Integrin-mediated Activation of Focal Adhesion Kinase Is Required for Signaling to Jun NH₂-terminal Kinase and Progression through the G1 Phase of the Cell Cycle

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Abstract. The extracellular matrix exerts a stringent control on the proliferation of normal cells, suggesting the existence of a mitogenic signaling pathway activated by integrins, but not significantly by growth factor receptors. Herein, we provide evidence that integrins cause a significant and protracted activation of Jun NH₂-terminal kinase (JNK), while several growth factors cause more modest or no activation of this enzyme. Integrin-mediated stimulation of JNK required the association of focal adhesion kinase (FAK) with a Src kinase, Fak, and the phosphorylation of p130Cas, the phosphorylation of p130Cas, and subsequently, the recruitment of Crk. Ras as and PI-3K were not required. FAK–JNK signaling was necessary for proper progression through the G1 phase of the cell cycle. These findings establish a role for FA K in both the activation of JNK and the control of the cell cycle, and identify a physiological stimulus for JNK signaling that is consistent with the role of Jun in both proliferation and transformation.

Key words: integrins • focal adhesion kinase • Jun NH₂-terminal kinase • Jun • cell cycle

NORMAL cells require adhesion to extracellular matrix components to proliferate in vitro, suggesting that integrins activate signaling pathways that are necessary for cell cycle progression (reviewed in Giancotti, 1997). In principle, integrins could cooperate with growth factor receptors to produce a synergistic stimulation of one or more mitogenic signaling pathways. Indeed, the results of several studies support this model (reviewed in Ruoslahti and Reed, 1994; Clark and Brugge, 1995; Parsons, 1996; Clark and Hynes, 1997; Schwartz, 1997; Yamada and Geiger, 1997; Howe et al., 1998; Schlaepfer and Hunter, 1998). However, such a mechanism does not readily explain the strict adhesion requirement for growth displayed by normal cells. In addition, or instead, integrins could activate a signaling pathway that is not significantly activated by growth factors, but is necessary for cell proliferation. The identification of such a pathway would provide a more complete understanding of the anchorage-dependent growth that is currently available.

Integrins activate common, as well as subgroup-specific, signaling pathways. A subset of integrins, which includes α1β1, α5β1, αvβ3, and αvβ4, is coupled to the Ras-extracellular signal-regulated kinase (ERK)1 signaling pathway by the adaptor protein Shc (Mainiero et al., 1995; Wary et al., 1996). Shc binds directly to the uniquely large cytoplasmic domain of β4 when this integrin undergoes tyrosine phosphorylation (Mainiero et al., 1995; 1997). In contrast, the recruitment of Shc by β1 and αv integrins is indirect, requiring the interaction of the integrin α subunit with the membrane adaptor caveolin-1 and associated tyrosine kinase, Fyn (Wary et al., 1998). Biochemical and genetic evidence suggest that integrins recruit Shc independently of focal adhesion kinase (FAK), and that this event is necessary and sufficient to activate the Ras-ERK pathway. In primary cells, inhibition of integrin-mediated Shc signaling results in cell cycle arrest, despite the presence of growth factors, suggesting that a combined stimulation of Ras as by Shc-linked integrins and growth factor receptors is required for progression through the G1 phase of the cell cycle (Wary et al., 1996, 1998).
Certain integrins appear to associate preferentially with specific growth factor receptors and contribute to their activation (Miyamoto et al., 1996; Schneller et al., 1999; Moro et al., 1998; Soldi et al., 1999). For example, αvβ3 combines with the platelet-derived growth factor (PDGF) receptor. Hence, fibroblasts show enhanced proliferation in response to PDGF when attaching to the αvβ3 ligand vitronectin than they do on the β1 integrin ligand collagen (Schneller et al., 1997). The selective association of integrins with growth factor receptors represents a second potential mechanism of integrin-specific growth control.

Whereas the aforementioned pathways are activated only by certain integrins, the tyrosine kinase FAK is activated by most integrins (Parsons, 1996). A ctsivated FAK undergoes autophosphorylation at Tyr 397 and thereby binds to the SH2 domain of the Src-family kinase Src or Fyn (Schaller et al., 1994; Schlaepfer et al., 1994). The Src-family kinase then phosphorylates a number of a FAK-associated proteins, including p130Cas and paxillin, which contain multiple docking sites for the adaptor proteins Crk and Nck (Schaller and Parsons, 1995; Richardson and Parsons, 1996; V uori et al., 1996; Schlaepfer et al., 1997). In addition, Src phosphorylates FAK at a tyrosine residue able to recruit Grb2 (Schlaepfer et al., 1994). It is possible that FAK contributes to the activation of the Ras-ERK cascade by these (Schlaepfer et al., 1994) and potentially other mechanisms (Chen et al., 1996; King et al., 1997; Lin et al., 1997; Lin et al., 1997; Renshaw et al., 1997). A through previous studies have provided direct evidence for a role of FAK in cell migration (I I i c et al., 1995; Fincham and Frame, 1998; Cary et al., 1998; K lemke et al., 1998) and protection from apoptotic cell death (Frisch et al., 1996b; I I i c et al., 1998). It is unclear whether FAK also regulates cell proliferation, and if so, by what mechanism.

Integrin-mediated adhesion activates not only E R K , but also J un H2-terminal kinase (J N K ; M iyamoto et al., 1995; Mainiero et al., 1997; M a cK enna et al., 1998). J N K is the final element of a mitogen-activated protein kinase (M A P K ) cascade known to be activated by stress stimuli, such as U V radiation, hyperosmolar conditions, and inflammatory cytokines (I p and D avis, 1998). U pon activation, J N K enters the nucleus, and phosphorylates and activates the transcription factors c-J un and activating transcription factor 2 (A T F2), thereby regulating A P-1-dependent transcription (K arin et al., 1997). B ecause there is evidence that c-J un is required for cell proliferation (R iabowol et al., 1992; Johnson et al., 1993), we sought to examine the mechanism by which integrins activate J N K and test the hypothesis that activation of this signaling pathway contributes to the control of cell cycle progression.

O ur results indicate that integrins cause a significant and protracted activation of J N K , while growth factors appear to be unable to do so. B y using various dominant-negative signaling molecules, we provide evidence that the activation of J N K by integrins is mediated by F A K and is necessary for cell cycle progression.

### Materials and Methods

#### Antibodies and Extracellular Matrix Proteins

The mAb M 2 to Fl a G tag was purchased from E astman-K odak and the anti-CD 2 mAb RPA-2.10 from Ph arMingen. The anti-H-R as mAb R 02120 (clone 18) and anti-p130Cas mAb P 27820 (clone 21) were from Transduction Laboratories. The origin and specificity of the affinity-puri fied antibodies to E R K 2, p hospho-E R K , S rc, and G S T and of the anti-5-bromodeoxyuridine (anti-BrdU) mAb were described previously (W a r y et al., 1996; 1998). The mAb 3C2 reacting with the gag portion of v-C r k was also described previously (Potts et al., 1997). H uman fibronectin was from G IB C O B RL and poly-γ-L-lysine from S ignma C hemical Co.

#### Cell Lines, Constructs, and Transfections

293 human embryonic kidney cells were cultured in D M E 10% F C S on gelatin-coated plates. N I H -3 T M mouse fibroblasts were cultured in D M E 10% calf serum (CS). Fibroblasts from Sc r/c- and Fyn/c- embryos were obtained from Philips Sporan (F red H utchinson C ancer Researc h C enter, S eattle, WA ) and cultured in D M E 10% CS. H uman umbilical vein endothelial cells (H U V E C s) were purchased from C lonetics and cultured on gelatin-coated dishes in human endothelial serum-free medium (S FM ; G IB C O B RL ) supplemented with 20% F C S (G IB C O B RL ), 10 ng/ml E G F , 20 ng/ml bFGF, and 1 μg/ml heparin (all from I ntrogen).

The reporter plasmid p co1l T R E -tk-L uc, in which the expression of L uciferase is driven by a single copy of the collagen gene 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element (T R E ) linked to the H epes simplex virus thymidine kinase minimal promoter, was described previously (G a l ien et al., 1994). V ectors expressing the F A K -tagged version of J N K 1, glutamine S-transferase (GST)-J N K , dominant-negative R as (N 77), and H A-tagged μ-galactosidase were described previously (M acK enna et al., 1998). T he CD M V promoter-based vectors encoding C D 2-F A K (wild-type), C D 2-F A K K 454R (kinase dead), and C D 2- F A K Y 397F were described previously (Ch an et al., 1994). A kinase dead version of chicken C r k was obtained from S ara C ourtneid e (E MB L, H eidelberg, G ermany) and subcloned into the cytomegalovirus (C MV ) promoter-based vector p K R 5. T he p E B G vectors expressing G T S-tagged M K K 4 (wild-type) and M K K 4 G 12V (kinase dead) from the human elongation factor 1α promoter were described previously (S u et al., 1997). T he M o loney L eukemia V irus (M L V )-L T R -based p M E X N E O vec tors encoding v-Cr k (wild-type), v-C r k R 273N (SH 2 mutant), and v-C r k D 386DRHAD (SH 3 insertional mutant) were described previously (A hli- n-G uitel et al., 1998). T he p E B G vectors encoding G T S-tagged r p 130Cas (short form) and its substrate region deleted form (S D , Δ213-221) were also described (M ayer et al., 1995). T he T AM -67 transactivation domain mutant form of c-J un (jun Δ33-122) was expressed from p CM V and previously characterized (B rown et al., 1993). T he dominant-negative version of paxillin used in this study carries three phenylalanine mutations at tyrosine 31, 118, and 187 and is unable to bind to C r k. T he M L V -L T R -based expression vector p s raf-22w encodes an activated version of c r a f 1 lacking an N H2-terminal segment of 365 amino acids (S tanton et al., 1989). T he vector encoding activated R as, p D C R-H-a r s (G 12V ), was kindly provided by J ohn W estwick (S ignal P harmaceuticals).

N I H -3 T M cells were transiently transfected with Lipofectamine according to the manufacturer’s instructions (G IB C O B RL ). 293 cells were plated at 6 × 104 per 15-cm dish for 8 h and then transfected overnight with various amounts of plasmid by the calcium phosphate method. A ll transfections were normalized to the same total amount of DNA with empty vector. C ell s were allowed to recover for 12 h before growth factor starvation.

#### Biochemical Methods

To monitor the activation of J N K and E R K during G 1, H U V E C s were synchronized in G 0 by a 24 h incubation in human endothelial S FM containing 0.2% F C S . T hey were then detached with 0.02% EDT A , collected in D M E containing 0.2% F C S , and kept in suspension at a density of 105/ml for 15 min at room temperature to recover. A liquots consisting of 1.5 × 106 cells were plated on 15-cm dish diam coated with 20 μg/ml fibronectin and postcoated with 0.2% heat-inactivated B SFM supplemented with I T S+1 (S ignma C hemical Co.), E G F (10 ng/ml), bFGF (20 ng/ml), and heparin (1 μg/ml) for the indicated times. C ell s from an identical aliquot were pelleted and lysed in suspension as a control. B efore biochemical analysis, N I H -3 T M cells were serum starved for 18 h and 293 cells were serum starved for 24 h in D M E containing 0.2% C S or F C S , respectively. A fter detachment with 0.02% E D TA , cells were collected in D M E containing 0.2% heat-inactivated B SFM, washed in the same medium, and kept in suspension at a density of 105/ml for 15 min at room temperature to recover. A liquots consisting of 1.5 × 106 cells were plated on 15-cm dish diam coated with 20 μg/ml fibronectin and postcoated with 0.2% heat-inactivated B SFM for the indicated times. C ell s from an identical aliquot were pelleted and lysed in sus-
The rapid activation of ERK at the onset of G1 may serve to attain detectable levels of c-Jun, but not c-Fos (Karin, 1995), later (Fig. 1). Since unstimulated cells are known to contain significant level during mid-G1, before the activation of the D-type cyclin-dependent kinase CDK4 (Fig. 1). Peak JNK activity was observed 4 h after entry into G1. In contrast, the activation of ERK was biphasic with a first peak 10 to 20 min after entry into G1 and a second minor peak 8 h later (Fig. 1). Since unstimulated cells are known to contain detectable levels of c-Jun, but not c-Fos (Karin, 1995), the rapid activation of ERK at the onset of G1 may serve to induce serum response element (SRE)-dependent transcription of the c-Fos gene before JNK-mediated transcriptional activation of c-Jun. The AP-1 transcription factor Fos/Jun may then promote the expression of genes necessary for G1 progression. The extent and timing of JNK activation during the cell cycle are thus consistent with a potential role in the control of G1 progression.

We next compared the ability of integrins and growth factor receptors to activate JNK in NIH-3T3 fibroblasts. Preliminary experiments showed that the activity of JNK was significantly higher in growth factor deprived, stably adherent cells than in cells that had been detached and immediately lysed (Fig. 2 A). In accordance with the previous observation that several growth factors cause a relatively modest activation of JNK (Kyrkikis et al., 1994; M inden et al., 1994), exposure to mitogenic concentrations of PDGF, bFGF, and insulin increased the activity of JNK only to a limited extent in growth factor starved, stably adherent NIH-3T3 cells. In contrast, PDGF, bFGF, and to a minor extent, insulin, caused a significant activation of ERK under the same conditions (Fig. 2 A).

To further examine the relative contribution of integrins and growth factor receptors to the activation of JNK, NIH-3T3 cells were either plated on fibronectin in the absence of growth factors or treated with various doses of PDGF while in suspension. As shown in Fig. 2 B, adhesion to fibronectin induced a rapid, strong, and protracted activation of JNK. By contrast, JNK activity increased only to a limited extent in growth factor starved, stably adherent NIH-3T3 cells. In contrast, PDGF, bFGF, and to a minor extent, insulin, caused a significant activation of ERK under the same conditions (Fig. 2 A).
Adhesion to fibronectin caused maximal stimulation of JNK and TRE-dependent transcription. (A) After serum starvation, NIH-3T3 cells were treated while adherent with 20 ng/ml of PDGF, 20 ng/ml bFGF, or 6.25 μg/ml insulin for 10 min, or left untreated and either detached (S) or adherent (Starved). JNK was precipitated from extracts containing equal amounts of total proteins and subjected to an in vitro kinase assay (GST-Jun). Total lysates containing the same amount of proteins were subjected to immunoblotting with antiphospho-ERK (Phospho-ERK). (B) Serum-starved NIH-3T3 cells were detached and either left in suspension (S) or plated on fibronectin (Fn) for the indicated times. Alternatively, they were left adherent and treated with the indicated concentrations of TNF-α for 15 min. JNK was precipitated from extracts containing equal amounts of total proteins and subjected to an in vitro kinase assay (GST-Jun). (C) As shown in Fig. 2, D and E, adhesion to fibronectin promoted TRE-dependent transcription with kinetics closely followed that of JNK activation. Simultaneous exposure to PDGF caused a delay in the transcriptional response to fibronectin, as observed for the activation of JNK. The induction of TRE-dependent transcription by integrins required the transcriptional activity of c-Jun because it was suppressed by the TATM-67 dominant-negative form of the transcription factor (93.3% inhibition). Integrin-mediated activation of ERK and SRE-dependent transcription of Fos (Wary et al., 1996; Mainiero et al., 1997) can increase the levels of AP-1 available for phosphorylation by JNK. However, TRE-dependent transcription could not have occurred in the absence of JNK-mediated phosphorylation of c-Jun.

Integrin-mediated Activation of JNK Requires FAK, Src, p130Cas, Crk, and MKK

The mechanism by which integrins activate JNK was examined by introducing dominant-negative versions of various signaling components into human embryonic kidney 293 cells. Since preliminary experiments suggested that the ability to activate JNK is shared by all integrins, irrespective of whether they are able or not to recruit Shc (data not shown), we decided to examine the role of FAK in this process. Inactivating mutations were introduced at either the Src SH2-binding site or the ATP-binding site of CD2-associated protein (CD2-AP). Since preliminary experiments suggested that the ability to activate JNK is shared by all integrins, irrespective of whether they are able or not to recruit Shc (data not shown), we decided to examine the role of FAK in this process. Inactivating mutations were introduced at either the Src SH2-binding site or the ATP-binding site of CD2-AP. Since preliminary experiments suggested that the ability to activate JNK is shared by all integrins, irrespective of whether they are able or not to recruit Shc (data not shown), we decided to examine the role of FAK in this process. Inactivating mutations were introduced at either the Src SH2-binding site or the ATP-binding site of CD2-AP.

Figure 2. Adhesion to fibronectin induces activation of JNK and TRE-dependent transcription. (A) After serum starvation, NIH-3T3 cells were treated while adherent with 20 ng/ml of PDGF, 20 ng/ml bFGF, or 6.25 μg/ml insulin for 10 min, or left untreated and either detached (S) or adherent (Starved). JNK was precipitated from extracts containing equal amounts of total proteins and subjected to an in vitro kinase assay (GST-Jun). Total lysates containing the same amount of proteins were subjected to immunoblotting with antiphospho-ERK (Phospho-ERK). (B) Serum-starved NIH-3T3 cells were detached and either left in suspension (S) or plated on fibronectin (Fn) for the indicated times. Alternatively, they were left adherent and treated with the indicated concentrations of PDGF for 5 min and subjected to JNK assay as above. (D) Suspended cells were lysed (S) or plated on fibronectin for the indicated times in SFM supplemented with or without 20 ng/ml PDGF during the last 5 min of adhesion (Fn+PDGF) before extraction. JNK assay was performed as above. (E) NIH-3T3 cells were transfected with pcoll TRE-tk-Luc. After serum starvation, the cells were detached and left in suspension (S) or plated on fibronectin (Fn) for the indicated times in SFM supplemented with or without 20 ng/ml PDGF. Cell lysates containing equal amounts of total proteins were subjected to luciferase assay. Values are expressed in arbitrary units. A fter cycloheximide-induced blockage of protein synthesis, luciferase activity decays with a half-life of ~1 h in serum starved NIH-3T3 cells replated on fibronectin (data not shown).

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tion of JNK was also blocked by a kinase inactive version of M KK4, one of the major enzymes that binds to and phosphorylates JNK (Fig. 4). In contrast, it was not inhibited by expression of dominant-negative Ras (Fig. 3) or by exposure to the PI-3K inhibitors Wortmannin (100 nM) and LY294002 (50 μM) (data not shown). These results indicate that the FAK/Src complex links integrins to M KK4 or a related enzyme, and thereby JNK. In addition, they suggest that Ras and its substrate, PI-3K, which can activate Rac and thus JNK (Rodriguez-Viciana et al., 1994; Nobes et al., 1995; Klippel et al., 1996), do not contribute to this process.

To examine the mechanism by which the FAK/Src complex activates JNK, we focused on the role of the docking/adaptor proteins p130CA and paxillin, which bind to the FAK/Src complex and become heavily phosphorylated on tyrosine residues upon integrin engagement (Schaller and Parsons, 1995; Richardson and Parsons, 1996; Vuori et al., 1996; Schlaepfer et al., 1997). The activation of JNK by integrins was inhibited to a significant extent by a mutant form of Crk carrying a deletion of the entire substrate region (SD-CAS; Fig. 4). In contrast, a mutant form of p130CA carrying a deletion of the entire substrate region (SD-CAS) or with 3, 9, and 27 μg of plasmids encoding CD2-FAK K454R (catalytically inactive), CD2-FAK Y397F (unable to bind to the SH2 domain of Src family kinases), and K-Src (kinase dead), or with 9 and 27 μg of vector encoding DN Ras (N17, dominant-negative). The cells were serum starved for 24 h, detached, and lysed immediately (S) or plated on fibronectin for 20 min (Fn). FLAG-tagged JNK was immunoprecipitated from extracts containing equal amounts of total proteins and subjected to an in vitro kinase assay with GST-Jun as a substrate (A). Total lysates containing the same amount of proteins were subjected to immunoblotting with antibodies to CD2, Src, and Ras to verify that the expression of the various dominant-negative proteins was proportional to the amount of DNA transfected (B). Immunoblotting with anti-FLAG antibodies was used to verify equal expression of FLAG-JNK in all samples (C).

We reasoned that an SH3 mutant form of the viral version of Crk, which is anchored to the membrane via its gag sequences and truncated before tyrosine 222, would have interacted efficiently, via its intact SH2 domain, with p130CA, but not with downstream target effectors. As shown in Fig. 4, expression of this mutant form of Crk effectively suppressed JNK activation in cells plated on fibronectin. Conversely, the introduction of a control construct with a mutated SH2 domain stimulated the activation of JNK, suggesting that the recruitment of Crk to the plasma membrane and its interaction with downstream target(s) via the SH3 domain are sufficient to activate JNK (Fig. 4). These observations indicate that the FAK/Src/p130CA complex activates JNK by recruiting Crk to the plasma membrane. They are also in agreement with previous studies implicating Crk in the activation of JNK (Tanaka et al., 1997).

Integrin-mediated JNK Signaling Controls Cell Cycle Progression

To examine whether FAK signaling to JNK was required for cell proliferation, NIH-3T3 fibroblasts were transiently transfected with various amounts of vectors encoding wild-type and mutant versions of the signaling components of this pathway, in combination with the marker proteins of the FAK/Src complex in integrin-mediated activation of JNK. 293 cells were transiently transfected with 3 μg of vector encoding FLAG-tagged JNK alone or in combination with 3, 7.5, and 15 μg of plasmid encoding a mutant form of p130CA carrying a deletion of the entire substrate region (SD-CAS), or with 3, 7.5, and 27 μg of plasmids encoding SH2-v-Crk (SH2 mutant), SH3-v-Crk (SH3 mutant), and GST-DN M KK4 (kinase inactive). The cells were serum starved for 24 h, detached, and solubilized immediately (S) or plated on fibronectin for 20 min (Fn). FLAG-tagged JNK was immunoprecipitated from extracts containing equal amounts of total proteins and subjected to an in vitro kinase assay with GST-Jun as a substrate (A). Total lysates containing the same amount of proteins were subjected to immunoblotting with antibodies to p130CA, gag, and GST to verify that the expression of the various dominant-negative proteins was proportional to the amount of DNA transfected (B). Immunoblotting with anti-FLAG antibodies was used to verify equal expression of FLAG-JNK in all samples (C).
β-galactosidase. The cells were synchronized in G0 and plated on fibronectin in the presence of PDGF. Entry of the transfected cells into S phase was evaluated by double staining with X-gal and anti-BrdU antibodies.

While CD2-FAK, which is constitutively active (Chan et al., 1994), promoted entry into the S phase to a limited extent, its kinase dead version suppressed it (Fig. 5). In both cases, the effects observed were dose dependent. In addition, whereas wild-type p130CAS did not affect progression through G1, a mutant version carrying a deletion of the substrate region, which includes all the Crk binding sites, partially inhibited transit through G1 (Fig. 5). The incomplete effect of this mutant may be due to residual, integrin-induced binding of Crk to paxillin (Schaller and Parsons, 1995). In accordance with this hypothesis, dominant-negative Crk suppressed entry into S phase as effectively as the kinase dead version of CD2-FAK. Finally, cell cycle progression was also suppressed by dominant-negative versions of M KK4 and Jun (Fig. 5). These findings suggest that integrin-mediated activation of the FAK-JNK pathway is necessary for progression through the G1 phase of the cell cycle.

Discussion

Although the details of FAK's interaction with a number of cytoskeletal and signaling components are known, the biological function of this kinase is incompletely understood. Our results suggest that FAK mediates activation of JNK and c-jun in response to integrin ligation, and by doing so, regulates progression through the G1 phase of the cell cycle.

What is the mechanism by which FAK activates JNK? Upon activation, FAK undergoes autophosphorylation at tyrosine 397 and combines with the SH2 domain of Src or Fyn (Parsons, 1996). The most prominent substrates of the FAK/Src complex are the docking adaptor proteins p130CAS and paxillin (Schaller and Parsons, 1995; Richmond and Parsons, 1996; Vouici et al., 1996). Both contain tyrosine phosphorylation sites conforming to the consensus for binding to the adaptor protein Crk. However, while paxillin has only two such sites and does not appear to associate efficiently with Crk in response to integrin ligation (Schaller and Parsons, 1995), p130CAS contains nine Crk-binding motifs and associates well with Crk in cells adhering to fibronectin (Vuori et al., 1996). Our results indicate that the expression of dominant-negative versions of FAK, Src, p130CAS, and Crk suppress the activation of JNK by integrins. Together with complementary results of a recent study (Dolfi et al., 1998), these findings provide evidence that integrin-mediated activation of JNK requires the association of FAK with Src (or Fyn) and p130CAS, and the recruitment of Crk. It is unlikely that the coupling of Ras to Rac mediated by PI-3K (Rodriguez-Viciana et al., 1994; Nobes et al., 1995; Lippel et al., 1996) contributes in a significant manner to the activation of JNK by integrins because a dominant-negative form of Ras and specific inhibitors of PI-3K did not interfere with activation of this pathway. Thus, it appears that the β1 and αv integrins activate JNK and ERK via two separate pathways (see Fig. 6 for a model). By contrast, the α6β4 integrin, which is presumably unable to activate FAK because it does not contain the sequences required for its recruitment, is coupled to JNK signaling via the Ras-PI-3K-Rac pathway (Mainiero et al., 1997).

The mechanism by which Crk activates JNK in response to integrin ligation remains to be examined. Crk is known to interact via one of its SH3 domains with the exchange factor C3G (Tanaka et al., 1994) and previous studies have indicated that C3G is required for activation of JNK by the viral oncoprotein v-Crk (Tanaka et al., 1997). Interestingly, the activation of JNK by v-Crk and C3G appears to require the sequential action of the mixed lineage kinases M KK3 and D LK, but not the activity of Rho family GTPases or PAK proteins (Tanaka and H anchusa, 1998). In addition, or instead, Crk may activate Rac, and thereby JNK, by promoting the association of C3G with D OCK 180 or mSOS (Dolfi et al., 1998).

The identity of genes regulated by JNK is largely unknown, but they must include genes important for cell proliferation. The evidence for this is several fold: first, deregulated expression of c-jun or its mutated viral version v-jun is sufficient to cause neoplastic transformation of primary avian and mammalian fibroblasts (Vogt, 1994); second, primary fibroblasts derived from c-jun−/− mice display a severe proliferation defect (Johnson et al., 1993); and third, several oncoproteins, including v-Src, activated Ras, v-Crk, Bcr-A bl, and Met, potently activate JNK and there is evidence to suggest that this activation is required to

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Figure 5. Integrin-mediated activation of JNK is required for progression through the G1 phase of the cell cycle. NIH-3T3 fibroblasts were transiently transfected with 1 μg of vector encoding β-galactosidase alone or in combination with the indicated amounts of plasmids encoding CD2-FAK, CD2-FAK K454R, GST-CAS (wild-type), GST-SD-CAS (substrate region deleted), v-Crk, SH3-Δ-Crk, GST-Wt M KK4 (wild-type), GST-Δn M KK4, Δn-Jun (Δ3-122), or Δn-RAS (N17). After synchronization in G0 by serum deprivation, the cells transfected only with the β-galactosidase plasmid were plated on coverslips coated with poly-L-lysine (PL, white bar) or fibronectin (Fn, black bar), while those cotransfected with vectors encoding wild-type and constitutively active proteins (stippled bars) or corresponding dominant-negative versions (gray bars) were plated only on fibronectin. After 16 h of incubation in defined medium supplemented with PDGF and BrdU, the cells were fixed and stained with anti-BrdU mAb. The number of transfected cells entering S phase was evaluated as described in Materials and Methods. Untransfected cells or those cotransfected with vectors encoding wild-type and constitutively active proteins (stippled bars) or corresponding dominant-negative versions (gray bars) were plated only on fibronectin (Vuori et al., 1996). Our results indicate that the expression of dominant-negative versions of FAK, Src, p130CAS, and Crk suppress the activation of JNK by integrins. Together with complementary results of a recent study (Dolfi et al., 1998), these findings provide evidence that integrin-mediated activation of JNK requires the association of FAK with Src (or Fyn) and p130CAS, and the recruitment of Crk. It is unlikely that the coupling of Ras to Rac mediated by PI-3K (Rodriguez-Viciana et al., 1994; Nobes et al., 1995; Lippel et al., 1996) contributes in a significant manner to the activation of JNK by integrins because a dominant-negative form of Ras and specific inhibitors of PI-3K did not interfere with activation of this pathway. Thus, it appears that the β1 and αv integrins activate JNK and ERK via two separate pathways (see Fig. 6 for a model). By contrast, the α6β4 integrin, which is presumably unable to activate FAK because it does not contain the sequences required for its recruitment, is coupled to JNK signaling via the Ras-PI-3K-Rac pathway (Mainiero et al., 1997).

The mechanism by which Crk activates JNK in response to integrin ligation remains to be examined. Crk is known to interact via one of its SH3 domains with the exchange factor C3G (Tanaka et al., 1994) and previous studies have indicated that C3G is required for activation of JNK by the viral oncoprotein v-Crk (Tanaka et al., 1997). Interestingly, the activation of JNK by v-Crk and C3G appears to require the sequential action of the mixed lineage kinases M KK3 and D LK, but not the activity of Rho family GTPases or PAK proteins (Tanaka and H anchusa, 1998). In addition, or instead, Crk may activate Rac, and thereby JNK, by promoting the association of C3G with D OCK 180 or mSOS (Dolfi et al., 1998).

The identity of genes regulated by JNK is largely unknown, but they must include genes important for cell proliferation. The evidence for this is several fold: first, deregulated expression of c-jun or its mutated viral version v-jun is sufficient to cause neoplastic transformation of primary avian and mammalian fibroblasts (Vogt, 1994); second, primary fibroblasts derived from c-jun−/− mice display a severe proliferation defect (Johnson et al., 1993); and third, several oncoproteins, including v-Src, activated Ras, v-Crk, Bcr-A bl, and Met, potently activate JNK and there is evidence to suggest that this activation is required to
cause neoplastic transformation (Derijard et al., 1994; Minden et al., 1995; Raitano et al., 1995; Johnson et al., 1995; Romer, 1996). Although additional mechanisms cannot be excluded, our observation that dominant-negative forms of FAK, FRNK and FAK-Y397F, inhibit ERK activation to a much lesser degree, but interfere with cell proliferation nonetheless (Zhao et al., 1998; see also Gillmore and Romer, 1996). It is possible that the lack of p53 or presence of middle T antigen bypasses the requirement for FAK during cell proliferation. In addition, it recently has been shown that FAK-"" cells have elevated levels of PYK-2, which may compensate, at least in part, for the lack of FAK (Sieg et al., 1998).

The mitogenic signaling pathway linking integrins to JNK is likely to be deregulated in, and to contribute to, the transformation of at least some neoplastic cells. Previous studies have provided evidence that FAK is overexpressed in invasive carcinomas (Owens et al., 1995) and that the constitutively active CD2-FAK induces anchorage-independent growth in MDCK cells (Frisch et al., 1996b). In addition, the viral version of Src is a potent oncogene capable of transforming a variety of cell types, and there is strong genetic evidence that p130CAS is a necessary substrate of v-Src-induced transformation (Honda et al., 1998). In accordance with these findings, we have observed that CD2-FAK and p130CAS cooperate with activated Raf to induce anchorage-independent growth in NIH-S3T3 cells (F. Liu and F. G. Giancotti, unpublished results). Finally, v-Crk and v-Jun are potent oncogenes (Mayer et al., 1988; Vogt, 1994). Taken together, these observations suggest that the FAK-JNK pathway can contribute to neoplastic transformation.

Figure 6. Model of anchorage-dependent cell growth. Shc-linked integrins and growth factors receptors cooperate to activate the Ras-ERK cascade and promote the transcription of c-Fos. All β1 and αv integrins stimulate the FAK-JNK pathway in the absence of a significant input from growth factor receptors and induce transcription of c-Jun, as well as activation of newly formed Fos/Jun dimers.

to localize to focal adhesions, interferes with both fibronectin-induced activation of ERK and progression through the cell cycle (Zhao et al., 1998). On the basis of these results, it has been argued that FAK regulates cell proliferation by stimulating ERK. However, an alternative explanation is that this truncated form of FAK acts as a cytoplasmic sink for all Src-family kinases and thus disrupts not only FAK, but also Shc signaling. In accordance with this hypothesis, two more specific dominant-negative forms of FAK, FRNK and FAK-Y397F, inhibit ERK activation to a much lesser degree, but interfere with cell proliferation nonetheless (Zhao et al., 1998; see also Gilmore and Romer, 1996). It is possible that the lack of p53 or presence of middle T antigen bypasses the requirement for FAK during cell proliferation. In addition, it recently has been shown that FAK-"" cells have elevated levels of PYK-2, which may compensate, at least in part, for the lack of FAK (Sieg et al., 1998).

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Our present results imply that FAK, which appears to be activated by all β1 and αv integrins, is required during G1 progression because of its ability to activate JNK. Recently, it has been shown that a mutant form of FAK, which is truncated at the COOH terminus and thus unable to localize to focal adhesions, interferes with both fibronectin-induced activation of ERK and progression through the cell cycle (Zhao et al., 1998). On the basis of these results, it has been argued that FAK regulates cell proliferation by stimulating ERK. However, an alternative explanation is that this truncated form of FAK acts as a cytoplasmic sink for all Src-family kinases and thus disrupts not only FAK, but also Shc signaling. In accordance with this hypothesis, two more specific dominant-negative forms of FAK, FRNK and FAK-Y397F, inhibit ERK activation to a much lesser degree, but interfere with cell proliferation nonetheless (Zhao et al., 1998; see also Gilmore and Romer, 1996). It is possible that the lack of p53 or presence of middle T antigen bypasses the requirement for FAK during cell proliferation. In addition, it recently has been shown that FAK-"" cells have elevated levels of PYK-2, which may compensate, at least in part, for the lack of FAK (Sieg et al., 1998).

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1998; Pozzi et al., 1998). These data suggest that integrin-mediated Shc signaling is necessary for cell cycle progression.

The results of this and previous studies support the model that integrins control cell cycle progression primarily by regulating immediate early gene expression (Fig. 6). While Shc-linked integrins and growth factor receptors cooperate to activate the Ras-ERK cascade and promote SR E-dependent transcription of c-Fos, all β1 and αv integrins appear to be able to stimulate the FAK–JNK pathway in the absence of a significant contribution from growth factor receptors. It is likely that, in response to integrin ligation, JNK not only acts on preexisting Jun/ATF2 dimers, thereby promoting the CRE-dependent transcription of c-jun, but also activates the Fos/Jun dimers formed in response to the coordinated action of both integrins and growth factor receptors. The existence of a signaling pathway activated by all integrins within a signaling network coordinate regulated by a subset of integrins and growth factor receptors ensures that the control of cell proliferation exerted by the extracellular matrix is both stringent and integrin-specific.

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Regulation of JNK and Cell Cycle Progression by FAK

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The adaptor protein Shc couples a class of integrins to the control of cell cycle and cell survival. In this study, we demonstrate that FAK, a non-receptor tyrosine kinase associated with integrins, can modulate Shc levels and phosphorylation in Swiss 3T3 cells. This effect is mediated by the activation of JNK, a mitogen-activated protein kinase that is involved in cell proliferation and apoptosis. Our results provide a new link between integrins and the mitogen-activated protein kinase pathway, and suggest that FAK plays a role in the regulation of cell cycle progression and cell survival.

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