and 450 μF. Cells were allowed to recover in complete medium for 12 hr. Efficiency approximated 35% as assessed by epifluorescence microscopy of cells transfected with pEGFP-F. We used expression vectors encoding GST-Rb (pGEX-2T-GST-Rb(40-252)) and GST-Pak (pGEX 4T-1 αPak42-61), Cyclin D1 (pRc-CMV-Cyclin D1), dominant negative Shc (pRK5-Shc- Y239F/Y317F), FAK (pH3-FRINK and pCDM6-CDF2-FAK-Y397F), Crk (pMEGNo-v-c-Rtk368DRHDH), Rac1 (pcDNA3 Rac-N71), Ras (p2Zip Ras-N71) and PI-3K (psGS5-p85/sIsh2-N2), HA-tagged Rac1 (pX1-HA-Rac1), activated Cdc42 (pzip CDC42-V12), Rac1 (pcDNA3 Rac1-61L, and pGCT Rac1-V12), RhoA (pcDNA3 RhoA-63L), PISK (pCG-PISK), MEK1 (pMCL-MEK1/S218/E222) and HA-tagged Ras (pDCR-HA-Ras-G12V), myristoylated Akt (pCMV-Myr-AKT-HA), SOS1 (pcDNA3-Myr-SOS1) and SOS1 carrying point mutations that inactivate the Dbll domain (pcDNA3-Myr-SOS-dbl-), and famesylated EGFP (pEGFP-F).

Measurement of Cell Cycle Progression

Cells were synchronized in G0 by growth factor deprivation, detached, and replated on coverslips coated with 15 μg/ml human plasma fibronectin or mouse laminin-1 (Collaborative Research). After incubation in Serum Free Medium (SFM, Gibco-BRL) supplemented with growth factors (20 ng/ml bFGF, 1 μg/ml heparin, 10 ng/ml EGF, 10 μg/ml insulin, 10 μg/ml transferrin, 1 μg/ml seleno acid) (complete SFM) and 10 μM BrdU (Boehringer) for 24 hr, the cells were fixed and stained with anti-BrdU Mab (Boehringer) and TRITC-conjugated anti-mouse IgG to estimate the percentage of GFP-positive cells that had incorporated BrdU. The ability of various signaling proteins to rescue entry in S phase on laminin-1 was expressed as percentage of rescue (R). To calculate this value, we used the formula R = (X – L) / (F – L) X 100 (where X is the percentage of cells expressing a given signaling protein that had incorporated BrdU on laminin-1, F is the percentage of cells expressing GFP alone that had incorporated BrdU on fibronectin, and L is the percentage of cells expressing GFP alone that had incorporated BrdU on laminin-1). The percentage of mock or GFP transfected cells entering in S phase on fibronectin ranged between 40% and 50%. Overexpression of Ras-V12 and Rac-L61 promoted entry into S-phase on laminin-1 beyond the value observed for mock or GFP transfected cells on fibronectin. Hence, the rescue exceeded 100% in this case.

Biochemical Analysis of the Cell Cycle

Immunoprecipitation and immunoblotting with antibodies to cyclin D1 (DCS-6, Neomarkers), cyclin E (C-19, Santa Cruz), and p27 (clone 57, Transduction Laboratories) were performed as previously described (Wary et al., 1998). CDK2 immunocomplex kinase assays were performed as described by Fang et al. (1996). CDK4 and CDK6 kinase assays were performed on immune complexes using antibodies to CDK4 (C-22, Santa Cruz) and CDK6 (C-21, Santa Cruz), and 4 μg of GST-Rb (residues 792–928) for pulse-chase analysis. Cells were synchronized in G0, detached, and plated on fibronectin or laminin-1 for 6 hr in complete SFM followed by 1 hr in methionine/cysteine-free complete SFM (GibcoBRL). They were then labeled for 1 hr with 200 μCi/ml [35S]-methionine/cysteine (Trans35S-Label™, ICN) and chased for the indicated times in complete SFM. The radioactivity incorporated by Cyclin D1 was quantified by Phos...
phorimer analysis (Molecular Dynamics). Northern-blotting of cyclin D1 was quantified by Phosphorimager.

Signaling Methods

To specifically ligate α5β1 and α2β1, we incubated 4 × 10^6 cells in suspension with 5 μg of anti-α2 or anti-α5 Mab (P1D6 and P1D6, Gibco-BRL) and, after washing, replated them on dishes coated with rabbit anti-mouse IgGs (Pierce) at 37°C for the indicated times. Activated Rac was pulled down from 800 μg total proteins with 40 μg GST-PKCS9 and detected by immunoblotting with anti-Rac Mab (Transduction Laboratories) (Manser et al., 1997). Activated Cdc42 was pulled down by using the same protocol and detected with anti-Cdc42 Mab (Transduction Laboratories). For dominant-negative studies, cells were transfected with the indicated amounts of each construct and 0.2 μg of PK-JA-HA-Rac1. Under this protocol, recombinant Rac was expressed at levels lower than endogenous Rac. Four hundred to six hundred micrograms total proteins (proportional to HA-Rac1 expression levels) were subjected to GST-PK pull-down followed by immunoblotting with anti-HA Mab. Activated Rho was pulled down from 500 μg total proteins with 40 μg GST-Rhotekin RBD and detected by immunoblotting with anti-Rho Mab (26c4, Santa Cruz) (Ren et al., 1999).

PKA phosphorylation was measured by immunoblotting FAK with anti-phospho-ERK's SC-7368 from Santa Cruz and #91065 from Cell Signaling Technology; anti-phospho-ERK's 473 Mab #9271 from Cell Signaling Technology). Blots were reprobed with antibodies to ERK-2 (SC-154, Santa Cruz) or total Akt-1 (SC-1618, Santa Cruz) to control for equal protein loading.

Expression of recombinant proteins was verified by immunoblotting with antibodies to the HA (HA-11, Babco), Myc (9E10), CD2 (RPA-2.10, PharMingen), Flag (M2, Eastman-Kodak), Glu-Glu (mAb, Babco), or Gag (mAb 3C2) epitope tag. The level of expression of each recombinant was also compared to that of endogenous protein by using antibodies reacting with both.

Immunofluorescent Methods

Cells were fixed with 3.7% paraformaldehyde and permeabilized by using antibodies reacting with both. regulate Rac binding. J. Biol. Chem. each recombinant was also compared to that of endogenous protein by using antibodies reacting with both.

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