

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

Maja Oktay,* Kishore K. Wary,* Michael Dans,* Raymond B. Birge,[‡] and Filippo G. Giancotti*

*Laboratory of Cell Adhesion and Signaling, Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York 10021; and [‡]Laboratory of Molecular Oncology, The Rockefeller University, New York 10021

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 and p130^{CAS}, the phosphorylation of p130^{CAS}, and

subsequently, the recruitment of Crk. Ras 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 FAK 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 than is currently available.

Integrins activate common, as well as subgroup-specific, signaling pathways. A subset of integrins, which includes

α 1 β 1, α 5 β 1, α v β 3, and α 6 β 4, 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 β 4 when this integrin undergoes tyrosine phosphorylation (Mainiero et al., 1995; 1997). In contrast, the recruitment of Shc by β 1 and α v 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 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).

1. Abbreviations used in this paper: ATF2, activating transcription factor 2; BrdU, 5-bromodeoxyuridine; CS, calf serum; CMV, cytomegalovirus; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GST, glutathione S-transferase; HUVECs, human umbilical vein endothelial cells; JNK, Jun NH₂-terminal kinase; PDGF, platelet-derived growth factor; SD, substrate region deleted; SFM, serum-free medium; SRE, serum response element; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA-responsive element.

Maja Oktay's present address is Department of Pathology, Yale University School of Medicine, New Haven, CT 06520.

Address correspondence to Filippo G. Giancotti, Memorial Sloan-Kettering Cancer Center, Box 216, 1275 York Avenue, New York, NY 10021. Tel.: (212) 639-7333. Fax: (212) 794-6236. E-mail: f-giancotti@ski.mskcc.org

Certain integrins appear to associate preferentially with specific growth factor receptors and contribute to their activation (Miyamoto et al., 1996; Schneller et al., 1997; Moro et al., 1998; Soldi et al., 1999). For example, $\alpha\text{v}\beta\text{3}$ combines with the platelet-derived growth factor (PDGF) receptor. Hence, fibroblasts show enhanced proliferation in response to PDGF when attaching to the $\alpha\text{v}\beta\text{3}$ 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). Activated 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 FAK-associated proteins, including p130^{CAS} and paxillin, which contain multiple docking sites for the adaptor proteins Crk and Nck (Schaller and Parsons, 1995; Richardson and Parsons, 1996; Vuori 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., 1997) and potentially other mechanisms (Chen et al., 1996; King et al., 1997; Lin et al., 1997; Renshaw et al., 1997). Although previous studies have provided direct evidence for a role of FAK in cell migration (Ilic et al., 1995; Fincham and Frame, 1998; Cary et al., 1998; Klemke et al., 1998) and protection from apoptotic cell death (Frisch et al., 1996b; Ilic et al., 1998), it is unclear whether FAK also regulates cell proliferation, and if so, by what mechanism.

Integrin-mediated adhesion activates not only ERK, but also Jun NH₂-terminal kinase (JNK; Miyamoto et al., 1995; Mainiero et al., 1997; MacKenna et al., 1998). JNK is the final element of a mitogen-activated protein kinase (MAPK) cascade known to be activated by stress stimuli, such as UV radiation, hyperosmolar conditions, and inflammatory cytokines (Ip and Davis, 1998). Upon activation, JNK enters the nucleus, and phosphorylates and activates the transcription factors c-Jun and activating transcription factor 2 (ATF2), thereby regulating AP-1-dependent transcription (Karin et al., 1997). Because there is evidence that c-Jun is required for cell proliferation (Riabowol et al., 1992; Johnson et al., 1993), we sought to examine the mechanism by which integrins activate JNK and test the hypothesis that activation of this signaling pathway contributes to the control of cell cycle progression.

Our results indicate that integrins cause a significant and protracted activation of JNK, while growth factors appear to be unable to do so. By using various dominant-negative signaling molecules, we provide evidence that the activation of JNK by integrins is mediated by FAK and is necessary for cell cycle progression.

Materials and Methods

Antibodies and Extracellular Matrix Proteins

The mAb M2 to FLAG tag was purchased from Eastman-Kodak and the anti-CD2 mAb RPA-2.10 from PharMingen. The anti-H-Ras mAb

R02120 (clone 18) and anti-p130^{CAS} mAb P27820 (clone 21) were from Transduction Laboratories. The origin and specificity of the affinity-purified rabbit antibodies to ERK2, phospho-ERK, Src, and GST and of the anti-5-bromodeoxyuridine (anti-BrdU) mAb were described previously (Wary et al., 1996; 1998). The mAb 3C2 reacting with the gag portion of v-Crk was also described previously (Potts et al., 1987). Human fibronectin was from GIBCO BRL and poly-L-lysine from Sigma Chemical Co.

Cell Lines, Constructs, and Transfections

293 human embryonic kidney cells were cultured in DME 10% FCS on gelatin-coated plates. NIH-3T3 mouse fibroblasts were cultured in DME 10% calf serum (CS). Fibroblasts from Src^{-/-} and Fyn^{-/-} embryos were obtained from Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA) and cultured in DME 10% CS. Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics and cultured on gelatin-coated dishes in human endothelial serum-free medium (SFM; GIBCO BRL) supplemented with 20% FCS (GIBCO BRL), 10 ng/ml EGF, 20 ng/ml bFGF, and 1 $\mu\text{g/ml}$ heparin (all from Intergen).

The reporter plasmid pcoll TRE-tk-Luc, in which the expression of luciferase is driven by a single copy of the collagen gene 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) linked to the Hepes simplex virus thymidine kinase minimal promoter, was described previously (Galien et al., 1994). Vectors encoding the FLAG-tagged version of JNK 1, glutathione S-transferase (GST)-Jun, dominant-negative Ras (N17), and HA-tagged β -galactosidase were described previously (Mainiero et al., 1997). The CMV promoter-based pCDM8 vectors encoding CD2-FAK (wild-type), CD2-FAK K454R (kinase dead), and CD2-FAK Y397F were described previously (Chan et al., 1994). A kinase dead version of chicken c-Src was obtained from Sara Courtneidge (EMBL, Heidelberg, Germany) and subcloned in the cytomegalovirus (CMV) promoter-based vector pRK5. The pEBG vectors expressing GST-tagged MKK4 (wild-type) and MKK4 K129R (kinase dead) from the human elongation factor 1- α promoter were described previously (Su et al., 1997). The Moloney Leukemia Virus (MLV)-LTR based pMEXneo vectors encoding v-Crk (wild-type), v-Crk R273N (SH2 mutant), and v-Crk D386DRHAD (SH3 insertional mutant) were described previously (Altun-Gultekin et al., 1998). The pEBG vectors encoding GST-tagged rat p130CAS (short form) and its substrate region deleted form (SD, Δ 213-514) were also described (Mayer et al., 1995). The TAM-67 transactivation domain mutant form of c-Jun (Jun Δ 3-122) was expressed from pCMV and previously characterized (Brown et al., 1993). The dominant-negative version of paxillin used in this study carries three phenylalanine permutations at tyrosine 31, 118, and 187 and is unable to bind to Crk. The MLV-LTR-based expression vector p Δ raf-22w encodes an activated version of c-Raf 1 lacking an NH₂-terminal segment of 305 amino acids (Stanton et al., 1989). The vector encoding activated Ras, pDCR-Ha-ras (G12V), was kindly provided by John Westwick (Signal Pharmaceuticals).

NIH-3T3 cells were transiently transfected with Lipofectamine according to the manufacturer's instructions (GIBCO BRL). 293 cells were plated at 6×10^6 per 15-cm diam dish for 8 h and then transfected overnight with various amounts of plasmid by the calcium phosphate method. All transfections were normalized to the same total amount of DNA with empty vector. Cells were allowed to recover for 12 h before growth factor starvation.

Biochemical Methods

To monitor the activation of JNK and ERK during G1, HUVECs were synchronized in G0 by a 24 h incubation in human endothelial SFM containing 0.2% FCS. They were then detached with 0.02% EDTA, collected in SFM containing 0.2% heat-inactivated BSA, washed in the same medium, and kept in suspension at a density of $10^6/\text{ml}$ for 15 min at room temperature to recover. Aliquots consisting of 1.5×10^7 cells were plated on 15-cm diam dishes coated with 20 $\mu\text{g/ml}$ fibronectin and postcoated with 0.2% heat-inactivated BSA in SFM supplemented with ITS+1 (Sigma Chemical Co.), EGF (10 ng/ml), bFGF (20 ng/ml), and heparin (1 $\mu\text{g/ml}$) for the indicated times. Cells from an identical aliquot were pelleted and lysed in suspension as a control. Before biochemical analysis, NIH-3T3 cells were serum starved for 18 h and 293 cells for 24 h in DME containing 0.2% CS or FCS, respectively. After detachment with 0.02% EDTA, cells were collected in DME containing 0.2% heat-inactivated BSA, washed in the same medium, and kept in suspension at a density of $10^6/\text{ml}$ for 15 min at room temperature to recover. Aliquots consisting of 1.5×10^7 cells were plated on 15-cm diam dishes, coated with 20 $\mu\text{g/ml}$ fibronectin and postcoated with 0.2% heat-inactivated BSA for the indicated times. Cells from an identical aliquot were pelleted and lysed in sus-

pension as a control. NIH-3T3 cells were treated with growth factors as indicated.

To analyze the activation of JNK, cells were extracted for 30 min on ice with 0.5 ml/dish of modified Triton lysis buffer (25 mM Hepes, pH 7.5, 300 mM NaCl, 0.1% Triton X-100, 0.2 mM EDTA, 20 mM β -glycerophosphate, 1.5 mM $MgCl_2$, and 0.5 mM DTT) containing phosphatase and protease inhibitors. Aliquots containing 0.5 mg of total proteins were brought to 0.8 ml with modified Triton lysis buffer and diluted to 1.2 ml with HBB buffer (20 mM Hepes, pH 7.7, 50 mM NaCl, 0.05% Triton X-100, 0.1 mM EDTA, 20 mM β -glycerophosphate, 2.5 mM $MgCl_2$, and 10 mM DTT) supplemented with phosphatase and protease inhibitors. Endogenous JNK was precipitated with 5 μ g of GST-Jun fusion protein coupled to glutathione agarose beads (Hibi et al., 1993). The beads were washed four times in HBB buffer, twice in kinase buffer (20 mM Hepes pH 7.5, 20 mM β -glycerophosphate, 10 mM $MgCl_2$, and 10 mM DTT), and incubated with 35 μ l of kinase buffer containing 10 μ Ci of γ [32 P]ATP (ICN) and 20 μ M cold ATP. Recombinant FLAG-tagged JNK 1 was immunoprecipitated with the anti-Flag mAb M2. The beads were washed as above and incubated with 35 μ l of kinase buffer containing 5 μ g of GST-Jun, 10 μ Ci of γ [32 P]ATP, and 20 μ M cold ATP. After 30 min of incubation at 30°C, the samples were boiled in sample buffer and separated by SDS-PAGE.

Immunoprecipitation and immunoblotting were performed essentially as described previously (Mainiero et al., 1995). Secondary reagents for immunoblotting included peroxidase-conjugated protein A and affinity-purified rabbit anti-goat IgGs.

To measure transcription from the collagen promoter TRE, NIH-3T3 cells were transiently transfected with the reporter plasmid pcoll TRE-tk-Luc. After 24 h of growth factor starvation, the cells were detached, kept in suspension for 30 min, and then solubilized or plated on dishes coated with 20 μ g/ml fibronectin for the indicated times in the absence or presence of 20 ng/ml PDGF. Luciferase activity in cell lysates was estimated as described previously (Mainiero et al., 1997).

Analysis of Cell Cycle Progression

NIH-3T3 cells were transiently transfected with vector encoding β -galactosidase in combination with various doses of the indicated constructs. The cells were allowed to recover in complete medium, synchronized in G0 by growth factor deprivation, and plated at low density on microtiter wells coated with 10 μ g/ml poly-L-lysine or 20 μ g/ml fibronectin in defined medium (DME supplemented with 6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, 0.625 ng/ml selenous acid, 1.25 mg/ml BSA, and 5.35 μ g/ml linoleic acid) supplemented with 20 ng/ml PDGF and 10 μ M BrdU. After 16 h, the cells were fixed and stained with X-gal followed by anti-BrdU mAb and AP-conjugated anti-mouse IgGs. The percentage of X-gal positive cells that had incorporated BrdU was evaluated microscopically after light counterstaining with hematoxylin.

Results

Integrins Activate JNK and TRE-dependent Transcription

Preliminary experiments were conducted to examine if JNK was physiologically activated in primary cells progressing through the G1 phase of the cell cycle. HUVECs were synchronized in G0 by growth factor deprivation and detached to simulate the cell rounding physiologically occurring at mitosis. They were then plated on fibronectin in the presence of growth factors to allow entry into and progression through G1.

In vitro kinase assays with the NH₂-terminal fragment of c-Jun as a substrate showed that JNK is activated to a 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

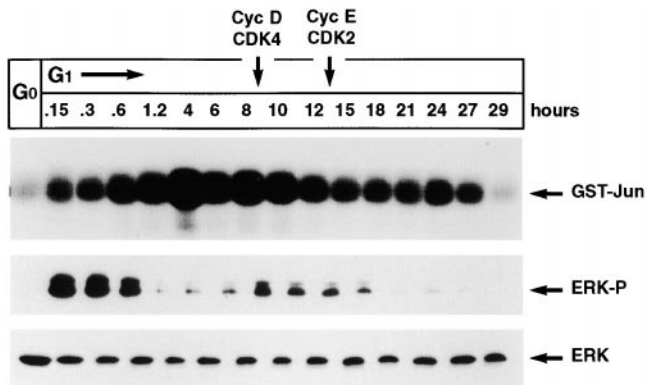


Figure 1. Activation of JNK and ERK during the G1 phase of the cell cycle in primary cells. HUVECs were synchronized in G0, detached, and then were lysed (G0) or replated on fibronectin for the indicated times in SFM supplemented with growth factors and then lysed. JNK was precipitated from extracts containing equal amounts of total proteins by using GST-Jun beads and subjected to an in vitro kinase assay. The position of phosphorylated GST-Jun is indicated. Total lysates containing the same amount of proteins were subjected to immunoblotting with anti-phospho-ERK (ERK-P). The blot was stripped and reprobbed with antibodies to ERK 1 to demonstrate equal loading (ERK). Peak activation of Cyclin D/CDK 4 and Cyclin E/CDK 2 occurs at the indicated times in HUVECs under these experimental conditions (F.G. Giancotti, unpublished results).

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 (Kyriakis et al., 1994; Minden 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 slowly and modestly over time in suspension, perhaps in response to the activation of a stress pathway, as observed by others (Frisch et al., 1996a; Khwaja and Downward, 1997). The activation of JNK caused by integrin ligation was comparable in intensity to that observed in cells treated with 5 to 10 ng/ml TNF- α (Fig. 2 B), a known acti-

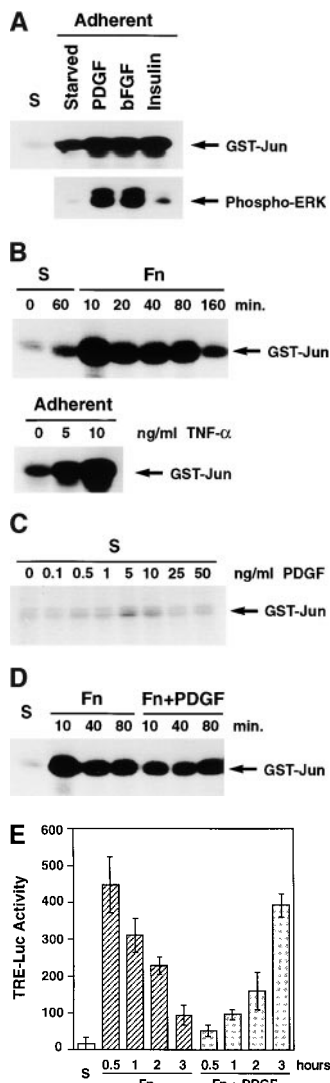


Figure 2. Adhesion to fibronectin induces activation of JNK and TRE-dependent transcription. (A) After serum starvation, NIH-3T3 cells were treated while adherent with 20 ng/ml of PDGF, 20 ng/ml bFGF, or 6.25 μ g/ml insulin for 10 min, or left untreated and either detached (S) or adherent (Starved). 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. (C) Suspended cells were 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. After 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).

vator of JNK (Kyriakis et al., 1994; Minden et al., 1994). Exposure to a wide range of PDGF concentrations caused little or no activation of JNK in suspended cells (Fig. 2 C). Whereas exposure to 1 μ g/ml lysophosphatidic acid (LPA), which is known to activate FAK (Chrzanowska-Wodnicka and Burridge, 1994), amplified the activation of JNK caused by integrin ligation by \sim 70% (data not shown), treatment with PDGF did not exert this effect. However, PDGF changed the time course of JNK activation in cells adhering to fibronectin. Specifically, while adhesion to fibronectin caused maximal stimulation of JNK in \sim 10 min, simultaneous exposure to PDGF significantly delayed the peak of activation of the kinase (Fig. 2 D).

This effect of PDGF may be related to its ability, when used at mitogenic concentrations as were used here, to transiently disrupt the cytoskeleton and thus delay integrin-mediated activation of FAK (Rankin and Rozengurt, 1994). Taken together, these observations indicate that JNK is activated by integrins, but only to a limited extent by PDGF, bFGF, and insulin. Exposure to growth factors may, however, contribute to sustain the activation of JNK caused by integrin ligation.

Phosphorylation of c-Jun by JNK is required for transcriptional activation of the dimeric transcription factor AP-1 and for the oncogenic cooperation between c-Jun and Ha-Ras (Smeal et al., 1991). To examine if the activation of JNK caused by integrin ligation could contribute to immediate early gene expression by promoting AP-1 dependent transcription from TRE, NIH-3T3 cells were transiently transfected with a vector encoding the luciferase gene under the transcriptional control of a TRE and plated on fibronectin in the presence or absence of PDGF. As shown in Fig. 2, D and E, adhesion to fibronectin promoted TRE-dependent transcription with kinetics that 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 TAM-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, p130^{CAS}, 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-FAK, a chimeric, membrane-anchored form of FAK that localizes efficiently to focal adhesions (Chan et al., 1994; Frisch et al., 1996b). We reasoned that membrane attachment would promote the interaction of these FAK mutants with focal adhesion components and thereby facilitate a dominant-negative effect.

As shown in Fig. 3, both CD2-FAK mutants exerted a dominant-negative effect on fibronectin-mediated activation of JNK, indicating that this process requires the kinase activity of FAK and its association with an Src family kinase. Accordingly, integrin-mediated activation of JNK was also suppressed by a kinase dead version of Src. In addition, we observed that the activation of JNK by integrins is partially defective in Src^{-/-} and in Fyn^{-/-} fibroblasts, in accordance with the notion that FAK can combine with both kinases (data not shown). Integrin-mediated activa-

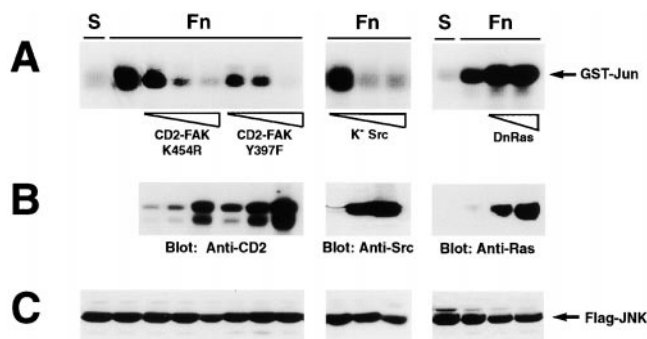


Figure 3. Role of the FAK/Src complex in integrin-mediated activation of JNK. 293 cells were transiently transfected with 3 μ g of vector encoding FLAG-tagged JNK alone or in combination with 3, 9, and 27 μ g of plasmids encoding CD2-FAK K454R (catalytically inactive), CD2-FAK Y397F (unable to bind to the SH2 domain of Src family kinases), and K⁻ Src (kinase dead), or with 9 and 27 μ g of vector encoding DnRas (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).

tion of JNK was also blocked by a kinase inactive version of MKK4, 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 μ M) (data not shown). These results indicate that the FAK/Src complex links integrins to MKK4 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 p130^{CAS} and paxillin, which bind to the FAK/Src complex and become heavily phosphorylated on tyrosine residues upon integrin engagement (Schaller and Parson, 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 p130^{CAS} carrying a deletion of the entire substrate region (SD-CAS; Fig. 4). In contrast, a mutant form of paxillin carrying phenylalanine substitutions at three tyrosine phosphorylation sites, including all the Crk binding sites, inhibited this event modestly and only when expressed at relatively high levels (data not shown). These results indicate that p130^{CAS} plays a major role, and paxillin perhaps a minor one, in integrin-mediated JNK activation.

9 out of 15 tyrosine phosphorylation sites within the substrate region of p130^{CAS} conform to the consensus motif for binding to the SH2 domain of Crk (Sakai et al., 1994). In addition to the SH2 domain, Crk contains either one or

two SH3 domains able to interact with downstream target-effectors (Mayer et al., 1988; Matsuda et al., 1992; ten Hoeve et al., 1993). Previous studies have indicated that the adaptor function of Crk is regulated positively by recruitment to the plasma membrane and negatively when tyrosine 222 becomes phosphorylated and associates intramolecularly with the SH2 domain (Mayer and Hanafusa, 1990; Feller et al., 1994).

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 p130^{CAS}, 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/p130^{CAS} 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

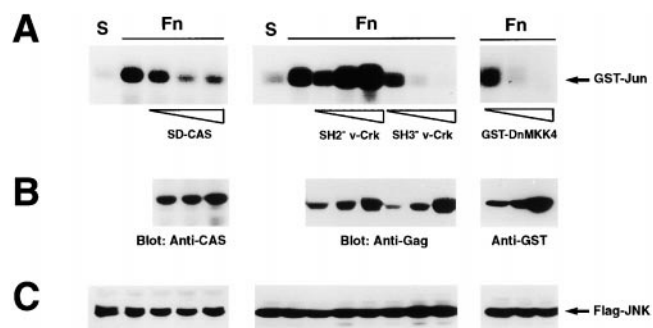


Figure 4. Role of p130^{CAS} and Crk in integrin-mediated activation of JNK. 293 cells were transiently transfected with 3 μ g of vector encoding FLAG-tagged JNK alone or in combination with 3.75, 7.5, and 15 μ g of plasmid encoding a mutant form of p130^{CAS} carrying a deletion of the entire substrate region (SD-CAS), or with 3, 9, and 27 μ g of plasmids encoding SH2⁻ v-Crk (SH2 mutant), SH3⁻ v-Crk (SH3 mutant), and GST-DnMKK4 (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 p130^{CAS}, 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 p130^{CAS} 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 MKK4 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

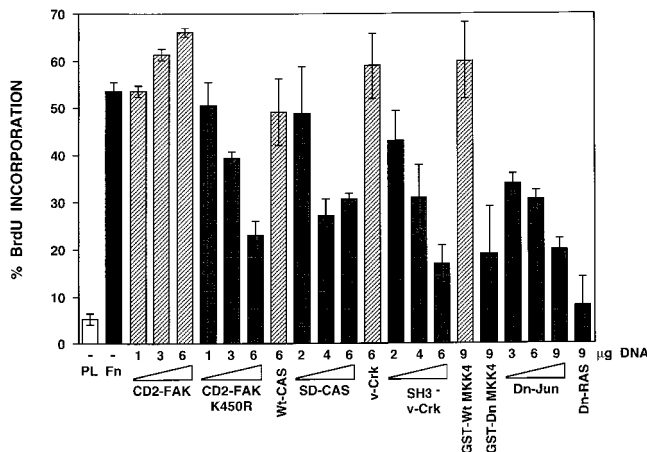


Figure 5. Integrin-mediated activation of JNK is required for progression through the G1 phase of the cell cycle. NIH-3T3 fibroblasts were transiently transfected with 1 μ g of vector encoding β -galactosidase alone or in combination with the indicated amounts of plasmids encoding CD2-FAK, CD2-FAK K454R, GST-CAS (wild-type), GST-SD-CAS (substrate region deleted), v-Crk, SH3⁻ v-Crk, GST-Wt MKK4 (wild-type), GST-Dn MKK4, Dn-Jun (Δ 3-122), or Dn-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. Under these conditions, none of the cells plated on fibronectin in the absence of PDGF enter into S phase. The diagram shows the mean value and standard deviation from triplicate samples.

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 p130^{CAS} and paxillin (Schaller and Parsons, 1995; Richardson and Parsons, 1996; Vuori 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), p130^{CAS} 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, p130^{CAS}, 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 p130^{CAS}, 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; Klippel 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 β 1 and α v integrins activate JNK and ERK via two separate pathways (see Fig. 6 for a model). By contrast, the α 6 β 4 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 MLK3 and DLK, but not the activity of Rho family GTPases or PAK proteins (Tanaka and Hanafusa, 1998). In addition, or instead, Crk may activate Rac, and thereby JNK, by promoting the association of C3G with DOCK 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-Abl, and Met, potentially activate JNK and there is evidence to suggest that this activation is required to

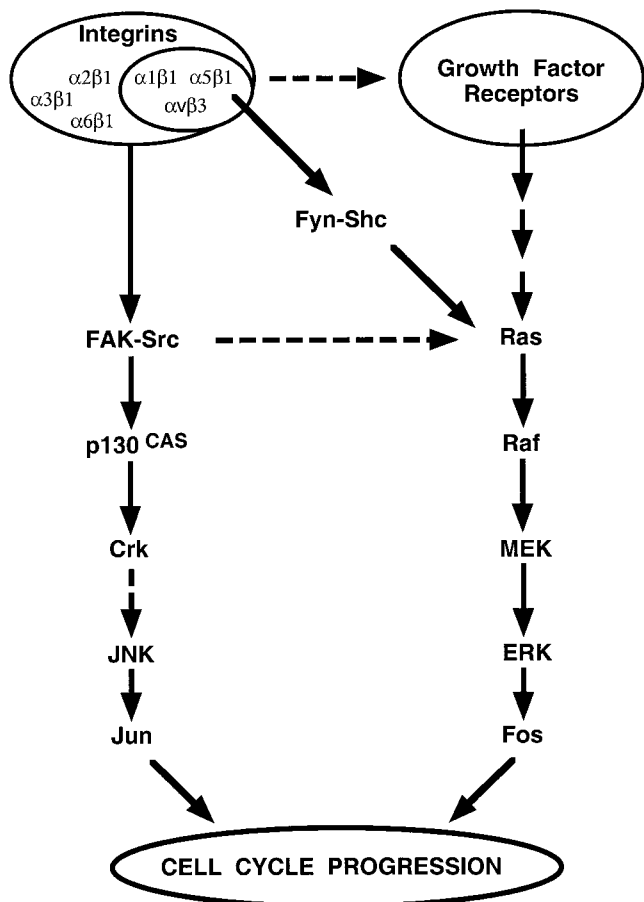


Figure 6. Model of anchorage-dependent cell growth. Shc-linked integrins and growth factors receptors cooperate to activate the Ras-ERK cascade and promote the transcription of c-Fos. All $\beta 1$ and αv integrins stimulate the FAK-JNK pathway in the absence of a significant input from growth factor receptors and induce transcription of c-Jun, as well as activation of newly formed Fos/Jun dimers.

cause neoplastic transformation (Derijard et al., 1994; Minden et al., 1995; Raitano et al., 1995; Johnson et al., 1996; Rodrigues et al., 1997; Tanaka et al., 1997).

Despite the clear requirement for c-Jun transcriptional activity in cell proliferation, it has been difficult to identify a physiological, nonstress stimulus for JNK consistent with its role in the regulation of AP-1 transcription. With the notable exception of EGF, mitogenic neuropeptides, and muscarinic receptor ligands, which indeed activate FAK or the related kinase PYK-2 (Zachary et al., 1992; Coso et al., 1995; Tokiwa et al., 1996; Yu et al., 1996; Cadwallader et al., 1997; Higashita et al., 1997; Logan et al., 1997; Slack, 1998), most growth factors cause a relatively modest activation of JNK (Kyriakis et al., 1994; Minden et al., 1994). Our results indicating that integrin ligation causes a significant activation of JNK and TRE-dependent transcription provide a physiological stimulus for JNK signaling that is consistent with its role in the control of cell proliferation.

Our present results imply that FAK, which appears to be activated by all $\beta 1$ 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). Although additional mechanisms cannot be excluded, our observation that dominant-negative forms of FAK, p130^{CAS}, Crk, MKK4, and Jun all inhibit entry into the S phase provides evidence that FAK regulates cell proliferation by activating JNK.

If FAK is required for cell cycle progression, why then do the FAK^{-/-} cells not display an obvious proliferation defect (Ilic et al., 1995)? There are two potential explanations. First, it is now apparent that the FAK^{-/-} cells originally examined by Ilic and colleagues also lack a functional form of the cell cycle regulator p53 (Furuta et al., 1995). Some of the cell lines generated more recently do have wild-type p53, but are transformed by the polyoma middle T antigen (Ilic et al., 1998). 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 p130^{CAS} 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 p130^{CAS} cooperate with activated Raf to induce anchorage-independent growth in NIH-3T3 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.

Previous studies have provided evidence that $\alpha 6\beta 4$ and a subset of $\beta 1$ and αv integrins activate the Ras-ERK signaling cascade by recruiting Shc (Mainiero et al., 1995; 1997; Wary et al., 1996; 1998). Mitogens and Shc-linked integrins synergize to promote transcription from the Fos SRE. Accordingly, ligation of integrins linked to Shc enables these cells to progress through the G1 phase of the cell cycle in response to mitogens, whereas adhesion mediated by other integrins results in growth arrest, despite the presence of mitogens. These mechanisms also appear to operate in vivo, as mice lacking the integrin $\alpha 1$ subunit or the cytoplasmic domain of $\beta 4$ display cell cycle defects consistent with the lack of Shc signaling (Murgia et al.,

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 SRE-dependent transcription of c-Fos, all $\beta 1$ 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 and ATF2/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 coordinately 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.

We are indebted to B. Binetruy, S. Courtneidge, E. Skolnik, K. Vuori, and J. Westwick for constructs. We thank P. Soriano for the Src^{-/-} and Fyn^{-/-} 3T3 fibroblasts. We are grateful to members of the Giancotti laboratory for discussions and comments on the manuscript.

This work was supported by National Institutes of Health grants R01 CA78901 (to F.G. Giancotti) and P30 CA08748 (to the Memorial Sloan-Kettering Cancer Center). F.G. Giancotti is an Established Investigator of the American Heart Association.

Received for publication 17 February 1999 and in revised form 25 May 1999.

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