Dual Stimulation of Ras/Mitogen-activated Protein Kinase and RhoA by Cell Adhesion to Fibronectin Supports Growth Factor–stimulated Cell Cycle Progression

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Abstract. In cellular transformation, activated forms of the small GTPases Ras and RhoA can cooperate to drive cells through the G1 phase of the cell cycle. Here, we show that a similar but substrate-regulated mechanism is involved in the anchorage-dependent proliferation of untransformed NIH-3T3 cells. Among several extracellular matrix components tested, only fibronectin supported growth factor–induced, E2F-dependent S phase entry. Although all substrates supported the mitogen-activated protein kinase (MAPK) response to growth factors, RhoA activity was specifically enhanced on fibronectin. Moreover, induction of cyclin D1 and suppression of $p21^{Cip/Waf}$ occurred specifically, in a Rho-dependent fashion, in cells attached to fibronectin. This ability of fibronectin to stimulate both Ras/MAPK- and RhoA-dependent signaling can explain its potent cooperation with growth factors in the stimulation of cell cycle progression.

Key words: fibronectin • cell adhesion • G1 cell cycle • integrin • Rho

Introduction

In tissues, cells are surrounded by a network of various proteins and polysaccharides that constitutes the extracellular matrix (ECM).[1] ECM components dramatically affect proliferation and differentiation of cells in culture (Roskelley et al., 1995; Assoian, 1997), and the ability of cells to grow relatively independently from cell–matrix interactions is a key event in malignant transformation (Freedman and Shin, 1974). Many cell–matrix interactions are mediated by the integrin family of cell adhesion receptors (Hynes, 1992; Sonnenberg, 1993). Integrin-mediated adhesion triggers cell spreading, rearrangement of the actin cytoskeleton, and organization of integrins and associated proteins into cell–matrix adhesions (Burridge and Chrzanowska-Wodnicka, 1996). These processes are regulated by small GTPases of the Rho family, and they ultimately affect multiple signaling pathways (Howe et al., 1998; Giancotti and Ruoslahti, 1999; Yamada and Danen, 2000).

Control of growth by cell anchorage takes place in the G1 phase of the cell cycle (Assoian, 1997). Progression through G1 occurs via sequential activation of cyclin D–cdk4/6 and cyclin E–cdk2 kinase complexes, phosphorylation of the Rb family of pocket proteins resulting in the release of the E2F transcription factor, followed by the transcription of several genes whose products are required for the initiation of S phase (Beijersbergen and Bernards, 1996). Cell adhesion has been reported to control the transcription and translation of cyclin D1 and the levels and/or distribution of the $p21^{Cip/Waf}$ and $p27^{Kip1}$ inhibitors of the cyclin E–cdk2 complex. As a consequence, phosphorylation of the pocket proteins, availability of free E2F, and induction of cyclin A early in S phase are anchorage dependent (Schulze et al., 1995; Zhu et al., 1996; Bohmer et al., 1996; Carstens et al., 1996; Fang et al., 1996; Kang and Krauss, 1996; Kramer et al., 1996; Bottazzi et al., 1999).

Currently, the mechanisms by which cell adhesion modulates growth factor signaling cascades are incompletely understood. Mitogen-activated protein kinase (MAPK) and Rho family members are both essential for cell cycle progression through G1 (Pages et al., 1993; Olson et al., 1995), and integrins can collaborate with growth factors to activate both pathways (Renshaw et al., 1997; Price et al., 1998; Aplin and Juliano, 1999; Ren et al., 1999). In the present study we find that cell spreading in general, which acts in synergy with growth factor signaling to activate extracellular signal–regulated kinase (ERK)-type MAPK, is not sufficient to promote cell cycle progression in NIH-3T3 cells stimulated with growth factors in the absence of

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[1]*Abbreviations used in this paper:* ECM, extracellular matrix; ERK, extracellular signal–regulated kinase; GST, glutathione *S*-transferase; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase.

serum. Adhesion to fibronectin specifically stimulates RhoA activity and supports cyclin D1 induction and p21$^{Cip/Waf}$ suppression in a RhoA-dependent fashion, a mechanism that we find to be instrumental in anchorage-dependent G1 cell cycle progression.

Materials and Methods

Antibodies and Adhesive Ligands

Polyclonal antibody C-15 (Santa Cruz Biotechnology, Inc.) and monoclonal antibody G3-245 (BD PharMingen) were used for immunoprecipitation and Western blotting of Rb. Antibodies to cyclin D1 and p21$^{Cip/Waf}$ were from Upstate Biotechnology. Paxillin, ERK1, and ERK2 monoclonal antibodies used for immunofluorescence and Western blotting were from Transduction Laboratories. Monoclonal antibodies against RhoA (Santa Cruz Biotechnology, Inc.) and Rac1 (Upstate Biotechnology) were used for Western blotting, as were monoclonal antibody 9E10 against the Myc epitope (BAbCo) and a monoclonal antibody against the phosphorylated Thr202/Tyr204 ERK activation epitope (New England Biolabs, Inc.). Fibronectin was purified from human plasma as described previously (Miekka et al., 1982), and its purity was confirmed by SDS-PAGE (not shown). Laminin 1 was purchased from Collaborative Biomedical Products, collagen type 1 was from Collagen Corporation, and poly-L-lysine was from Sigma-Aldrich.

Plasmids and Retroviral Expression Constructs

Luciferase reporter constructs for cyclin A (cyclin A gene promoter region from −7300 to +11 relative to the major transcription start site) (Schulze et al., 1995), cyclin A with a mutated E2F-binding site (harboring a mutated E2F site at −37 to −33) (Schulze et al., 1995), cyclin D1 (promoter region from −973 to +139), and for p21$^{Cip/Waf}$ (2.4-kb p21 promoter region) were gifts from Berthold Henglein (Institut Curie, Paris, France). Rb(NPC−) cDNA encoding a pRb mutant lacking 14 phosphorylation sites, rendering it insensitive to inactivation by G1 cyclin–cdk complexes (Chew et al., 1998), was a gift from Sibylle Mittnacht (Institute for Cancer Research, London, UK). An expression plasmid for *Clostridium botulinum* C3 toxin pCEF/C3 was a gift from J. Silvio Gutkind (National Institutes of Health). The dominant inhibitory MAPK kinase (MEK) plasmid pMCL⊕HA/MEK K97M (Mansour et al., 1994) was provided by Natalie Ahn (University of Colorado, Boulder, CO). The mouse cyclin D1 and p21$^{Cip/Waf}$ probes were gifts from Charles Sherr (St. Jude Children's Research Hospital, Memphis, TN), Wade Harper (Baylor College of Medicine, Houston, TX), and Konrad Huppi (National Institutes of Health). The puromycin resistance plasmid pHA262pur was provided by Hein te Riele (The Netherlands Cancer Institute, Amsterdam). Supernatants from the Phoenix packaging cell line transfected with Myc epitope–tagged N19RhoA or N17Rac1 (Leeuwen et al., 1997) were a gift from John Collard (The Netherlands Cancer Institute, Amsterdam).

Cell Culture, cDNA Transfections, and Retroviral Transductions

NIH-3T3 cells obtained from the American Type Culture Collection were maintained in DME supplemented with 10% calf serum, penicillin, and streptomycin. All luciferase reporter lines were generated by cotransfection of the reporter constructs (6 μg each) with empty pcDNA vector (3 μg) using Lipofectamine (Life Technologies). Stable transfectants were selected with G418 (0.8 mg/ml; Life Technologies) for 2 wk, and single-cell clones were isolated by limiting dilution. For each reporter, three independent clones were used for initial characterization of the patterns of suppression and activation by serum starvation and stimulation with growth factors. One representative clone was used for further experiments. Transient transfections with pcDNA/Rb(NPC−) and pCEF/C3 were done by electroporation (170 V, 960 μFd; 20 μg of each of these plasmids) together with 10 μg pHA262pur, followed by selection in media containing puromycin (1 μg/ml; Sigma-Aldrich) for 3 d. Retroviral infections were performed by culturing 10^5 cells for 8 h with 1 ml cell-free Phoenix supernatant in the presence of 10 μg/ml DOTAP (Boehringer). Cells were then maintained overnight in fresh medium and subsequently selected in the presence of zeocin (0.2 mg/ml; Invitrogen) for 10 d. For transient transfections as well as retroviral infections, selected cells were maintained in the absence of the selection drug for 2 d before they were used in experiments.

Serum Starvation, Replating, and Growth Factor Stimulation

For synchronization in G0, subconfluent NIH-3T3 cultures were incubated for 24 h in DME without serum (see Fig. 1 for a schematic representation of the experimental procedure). Cells were then detached with 0.05% trypsin-EDTA and collected in DME containing 2% BSA and 0.5 mg/ml soybean trypsin inhibitor (Sigma-Aldrich), washed in DME, and finally collected in DME containing 0.2% BSA and counted. For suspension cultures, cells were replated on wells that had been coated for 1 h at room temperature with 1% agar in PBS and subsequently been incubated overnight in DME. For adherent cultures, cells were replated on wells that had been precoated overnight at 4°C with 10 μg/ml fibronectin, 20 μg/ml laminin, 20 μg/ml collagen or 100 μg/ml poly-L-lysine and subsequently been blocked with 2% heat-denatured BSA for 2 h at 37°C. After 16 h incubation on the various substrates, growth factors were added to a final concentration of 15 ng/ml bFGF (a gift from Gera Neufeld, Technion-Israel Institute of Technology, Haifa, Israel), 10 ng/ml EGF (Sigma-Aldrich), 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenous acid, 5.35 μg/ml linoleic acid (ITS1; Collaborative Biomedical Products), and 1 μg/ml heparin (Sigma-Aldrich).

Immunofluorescence Microscopy and Flow Cytometry

Serum-starved cells were plated in DME containing 0.2% BSA on coverslips precoated with substrates as described above. For detection of integrins, paxillin, and actin, the cells were fixed in 2% paraformaldehyde for 15 min and permeabilized in 0.2% Triton X-100 for 5 min. For detection of MAPK, cells were fixed in 70% methanol/30% acetone for 10 min on dry ice. For immunofluorescence staining, the coverslips were then blocked with 10% heat-inactivated calf serum for 1 h and incubated with primary antibodies (5 μg/ml) for 1 h at room temperature. After washing with PBS, cells were incubated with Texas red–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) or Oregon green–conjugated phalloidin (Molecular Probes) for 1 h at room temperature. Preparations were then washed in PBS, mounted in Vectashield (Vector Laboratories), and analyzed with a confocal Leica TCS NT microscope.

Luciferase Assays

Serum-starved cells were collected, plated at a subconfluent density of 10^4 cells per well in precoated 48-well plates, and stimulated with growth factors as described above. At the indicated time points, medium was aspirated from adherent cultures and suspended cells were collected with a microcentrifuge. Cells were then lysed in 50 μl Passive Lysis Buffer® (Promega) and assayed for luciferase activity (Luciferase Assay System; Promega). Results are presented as the mean ± SD of data from two or more independent experiments, each of which was done in triplicate.

Immunoprecipitation and Western Blotting

Serum-starved cells were collected, plated at a subconfluent density of 10^5 cells per well in precoated 6-well plates, and stimulated with growth factors as described above. For analysis of total cell lysates, cells were lysed at the indicated time points in boiling Laemmli sample buffer. For immunoprecipitation, cells were lysed in 750 μl modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% Triton X-100, 2 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF) and lysates were clarified by centrifugation at 14,000 *g* for 20 min at 4°C. Clarified lysates were then incubated overnight at 4°C with 2 μg/ml of the antibody. Immune complexes were collected with 10 μl GammaBind (Amersham Pharmacia Biotech) for 2 h at 4°C, washed three times with lysis buffer, and solubilized in Laemmli sample buffer. Total cell lysates or immunoprecipitates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and analyzed by Western blotting followed by enhanced chemiluminescence using the SuperSignal system (Pierce Chemical Co.).

Biochemical Assays for Activity of RhoA and Rac1

Activity assays were based on the fact that GTPase effector proteins bind their specific partner GTPases only when they are in the GTP-bound (active) form, and the assays were performed essentially as described previously (Sander et al., 1998; Ren et al., 1999). Serum-starved cells were collected, plated at a subconfluent density of 2×10^6 cells per precoated 15-cm dish, and stimulated with growth factors as described above. For

each indicated time point, the cells in two dishes were lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM sodium chloride, 1% NP-40, 10% glycerol, 2 mM magnesium chloride, 10 mM sodium fluoride, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF), and lysates were clarified by centrifugation at 14,000 *g* for 20 min at 4°C. A 5% aliquot was removed for determination of total quantities of the GTPase being analyzed. Clarified lysates were then incubated for 1 h at 4°C with glutathione *S*-transferase (GST) fusion proteins (provided by John Collard, The Netherlands Cancer Institute, Amsterdam) that had been precoupled to Sepharose-glutathione beads (Amersham Pharmacia Biotech), and bound complexes were washed three times in NP-40 lysis buffer. The RhoA activity assay used a GST fusion protein with the Rho-binding domain of the Rho effector protein Rhotekin. For the Rac1 activity assay, a GST fusion protein of the binding domain of the Rac effector protein PAK1b was used. The samples were analyzed by SDS-PAGE and Western blotting using RhoA and Rac1 antibodies to detect bound activated GTPases.

Northern Blotting

Serum-starved cells were collected, plated at a subconfluent density of 10^6 cells per precoated 10-cm dish, and stimulated with growth factors as described above. At the indicated time points, cells were lysed in 1 ml TRIzol (Life Technologies) and RNA was isolated according to the manufacturer's protocol. 5 μg RNA of each sample was denatured, mixed with RNA loading buffer, and resolved on a 1.5% MOPS/formaldehyde/agarose gel in MOPS buffer according to standard procedures (Sambrook et al., 1989). The RNAs were transferred to a Hybond membrane (Amersham Pharmacia Biotech), hybridized with random-primed cDNA probes in ExpressHyb mix (CLONTECH Laboratories, Inc.) as described in the manufacturer's protocol, washed sequentially with 1× SSC/0.1% SDS and 0.1× SSC/0.1% SDS at 55°C, and used for autoradiography.

Results

Fibronectin Specifically Supports Rb-controlled S Phase Entry

Serum-starved NIH-3T3 cells were detached and seeded overnight in the absence of serum or growth factors on agar (suspended cells), polylysine (cells spread on a nonintegrin binding substrate), or fibronectin, laminin, or collagen (cells spread using different integrins on each ECM component) (Fig. 1). The cells were well spread on polylysine, fibronectin, and collagen, whereas cells that attached to laminin remained poorly spread (not shown). Growth factors were added 16 h after plating to avoid any transient signaling activated by trypsinization and replating. Subsequently, cells were assayed for Rb phosphorylation status or activation of a cyclin A luciferase reporter construct. Hyperphosphorylation of Rb was detected 16 h after growth factor stimulation only in cells plated on fibronectin (Fig. 2 A). Similarly, cyclin A promoter activity was strongly induced after 20 h in cells plated on fibronectin, whereas it remained weak or absent in cells plated on the other substrates (Fig. 2 B). Addition of 10% calf serum to cells plated on polylysine rescued Rb hyperphosphorylation and cyclin A induction (Fig. 2, A and B), confirming that the cells were viable and responsive. Consistent with these findings, BrdU labeling on coverslips and propidium iodide staining for FACS® showed that cells plated on fibronectin could enter S phase, whereas cells maintained in suspension or plated on other substrates did not (not shown).

Phosphorylation of Rb family members allows the E2F-dependent transcription of genes such as cyclin A that are involved in initiating S phase, although a pathway independent of E2F has also been described (Lukas et al., 1997; Chew et al., 1998). Therefore, we tested whether the

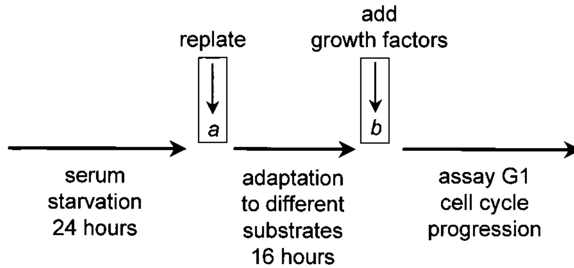

Figure 1. Experimental design. After serum starvation for 24 h, cells were trypsinized and collected in DME containing 2% BSA, washed, and plated in DME containing 0.2% BSA on various different substrates (point a). Growth factors were added 16 h later (point b), and the ability of each substrate to support progression through the G1 phase of the cell cycle was compared.

inactivation of Rb family members was essential in our experimental system. A cyclin A reporter construct harboring a mutated E2F site that was only weakly suppressed by serum starvation (Schulze et al., 1995, 1996) responded poorly to growth factor stimulation on each of the substrates tested (Fig. 2 B). This result suggested that the poor responsiveness of the wild-type cyclin A promoter in cells plated on substrates other than fibronectin was indeed due to repression by E2F in a complex with an Rb family member. To test this interpretation further, cells expressing the wild-type cyclin A reporter were transiently transfected with a cDNA encoding an Rb mutant that cannot be phosphorylated by cyclin–cdk complexes and therefore acts as a dominant inhibitory mutant that is constitutively associated with E2F (Chew et al., 1998). Consistent with a critical role for E2F-mediated transcriptional regulation of cyclin A in our system, this mutant Rb blocked the induction of the cyclin A promoter in cells plated on fibronectin (Fig. 2 C).

Thus, fibronectin specifically supports the activation by growth factors of a signal transduction cascade that leads to inactivation of Rb and subsequent induction of cyclin A at entry into S phase.

RhoA Activity Is Required for G1 Cell Cycle Progression and Is Rescued in Serum-starved Cells by Replating on Fibronectin

The organization of the actin cytoskeleton is thought to play an important role in the propagation of signal transduction cascades (Chen et al., 1994; Morino et al., 1995; Zhu and Assoian, 1995). In spite of the prolonged period of serum starvation (24 h plus an additional 16 h after replating), actin stress fibers spanning the cells were present in cells plated on fibronectin (Fig. 3 A). In contrast, cells plated on all of the other substrates showed actin diffusely distributed in the cytoplasm and in a cortical ring, even though the cells had spread extensively on polylysine and collagen. The cells that were spread on laminin (many were poorly spread) also did not contain stress fibers. Similarly, staining for paxillin revealed the presence of cell–matrix adhesions in cells plated on fibronectin, whereas the staining pattern was relatively diffuse in cells plated on each of the other substrates tested (Fig. 3 A).

Because the organization of actin stress fibers and the formation of cell–matrix adhesions are regulated by RhoA,

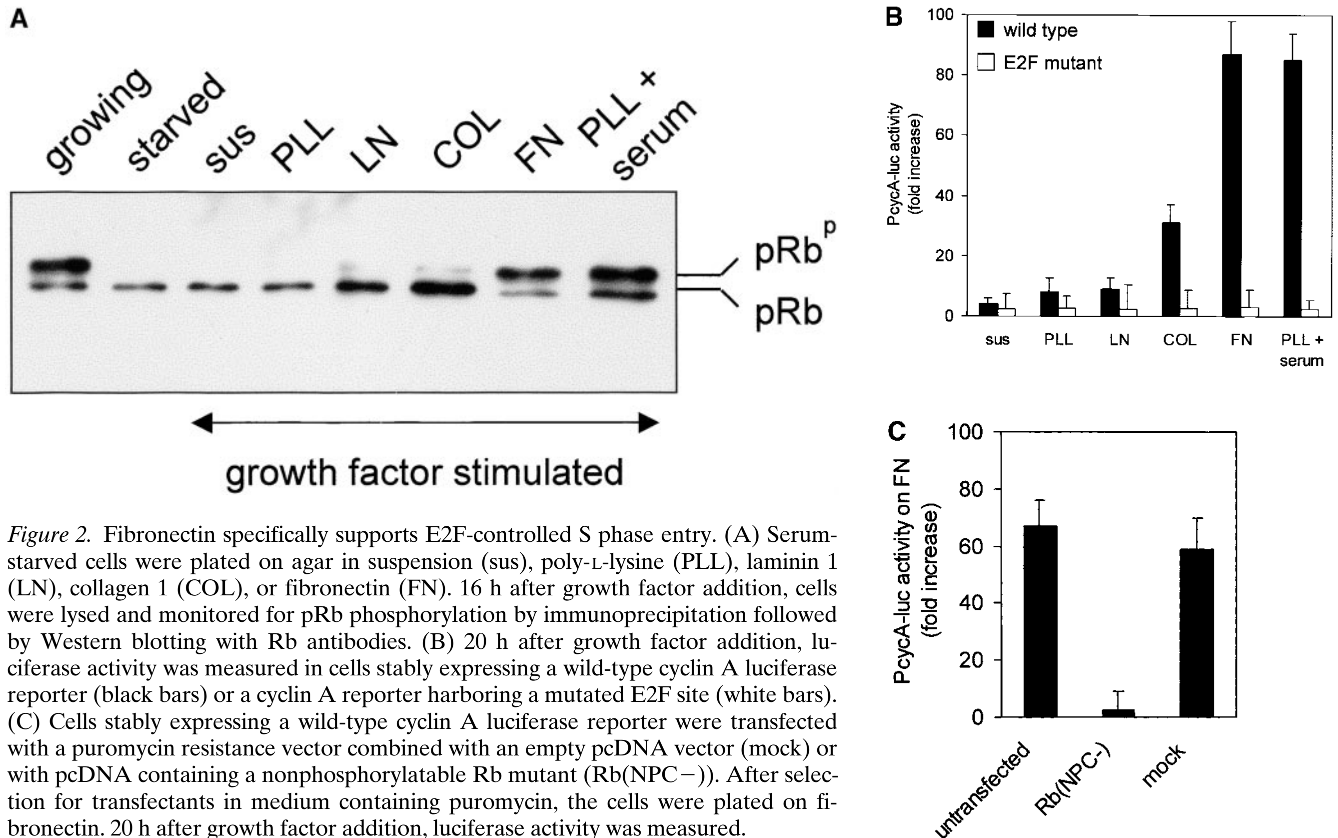

Figure 2. Fibronectin specifically supports E2F-controlled S phase entry. (A) Serum-starved cells were plated on agar in suspension (sus), poly-L-lysine (PLL), laminin 1 (LN), collagen 1 (COL), or fibronectin (FN). 16 h after growth factor addition, cells were lysed and monitored for pRb phosphorylation by immunoprecipitation followed by Western blotting with Rb antibodies. (B) 20 h after growth factor addition, luciferase activity was measured in cells stably expressing a wild-type cyclin A luciferase reporter (black bars) or a cyclin A reporter harboring a mutated E2F site (white bars). (C) Cells stably expressing a wild-type cyclin A luciferase reporter were transfected with a puromycin resistance vector combined with an empty pcDNA vector (mock) or with pcDNA containing a nonphosphorylatable Rb mutant (Rb(NPC−)). After selection for transfectants in medium containing puromycin, the cells were plated on fibronectin. 20 h after growth factor addition, luciferase activity was measured.

we tested the hypothesis that fibronectin stimulates or rescues RhoA activity that is downregulated in serum-starved cells. Initial support for this notion was provided by the restoration of growth factor responsiveness in cells plated on polylysine by serum, which contains the Rho activator lysophosphatidic acid (LPA) (Ridley and Hall, 1992; Ren et al., 1999) (Fig. 2, A and B). After serum starvation and suspension for 2 h, GTP-bound RhoA was barely detectable (Fig. 3 B). Plating cells on fibronectin, but not on polylysine or laminin, for 2 h resulted in a substantial increase in the level of active RhoA. The activation level of Rac1, another member of the Rho family of small GTPases, was low in serum-starved cells kept in suspension, and plating on fibronectin as well as polylysine resulted in elevated levels of GTP-bound Rac1. Activation of Rac1 was somewhat lower in cells plated on laminin. After overnight incubation, active RhoA was undetectable in cells plated on polylysine or laminin, whereas a low level remained present in cells plated on fibronectin (not shown). After growth factor stimulation, the level of GTP-bound RhoA was high in cells plated on fibronectin, whereas it remained low in cells plated on polylysine or laminin (Fig. 3 C). Rac1 activity disappeared after overnight incubation on each of the substrates (not shown). After stimulation with growth factors, GTP-bound Rac1 was minimal in cells plated on laminin whereas GTP-bound Rac1 was precipitated from cells plated on both fibronectin and polylysine. We observed that cells plated on laminin rounded up and detached after growth factor stimulation (not shown). Thus, replating on fibronectin specifically stimulated the activity of RhoA, whereas cell spreading in general appeared to be associated with Rac1 activity.

To test directly whether Rho activity was crucial for the supportive action of fibronectin, cells were treated with LPA to activate Rho, or they were transiently transfected with a cDNA encoding C3 toxin to inhibit Rho (Sekine et al., 1989; Paterson et al., 1990; Ridley and Hall, 1992; Ren et al., 1999). Addition of LPA did not induce a response in suspended cells, but in cells plated on polylysine it supported weak activation of the cyclin A promoter, which was blocked in cells transiently transfected with the C3 cDNA (Fig. 3 D). The fact that the effect of LPA was much weaker than serum indicates the involvement of other serum components, probably including fibronectin. Importantly, in cells plated on fibronectin, cyclin A promoter induction was strongly diminished by C3, indicating a requirement for endogenous Rho activity. As a further test, we transiently expressed the dominant inhibitory cDNAs N19RhoA and N17Rac1 using retroviral transduction. In agreement with the results obtained with the C3 cDNA, N19RhoA inhibited cyclin A promoter activation in cells plated on fibronectin (Fig. 3 E). Expression of N17Rac1 was also partially inhibitory, suggesting that activity of both GTPases is required.

We conclude that endogenous RhoA activity is specifically stimulated in serum-starved cells plated on fibronectin and is required for cell cycle progression.

Activation of MAPK Is Essential for G1 Progression but Does Not Require RhoA-mediated Actin Cytoskeletal Organization

One of the first steps in the progression through G1 is the induction of cyclin D1 through a MAPK-dependent mechanism (Lavoie et al., 1996). Indeed, the induction of a sta-

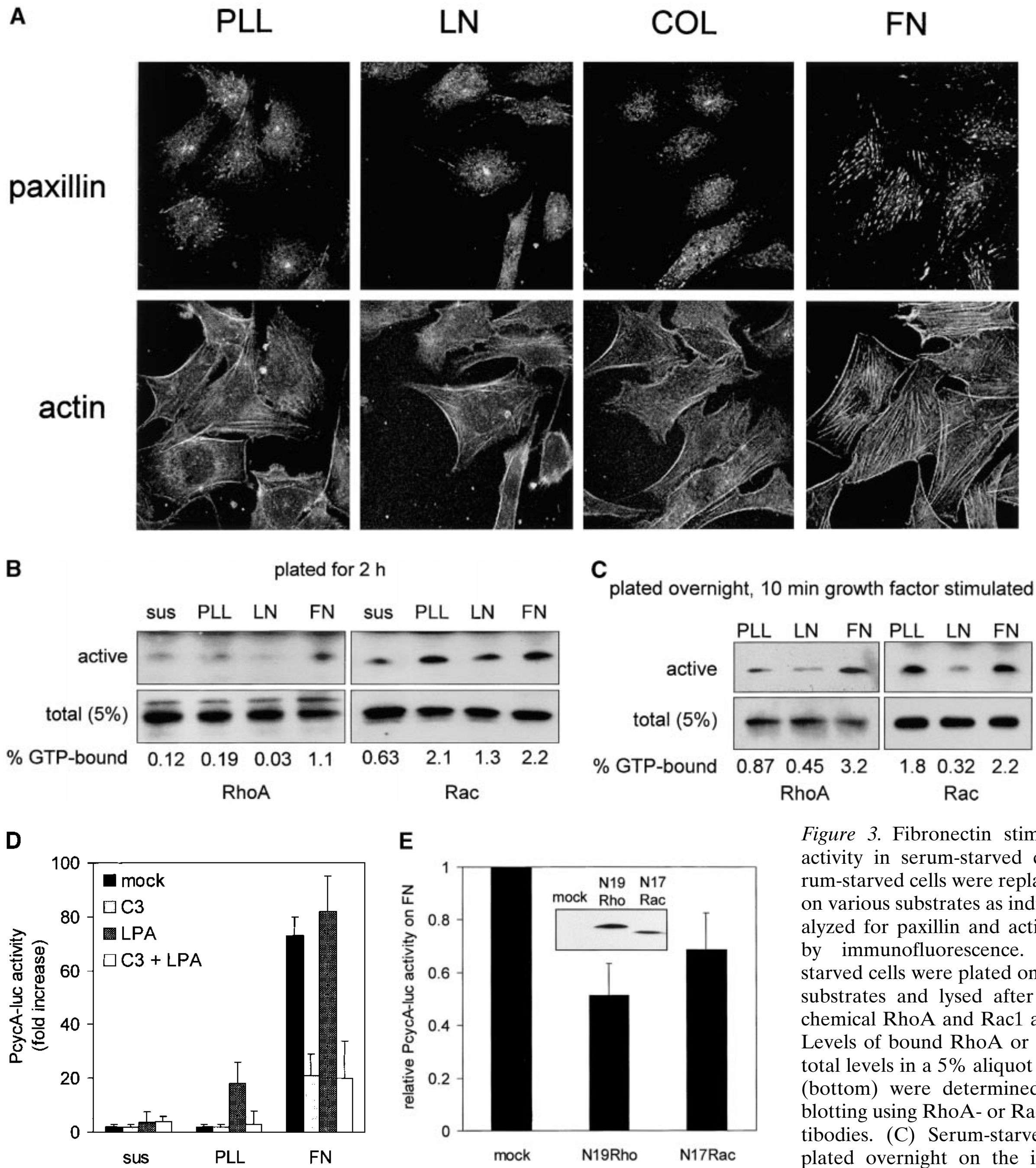

Figure 3. Fibronectin stimulates RhoA activity in serum-starved cells. (A) Serum-starved cells were replated overnight on various substrates as indicated and analyzed for paxillin and actin localization by immunofluorescence. (B) Serum-starved cells were plated on the indicated substrates and lysed after 2 h for biochemical RhoA and Rac1 activity assays. Levels of bound RhoA or Rac1 (top) or total levels in a 5% aliquot of each lysate (bottom) were determined by Western blotting using RhoA- or Rac1-specific antibodies. (C) Serum-starved cells were plated overnight on the indicated substrates followed by incubation for 10 min in the presence of growth factors, then lysed for biochemical RhoA and Rac1 activity assays. In B and C, the percentage of GTP-bound RhoA and Rac1 was determined according to densitometry of autoradiograms. (D) Cells stably expressing a wild-type cyclin A luciferase reporter were transfected with a puromycin resistance vector combined with an empty vector (mock) or with a C3 expression plasmid and selected in medium containing puromycin. Subsequently, the cells were plated in suspension, on polylysine, or on fibronectin, and 20 h after growth factor addition, luciferase activity was measured. (E) Cells stably expressing a wild-type cyclin A luciferase reporter were transduced with dominant inhibitory RhoA or Rac1 or control retrovirus and selected in media containing zeocin. Subsequently, the cells were plated on fibronectin, and 20 h after growth factor addition luciferase activity was measured. The level of activity in mock-transduced cells was set at 1. The inset shows Western blot analysis of the Myc-tagged constructs.

bly expressed cyclin D1 luciferase reporter after 4 h of growth factor stimulation, as well as the more delayed induction of cyclin A (20 h), was inhibited by a dominant inhibitory MEK plasmid (Fig. 4 A). Integrin-mediated cell spreading and growth factors cooperate in the activation of ERK-type MAPK (Renshaw et al., 1997; Aplin and Juliano, 1999). Therefore, we tested if the requirement for Rho-mediated cytoskeletal organization in G1 progression was at the level of MAPK activation. After trypsinization, a transient peak of activation of ERK1 and 2 could be detected that was rapidly attenuated in cells maintained in suspension or plated on polylysine and that decreased at a slower rate in cells plated on fibronectin in serum-free medium (not shown). After overnight plating in the absence

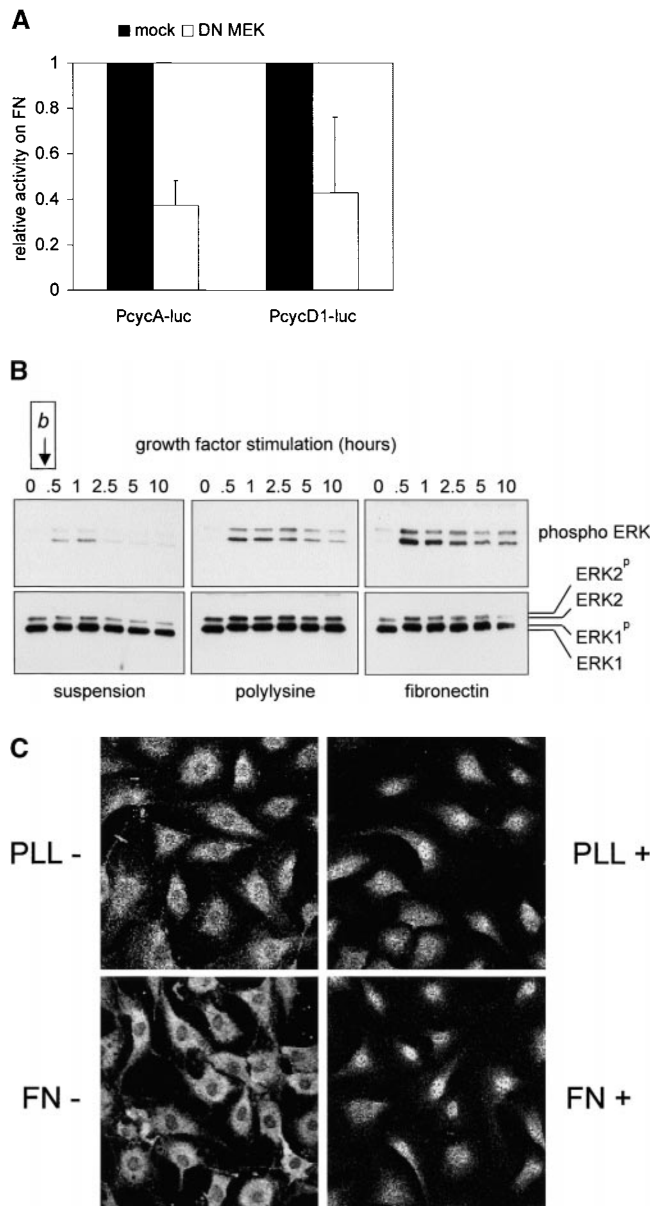

Figure 4. Regulation of ERK-type MAPKs. (A) Cells stably expressing a luciferase reporter for cyclin A or for cyclin D1 were transfected with a puromycin resistance vector combined with an empty vector (mock) or with a dominant inhibitory MEK expression plasmid and selected in medium containing puromycin. Subsequently, the cells were plated on fibronectin and lysed for luciferase activity assays 4 h (in the case of the cyclin D1 reporter) or 20 h (cyclin A reporter) after growth factor addition. (B) Serum-starved cells were plated overnight in suspension or on polylysine or fibronectin. They were then stimulated with growth factors and lysed at the indicated time points for Western blotting with antibodies against total ERK1 and ERK2 or with a phospho-ERK antibody. (C) Serum-starved cells were plated overnight on the indicated substrates and fixed for analysis of ERK2 localization by immunofluorescence before (−) or 2.5 h after growth factor stimulation (+).

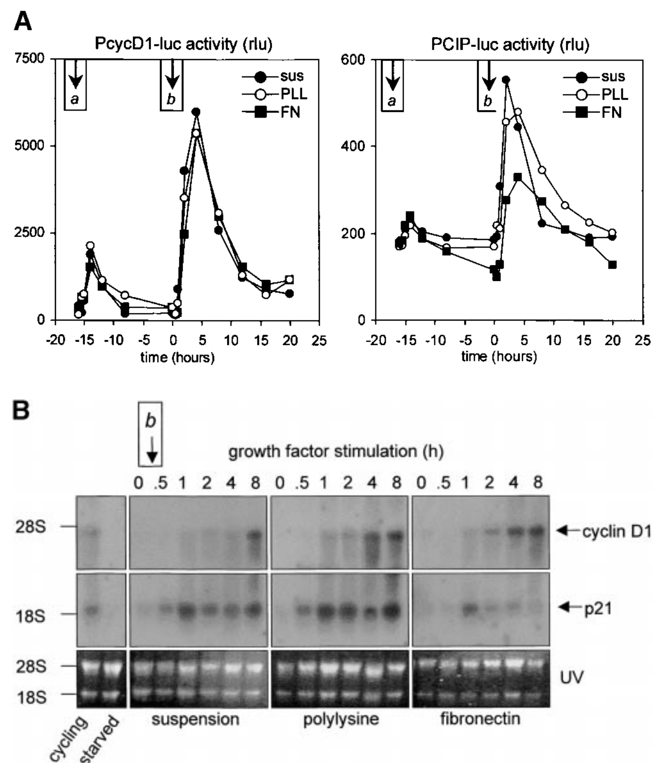

Figure 5. Regulation of cyclin D1 and $p21^{Cip/Waf}$ RNA. (A) Cells stably expressing a cyclin D1 (left) or a $p21^{Cip/Waf}$ (right) luciferase reporter were plated in suspension (sus, filled circles), on polylysine (PLL, open circles), or on fibronectin (FN, filled squares) (time a) and stimulated with growth factors 16 h later (time b). At the indicated time points, luciferase activity was measured. (B) Cells were starved, plated overnight in suspension or on polylysine or fibronectin, and subsequently stimulated with growth factors. At the indicated time points, cells were lysed for Northern blotting with probes against mouse cyclin D1 or $p21^{Cip/Waf}$.

of growth factors, no activated MAPK was detectable in cells incubated on any of the substrates tested (Fig. 4 B, time point 0). After subsequent stimulation with growth factors, active ERK1 and 2 were detectable on each of the substrates. In suspended cells, the growth factor–stimulated activity of ERK was rapidly lost, whereas activation was sustained for at least 10 h in cells attached to either polylysine or fibronectin. Furthermore, nuclear translocation of ERK in response to growth factors could be observed in cells plated on fibronectin or polylysine (Fig. 4 C) as well as other substrates tested (not shown).

From these findings, we conclude that the activation and nuclear localization of ERK are essential for G1 progression and depend on cell adhesion/spreading but not on RhoA-mediated actin cytoskeletal organization.

Fibronectin Stimulates RhoA-dependent Suppression of $p21^{Cip/Waf}$ and Induction of Cyclin D1 Protein

It has been reported that the induction of cyclin D1 requires cytoskeletal integrity (Bohmer et al., 1996). To test if RhoA-dependent actin cytoskeletal organization was required for cyclin D1 induction, we studied the regulation of a stably expressed cyclin D1 luciferase reporter, endogenous cyclin D1 RNA, and cyclin D1 protein levels in suspended cells and in cells plated on polylysine or fibronectin. In each condition, a transient 7-fold activation of the luciferase reporter was induced after trypsinization and replating, and a stronger (20-fold) activation occurred after growth factor stimulation that peaked after 4 h and decreased to baseline levels after ~12 h (Fig. 5 A, left). Similarly, in cells plated on polylysine or fibronectin, accumu-

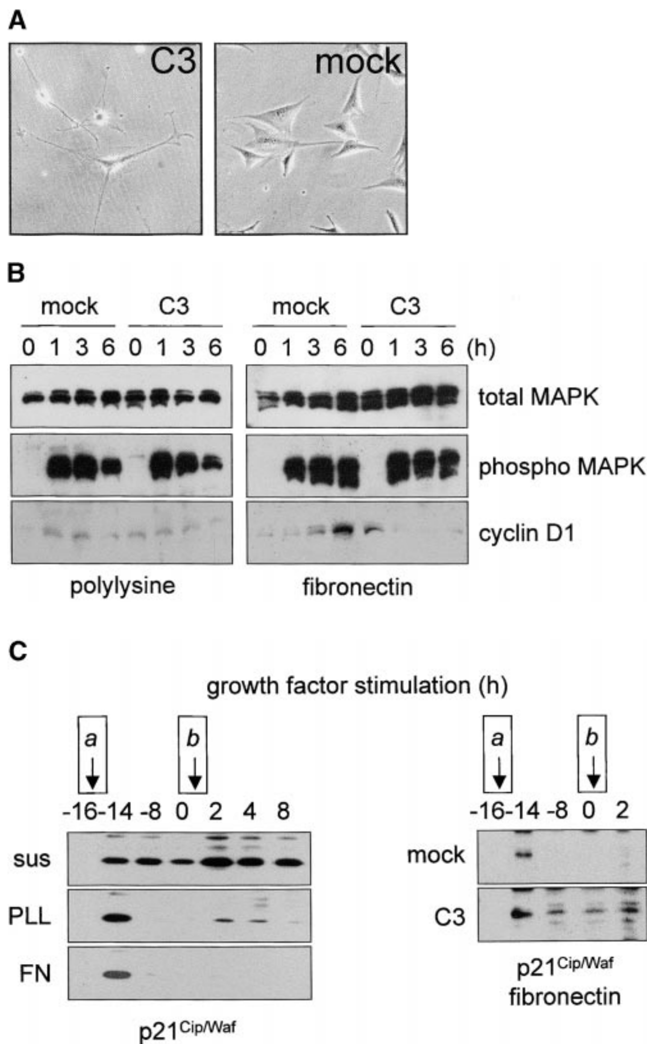

Figure 6. Regulation of cyclin D1 and p21$^{Cip/Waf}$ protein. (A) Morphology of pCEF/C3-transfected cells. (B) Cells were transfected with a puromycin resistance vector combined with empty vector (mock) or with a C3 toxin expression plasmid and selected for transfectants in medium containing puromycin. Subsequently, the cells were serum-starved, plated on polylysine or fibronectin, and stimulated with growth factors 16 h later and lysed at the indicated time points for Western blotting with antibodies against MAPK, phospho-MAPK, or cyclin D1. (C) Untransfected cells (left) or mock or pCEF/C3 transfected cells (right) were serum starved and plated on the indicated substrates. 16 h later they were stimulated with growth factors and lysed at the indicated time points for Western blotting with antibodies against p21$^{Cip/Waf}$.

lation of endogenous cyclin D1 RNA was detectable 1 h after growth factor stimulation and became maximal after 4 h (Fig. 5 B). In suspended cells, accumulation of cyclin D1 RNA was delayed, but it reached similar levels as those observed in attached cells. In contrast, while expression of cyclin D1 protein was not detectable in suspended cells (not shown) and poorly induced in cells plated on polylysine, it was strongly induced in cells plated on fibronectin (Fig. 6 B). To test whether the stimulation of cyclin D1 protein levels by fibronectin depended on the enhanced Rho activity observed in cells attached to fibronectin, the effect of C3 toxin was evaluated. In cells expressing the C3 cDNA, the induction of cyclin D1 was suppressed in cells plated on fibronectin, indicating that endogenous Rho activity was required for this response. Probing of the same blot for phospho- and total MAPK levels indicated that this effect of C3 was not due to inhibition of sustained MAPK activation. The low levels of cyclin D1 seen in cells plated on polylysine were not affected by C3. Parenthetically, we noted that in C3-expressing cells, cyclin D1 was not always completely absent in the cells before growth factor stimulation.

RhoA has previously been reported to downmodulate p21$^{Cip/Waf}$ levels in cells transformed by oncogenic Ras (Olson et al., 1998). Such a mechanism may also play a role in the stimulation of cell cycle progression by fibronectin that we observed. Therefore, we tested whether stimulation of RhoA activity by fibronectin would result in suppression of p21$^{Cip/Waf}$. In cells stably expressing a p21$^{Cip/Waf}$ luciferase reporter construct, weak activation was observed after trypsinization and replating that returned to baseline levels in cells in suspension or plated on polylysine; however, levels were further suppressed in cells plated on fibronectin (Fig. 5 A, right). Upon subsequent stimulation by growth factors, a threefold induction was observed on all substrates tested that was maximal after 2 h and then returned to baseline levels over the following 18 h. Similarly, endogenous p21$^{Cip/Waf}$ RNA became detectable after 30–60 min in suspended as well as attached cells. However, in suspended cells and in cells plated on polylysine, p21$^{Cip/Waf}$ RNA remained present for at least 8 h whereas in cells plated on fibronectin, it was suppressed at later time points (Fig. 5 B). We next determined the levels of p21$^{Cip/Waf}$ protein under these conditions. No p21$^{Cip/Waf}$ could be detected in serum-starved cells, but its expression was induced after trypsinization and replating of the cells (Fig. 6 C, left). This induction of p21$^{Cip/Waf}$ was rapidly suppressed in adherent cells, whereas protein expression remained high in suspended cells for up to 16 h. Subsequent stimulation with growth factors induced p21$^{Cip/Waf}$ expression in cells plated on polylysine, albeit at low levels, whereas expression remained undetectable in cells plated on fibronectin. Furthermore, expression of C3 cDNA neutralized the suppressive effect of fibronectin, demonstrating that endogenous RhoA activity was required for this suppressive signal from fibronectin (Fig. 6 C, right). The moderate levels of p21$^{Cip/Waf}$ that were induced after growth factor stimulation of cells plated on polylysine were not affected by C3 transfection (not shown).

We conclude that activation of RhoA by fibronectin stimulates the accumulation of cyclin D1 protein, whereas it suppresses p21$^{Cip/Waf}$ RNA levels during G1 cell cycle progression.

Discussion

Like most untransformed cells, NIH-3T3 fibroblasts only proliferate in response to growth factors when they are attached to ECM via their integrins. In suspension, they cannot pass through the restriction point in late G1, but once the cells have passed this point they no longer require growth factors or adhesion and are irreversibly committed to DNA synthesis (Pardee, 1989; Assoian, 1997). Cell adhesion sustains the MAPK response to growth factor stimulation, which in turn is needed for the induction of cyclin D1 (Bohmer et al., 1996; Weber et al., 1997a; Roovers et al.,

1999). In addition, p21$^{Cip/Waf}$ and p27^{Kip1} levels are suppressed in adherent cells resulting in increased activity of the cyclin E–cdk2 complex (Fang et al., 1996; Zhu et al., 1996; Bottazzi et al., 1999). Integrin-mediated adhesion regulates multiple growth factor–induced signal transduction cascades that are relevant to cell growth (Assoian, 1997; Howe et al., 1998). Fibronectin is particularly efficient in promoting anchorage-dependent survival and proliferation (Giancotti and Ruoslahti, 1999; Yamada and Danen, 2000), and the fibronectin receptor, integrin $\alpha5\beta1$, is required to propagate the mitogenic stimulus from fibronectin into the cell (Varner et al., 1995; Davey et al., 1999; Roovers et al., 1999). Here, we find that under serum-free conditions, fibronectin is in fact unique in its support of proliferation, due to its ability to stimulate the small GTPase RhoA in addition to the Ras/MAPK cascade.

RhoA, Rac, and Cdc42 are all required for Ras-induced transformation (Qiu et al., 1995a,b, 1997; Olson et al., 1998), and microinjection of activated forms of any of them can stimulate S phase entry (Olson et al., 1995). RhoA activity is required in G1 progression for the degradation of p27^{Kip1} and suppression of p21$^{Cip/Waf}$ levels (Hirai et al., 1997; Weber et al., 1997b; Adnane et al., 1998; Hu et al., 1999), and RhoA counteracts the induction of p21$^{Cip/Waf}$ by oncogenic Ras (Olson et al., 1998). Rho family GTPases can be activated by ECM as well as by growth factors (Barry et al., 1997; Bourdoulous et al., 1998; Price et al., 1998; Ren et al., 1999). In the present study, we find that cell adhesion to fibronectin specifically stimulates the formation of actin stress fibers and cell–matrix adhesions (structures that are dependent on RhoA activity) in serum-starved cells. Although several substrates stimulate activation of MAPK and Rac, only fibronectin stimulates activation of RhoA and supports G1 cell cycle progression in a Rho-dependent manner. Our findings confirm and extend the notion that RhoA regulates p21$^{Cip/Waf}$ levels. It has been previously reported that p21$^{Cip/Waf}$ is induced by ERK activation and is suppressed later in G1 by cell attachment through an unidentified mechanism (Bottazzi et al., 1999). Our finding that trypsinization/replating transiently activates ERK and induces p21$^{Cip/Waf}$ expression is in agreement with this report. Moreover, we find that subsequent suppression of p21$^{Cip/Waf}$ does not result from a general effect of cell adhesion and spreading, but instead requires the activation of RhoA, which is specifically stimulated by fibronectin under serum-free conditions. Although fibronectin only weakly suppresses transcription of a p21$^{Cip/Waf}$ reporter, endogenous p21$^{Cip/Waf}$ RNA levels are specifically and strongly suppressed in cells on fibronectin, suggesting that p21$^{Cip/Waf}$ RNA degradation may be enhanced in cells on fibronectin. Furthermore, the difference in p21$^{Cip/Waf}$ protein levels in cells kept in suspension or plated on polylysine suggests further regulation at the protein level.

Our finding that the level of MAPK activation induced by growth factor stimulation is reduced in cells kept in suspension for 16 h is in agreement with a previous report showing that the time of preincubation in suspension (varying from 1 to 24 h) determines the effectiveness of ERK activation (Renshaw et al., 1997). Stimulation of MAPK by growth factors has previously been shown to be supported by the interaction of various different integrins with ECM components (Aplin et al., 1999). Our findings are in agreement, except that in contrast to an earlier report (Chen et al., 1994), we demonstrate in our system that growth factors can activate MAPK even in cells plated on the nonintegrin substrate polylysine. This may be related to the fact that in our hands, cells plated on polylysine can activate Rac1 and spread. The MAPK activation induced by plating cells on fibronectin depends on integrity of the actin cytoskeleton (Chen et al., 1994; Morino et al., 1995; Zhu et al., 1995), whereas treatment with cytochalasin D to disrupt actin filaments does not affect MAPK activation by various types of soluble growth factors (Seufferlein et al., 1996). In a later study, it was shown that low concentrations of cytochalasin D that disrupt actin stress fibers and focal adhesions, but leave cortical actin structures intact, permit efficient EGF signaling to ERK-type MAPKs (Aplin and Juliano, 1999). Thus, our finding that cells plated in the absence of serum on polylysine, laminin, or collagen do show organization of actin in a cortical ring may explain the efficient MAPK activation by growth factors that we observe in these cells.

ERK-type MAPKs are crucial for growth factor–induced proliferation in fibroblasts (Pages et al., 1993), and the capacity to induce a sustained pattern of MAPK activation (over a 2-h period) has been correlated with the mitogenic effects of growth factors (Kahan et al., 1992). The activation of ERK-type MAPKs is followed by their redistribution from the cytoplasm to the nucleus, where they activate multiple transcription factors including Elk-1 (Treisman, 1996). In hamster embryonic fibroblasts, the mitogenic effect of PDGF was found to depend on its ability to induce a sustained pattern of MAPK activation (which can last up to 24 h), and this induction was linked to the continued expression of cyclin D1 (Weber et al., 1997a). It was later shown that integrin $\alpha5\beta1$–mediated adhesion could sustain the growth factor–induced MAPK response in fibroblasts, resulting in the expression of cyclin D1 (Roovers et al., 1999). Our data also show that the MAPK response to growth factor stimulation is transient in suspended cells but, in contrast to the results described by Roovers et al., we find that in polylysine-attached cells, growth factor stimulation can give rise to a sustained pattern of MAPK activation and induce the translocation of ERK into the nucleus. Thus, sustained activation and nuclear translocation of MAPK may be required, but is not sufficient for G1 phase progression and S phase entry. Furthermore, the regulation of a cyclin D1 luciferase reporter is identical in suspended and attached cells and the accumulation of endogenous cyclin D1 RNA is delayed but detectable in suspended cells. Thus, cyclin D1 transcription can occur under conditions where MAPK is only transiently activated. However, accumulation of cyclin D1 protein occurs only in attached cells and its induction is most efficient in cells plated on fibronectin. Thus, an important level of regulation appears to be the accumulation of cyclin D1 protein (either via control of translation or stability). Finally, we show that Rho activity is involved in this regulatory mechanism since C3 inhibits cyclin D1 induction.

Active Cdc42 has been reported to enhance the MAPK response to EGF in suspended cells, whereas active Rac1 or RhoA had no effect (Aplin and Juliano, 1999). In addition, constitutively activated Rac1 and Cdc42, but not

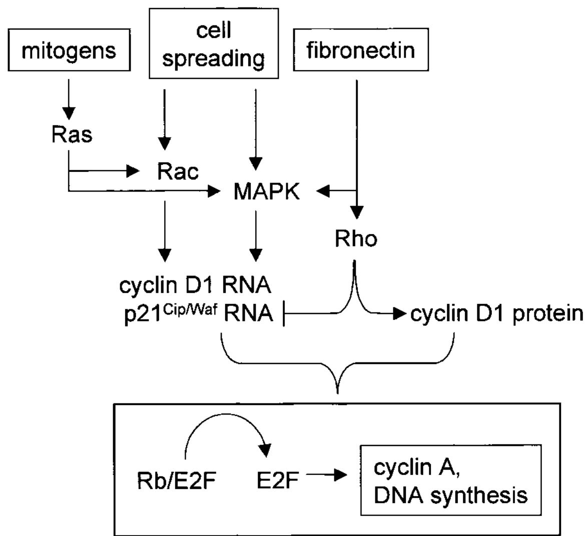

Figure 7. Model depicting proposed roles of growth factors and adhesion to fibronectin in regulation of G1 cell cycle progression. Cell adhesion and spreading on various substrates collaborates with soluble mitogens to stimulate activity of Rac and MAPK, which may be connected at the level of Ras. Growth factors and cell spreading can both induce transcription of cyclin D1 and p21$^{Cip/Waf}$. Cell adhesion to fibronectin, in addition to these pathways, also activates RhoA, which is required for the suppression of p21$^{Cip/Waf}$ and the accumulation of cyclin D1 later in G1. We show that adhesion to fibronectin suppresses p21$^{Cip/Waf}$ RNA levels whereas the stimulation of cyclin D1 induction occurs at the protein level. Together, these signaling pathways stimulate the hyperphosphorylation of Rb pocket proteins, cyclin A induction, and DNA synthesis.

RhoA, can induce E2F transcriptional activity and anchorage-independent cyclin A induction (Gjoerup et al., 1998; Philips et al., 2000). On the other hand, overexpression of Cdc42 can inhibit proliferation via activation of p38 MAPK (Molnar et al., 1997), and disassembly of a fibronectin matrix leading to decreased cyclin E–cdk2 activity was reported to be accompanied by increased Cdc42 and p38 MAPK activities and decreased RhoA activity (Bourdoulous et al., 1998). We also find that in NIH-3T3 cells active Rac1 can stimulate S phase entry in suspended cells or cells plated on polylysine whereas RhoA has no effect (not shown). However, the findings presented in this study demonstrate that endogenous RhoA activity is required for G1 progression to occur and that integrin-mediated adhesion to fibronectin stimulates this pathway. The seemingly contradictory results seen with overexpression of activated GTPases versus those seen with inhibition of endogenous RhoA family members could be due to activation of endogenous RhoA by overexpression of activated Cdc42 or Rac1 (Mackay and Hall, 1998). Activity of all three Rho family small GTPases appears to be required, and in cells expressing active RhoA, anchorage may still be needed for the activation of Cdc42 and Rac1. Moreover, whereas the stimulation of modest levels of RhoA activity by matrix adhesion and growth factors is required, expression of too high levels of dominant-active RhoA interferes with cell spreading and actually inhibits cell cycle progression (not shown). Thus, the inhibition of endogenous activity is probably a more accurate way to evaluate the pathways involved than the expression of high levels of activated forms of the small GTPases.

In conclusion, integrin-mediated cell adhesion to fibronectin cooperates with growth factors to stimulate a mitogenic signal transduction pathway (Fig. 7). Transient MAPK activation by cell attachment or growth factors results in the transcriptional activation of cyclin D1 and p21$^{Cip/Waf}$ genes. Cell spreading in general is associated with sustained MAPK activation and Rac1 activity, whereas cell adhesion to fibronectin specifically activates RhoA and suppresses p21$^{Cip/Waf}$ and induces cyclin D1 later in G1, in a Rho-dependent manner. Suppression of p21$^{Cip/Waf}$ in fibronectin-adherent cells occurs through reduced RNA levels, whereas the specific induction of cyclin D1 in cells plated on fibronectin occurs at the protein level (through enhanced translation or protein stability). This fibronectin-regulated mechanism is apparently subverted in malignancy, where constitutively dysregulated signaling of Ras/MAPK and Rho family GTPases can cooperate in oncogenic transformation.

We thank Natalie Ahn, John Collard, J. Silvio Gutkind, Wade Harper, Berthold Henglein, Konrad Huppi, Sybille Mitnacht, Hein te Riele, and Charles Sherr for providing cDNA constructs. We thank Gera Neufeld for providing bFGF, members of the Collard lab for providing GST fusion proteins, and Marc Vooijs for assistance with the preparation of the RNA blot. We thank Arjan van der Flier, Cecile Geuijen, Leo Price, Ed Roos, and Marc Vooijs for providing critical comments on the manuscript.

This work was supported by a Fogarty International Fellowship from the National Institutes of Health (E.H.J. Danen), a Dutch Cancer Society Fellowship (E.H.J. Danen), and by grant NKI 99-2117 from The Dutch Cancer Society (E.H.J. Danen, P. Sonneveld, and A. Sonnenberg).

Submitted: 10 May 2000
Revised: 16 October 2000
Accepted: 6 November 2000

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