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Cell Attachment to the Extracellular Matrix Induces Proteasomal Degradation of p21^{CIP1} via Cdc42/Rac1 Signaling

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The cyclin-dependent kinase 2 (Cdk2) inhibitors p21^{CIP1} and p27^{KIP1} are negatively regulated by anchorage during cell proliferation, but it is unclear how integrin signaling may affect these Cdk2 inhibitors. Here, we demonstrate that integrin ligation led to rapid reduction of p21^{CIP1} and p27^{KIP1} protein levels in three distinct cell types upon attachment to various extracellular matrix (ECM) proteins, including fibronectin (FN), or to immobilized agonistic anti-integrin monoclonal antibodies. Cell attachment to FN did not rapidly influence p21^{CIP1} mRNA levels, while the protein stability of p21^{CIP1} was decreased. Importantly, the down-regulation of p21^{CIP1} and p27^{KIP1} was completely blocked by three distinct proteasome inhibitors, demonstrating that integrin ligation induced proteasomal degradation of these Cdk2 inhibitors. Interestingly, ECM-induced proteasomal proteolysis of a ubiquitination-deficient p21^{CIP1} mutant (p21K6R) also occurred, showing that the proteasomal degradation of p21^{CIP1} was ubiquitin independent. Concomitant with our finding that the small GTPases Cdc42 and Rac1 were activated by attachment to FN, constitutively active (ca) Cdc42 and ca Rac1 promoted down-regulation of p21^{CIP1}. However, dominant negative (dn) Cdc42 and dn Rac1 mutants blocked the anchorage-induced degradation of p21^{CIP1}, suggesting that an integrin-induced Cdc42/Rac1 signaling pathway activates proteasomal degradation of p21^{CIP1}. Our results indicate that integrin-regulated proteasomal proteolysis might contribute to anchorage-dependent cell cycle control.

Anchorage to the extracellular matrix (ECM) is required for proliferation of all untransformed tissue cells (38). To control cell proliferation, anchorage regulates key cell cycle components mainly occurring in the G_1 phase (31). Progression through the G_1 phase is mediated by the cyclin-dependent kinases Cdk4/6 and Cdk2, whose activities are controlled by their associated cyclins and Cdk inhibitors (43). To this end, attachment to ECM results in up-regulation of cyclin E-Cdk2 activity and down-regulation of the Cdk2 inhibitors p21^{CIP1} and p27^{KIP1} (11, 45, 52). Importantly, cells in suspension display higher levels of the Cdk2 inhibitors p21^{CIP1} and p27^{KIP1} than do attached cells, leading to impaired cyclin E-Cdk2 activity in suspended cells concomitant with a cell cycle block in late G_1 phase (11, 45, 52). In fact, cyclin E-Cdk2 activity is considered the last control in the G_1 phase, since activated cyclin E-Cdk2 triggers cyclin A synthesis and thereby promotes S-phase entry. This suggests that the regulation of cyclin E-Cdk2 activity in late G_1 phase by anchorage may represent a control stage through which attached cells must maintain low levels of Cdk2 inhibitors until this point is passed. Therefore, the regulation of the Cdk2 inhibitors p21^{CIP1} and p27^{KIP1} appears to be an important step for anchorage-dependent G_1-phase progression of the cell cycle.

Proteasome-dependent proteolysis represents a cellular pathway for rapid down-regulation of specific proteins for which distinct temporal expression is required. Importantly, many cell cycle components are targets for proteasomes, including cyclins (A, B, D, and E), Rb, E2F, and the Cdk2 inhibitors p21^{CIP1} and p27^{KIP1}, as well as p53, and proteasomal degradation of these components determines the periodicity of the cell cycle (1, 3, 16, 20, 21, 32, 41). In most of the cases studied, proteasomal degradation is preceded by ubiquitination. However, although p21^{CIP1} can be ubiquitinated, proteasomal degradation of p21^{CIP1} does not require ubiquitination (42), which may be related to the capacity of the C8-α subunit of the 20S proteasome to directly interact with the C terminus of p21^{CIP1}, leading to rapid degradation of p21^{CIP1} (47). However, it is not known if anchorage to ECM might regulate proteasome-dependent proteolysis.

Integrins are the major cell surface receptors mediating cell anchorage to ECM proteins. Integrins activate a variety of signaling cascades, including focal adhesion kinase (FAK), mitogen-activated protein kinases of the ERK and JNK types, phosphoinositide 3-kinase (PI-3K), and integrin-linked kinase, all of which affect cell proliferation (13). These integrin-induced signals regulate key G_1-phase components, including induction of cyclin D1 mRNA and protein, thereby promoting G_1-phase progression of the cell cycle (8, 35, 51).

The small GTPases Cdc42, Rac, and Rho are also involved in integrin-activated signaling events (7, 9, 33). These small GTPases functionally switch intracellular signaling pathways by cycling between an inactive GDP-bound conformation and an active GTP-bound conformation. The GTP/GDP cycle is controlled by guanine nucleotide exchange factors, GTPase-activating proteins, and guanine nucleotide dissociation inhibitors. One basic function of these small GTPases is to regulate cytoskeleton polymerization and cell morphology and motility (17, 34). Furthermore, Rho, Cdc42, and Rac play critical roles in cell cycle progression through the G_1 phase (23, 25, 29, 49, 50). To this end, ectopic overexpression of constitutively active

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(ca) Rac or ca Cdc42 can induce cyclin D1 expression, pRB hyperphosphorylation, and E2F transcriptional activity and promote G_1-phase progression and subsequent DNA synthesis in the absence of growth factors (14). In addition, a recent study indicates that integrin-mediated activation of Rac1 controls G_1-phase progression of the cell cycle by promoting cyclin D1 synthesis (25) while Rho appears to maintain the correct timing of cyclin D1 expression in G_1 phase for control of cell cycle progression (50). In fact, within the G_1 phase of the cell cycle, the small GTPases not only regulate the expression of cyclins to control Cdk activity but also influence levels of Cdk2 inhibitors. To this end, ca Ras induces the cyclin-dependent kinase inhibitor $p21^{CIP1}$ while Rho signaling suppresses the induction of $p21^{CIP1}$ by Ras and thereby promotes DNA synthesis (30). In addition, the RhoA protein has been implicated in the mid- to late-G_1 phase and the down-regulation of the Cdk inhibitor $p27^{KIP1}$ (15). However, it is not clear if endogenous Cdc42 and/or Rac signaling induced by integrins may also affect Cdk2 inhibitors.

Our study aimed to elucidate if and how integrin-induced signaling, per se, could regulate the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$. To this end, we found that cell attachment to ECM through integrins induced a rapid reduction of the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ in three distinct cell types, caused by proteasomal proteolysis. In addition, we identified an integrin-to-Cdc42/Rac1 signaling pathway that mediated anchorage-induced proteasomal $p21^{CIP1}$ degradation. Our results indicate that integrin-regulated proteasomal proteolysis might contribute to the control of cell proliferation by anchorage.

MATERIALS AND METHODS

Antibodies. The following antibodies were utilized in this study. Anti-$p21^{CIP1}$ (Ab-5) polyclonal antibody (PAb) and anti-p53 (Ab-7) PAb were from Oncogene (Cambridge, Mass.), while anti-$p21^{CIP1}$ PAb (C19), anti-$p27^{KIP1}$ (F-8) monoclonal antibody (MAb), anti-cyclin E (M-20) PAb, anti-Myc (9E10) MAb, and anti-Cdc42 (P1) PAb were from Santa Cruz Biotechnology (Santa Cruz, Calif.). We purchased anti-$p21^{CIP1}$ (SX118) MAb from Pharmingen (San Diego, Calif.), anti-HA (12CA5) MAb from Boehringer Mannheim (Mannheim, Germany), anti-FLAG (M2) MAb from Sigma (St. Louis, Mo.), anti-Rac1 (clone 102) MAb from Transduction Laboratories (Lexington, Ky.), anti-FAK (pY^{397}) phosphospecific PAb from Biosource (Camarillo, Calif.), and anti-p44/42MAP kinase (Thr202/Thr204) phosphospecific PAb from New England BioLabs (Beverly, Mass.). Anti-actin (JLA 20) MAb was developed by J. J.-C. Lin and obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Secondary antibodies for horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin were from Jackson Labs (West Grove, Pa.).

Cell culture. Human primary umbilical vein endothelial cells (HUVECs) were obtained from Clonetics Corp. (San Diego, Calif.) and cultured in M199 medium containing 20% fetal bovine serum, 100 μg of gentamicin per ml, 4 mM L-glutamine, and 0.9 mg of endothelial cell growth supplements (Upstate Biotechnology, Lake Placid, N.Y.) per ml. Before experiments, HUVEC cultures were grown to confluence and used between passages three and five. ECV 304/T24 human bladder carcinoma cells (4) (American Type Culture Collection, Rockville, Md.) and murine NIH 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, Md.) containing 10% fetal calf serum and 5 μg of gentamicin per ml at 37°C in 5% CO_2.

Prior to the experiments, ECV 304 and NIH 3T3 cells were grown to confluence and starved for 12 h in serum-free medium in order to silence any sustained effects from growth factor signaling. After washing and trypsinization for 2 min, trypsin was neutralized with soybean trypsin inhibitor (1 mg/ml). Cells were then washed and suspended in DMEM containing 2% bovine serum albumin (BSA) at 37°C for 45 min as previously described to silence active anchorage-dependent signaling (6). For preparation of ECM-coated dishes, cell culture suspension dishes were coated with 10 μg of fibronectin (FN; cell binding fragment; Upstate Biotechnology) per ml, 10 μg of collagen type I (ColI; Upstate Biotechnology) per ml, 5 μg of vitronectin (VN) per ml, 5 μg of laminin (LM; Sigma) per ml, or 100 μg of poly-L-lysine (P-L-L; Sigma) per ml in phosphate-buffered saline (PBS) overnight at 4°C and then blocked with 1% heat-denatured BSA (Sigma) in PBS (pH 7.4) for 1 h at 37°C. For preparation of specific-integrin-coated dishes, cell culture suspension dishes were first coated with 25 μg of goat anti-mouse PAb (Jackson Labs) per ml at 37°C for 2 h and then blocked with 1% heat-denatured BSA for 1 h at 37°C. Anti-α5β1 integrin MAb JBS5 (Chemicon Int., Temecula, Calif.), anti-β1 integrin MAb P4C10 (Life Technologies), or anti-αvβ3 integrin MAb LM609 (Chemicon) was then allowed to bind to immobilized anti-mouse antibody overnight at 4°C. In certain experiments, we maintained cells in suspension by precoating the dishes with 1% heat-denatured BSA. When proteasome inhibitors were used, ECV 304 cells were pretreated with 10 μM *clasto*-lactacystin β-lactone (β-lactone; Calbiochem-Novabiochem Corp., La Jolla, Calif.), 10 μM lactacystin, or 50 μM *N*-acetyl-Leu-Leu-norleucinal (LLnL; Calbiochem-Novabiochem Corp.) in serum-free medium for 2, 4, or 10 h, respectively. The cells were then plated and incubated at 37°C for various times. Finally, the cells were harvested and rinsed in cold PBS and then frozen at −20°C.

DNA construct and transient transfections. An N-terminally hemagglutinin (HA) epitope-tagged human $p21^{CIP1}$ cDNA was generated by PCR based on a $p21^{CIP1}$ cDNA template provided by Steven I. Reed, and then this HA-$p21^{CIP1}$ cDNA was subcloned into the *Hin*dIII and *Eco*RI sites of pCDNA3. p21K6R cDNA was a generous gift from Markus Welcker that was subcloned into the *Hin*dIII and *Xba*I sites of the p3XFLAG-CMV-10 vector (Sigma). ECV 304 cells were transiently transfected with 4 μg each of various expression plasmids by using Lipofectamine Plus (Life Technologies) in accordance with the manufacturer's protocols and used at 36 h after transfection. Typically, 70% of ECV 304 cells were transfected this way, as detected by flow cytometry (data not shown). In some experiments, ECV 304 cells were transfected with 20 μg of a plasmid by using Lipofectamine 2000 (Life Technologies), resulting in approximately 80% transfection efficiency. Vector constructs for dominant negative (dn) N17 Cdc42, dn N17 Rac1, ca L61Rac1, ca L61Cdc42, and wild-type Cdc42 were kindly provided by Pontus Aspenström. His-tagged ubiquitin cDNA was a gift from Markus Welcker.

Analysis of active GTPases. Cdc42 and Rac1 activities were analyzed by a glutathione *S*-transferase (GST)-PAK pull-down assay as previously described (39). The bacterial strain that expresses the GST-PAK-CRIB domain (GST-PAK CD) fusion protein was kindly provided by John G. Collard. ECV 304 cells were harvested and kept in serum-free medium for 3 h to silence endogenous active GTPases. Cells were then plated on FN-coated dishes for 20 min. After cells were lysed, lysates were pulled down by glutathione-Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden) saturated with GST-PAK-CD. Finally, eluates were analyzed by Western blot analysis to detect GTP-Rac1 or GTP-Cdc42. Total Cdc42, Rac1, and actin levels in the primary lysates were detected in parallel.

Western blotting. Cells were lysed in a PBS-TDS buffer (PBS with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1× complete inhibitor cocktail [Boehringer Mannheim]). Protein concentrations in lysates were measured with a bicinchoninic acid protein quantification kit (Pierce, Rockford, Ill.) with BSA as the standard. In most of cases, 15 to 30 μg of total proteins was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to an Immobilon PVDF membrane (Millipore, Bedford, Mass.). After blocking of membranes with 10% dry milk in TBS-T (20 mM Tris-HCl, 0.5 M NaCl, 0.2% Tween 100), blots were incubated with primary antibodies for 1 h at room temperature. Following washes and 30 min of incubation with horseradish peroxidase-conjugated secondary antibodies, blots were visualized by an enhanced chemiluminescence detection system (NEN Life Science Products, Boston, Mass.). Actin protein levels were analyzed as a control for constant loading and transfer. Densitometry was determined by Kodak Digital Science 1D Image Analysis Software.

Pulse-chase analysis. After 24 h of transfection with HA-tagged $p21^{CIP1}$, ECV 304 cells were starved for 12 h in serum-free medium. Cells were washed with PBS and incubated for 40 min in DMEM depleted of methionine (DMEM-meth; ICN Biomedicals). Following trypsinization and neutralization, cells were suspended in DMEM-meth containing 2% BSA and 300 μCi of [^{35}S]methionine (Tran^{35}S-label; ICN Biomedicals) per ml for 40 min, allowing the [^{35}S]methionine to be incorporated. Unincorporated [^{35}S]methionine was removed by three washes with 20 ml of DMEM, and cells were subsequently resuspended in DMEM containing 2 mM unlabeled methionine (time point zero). The cells were immediately put onto dishes precoated with FN or BSA as described above. At the indicated times, cells were washed in cold PBS and frozen at −70°C. For immunoprecipitation, cells were lysed in PBS-TDS buffer for 15 min on ice and

then lysates were clarified by centrifugation at 13,000 rpm (Biofuge Fresco; Heraeus) for 20 min at 4°C. Furthermore, lysates were precleared for 1 h with 25 μl of protein G-Sepharose beads (Santa Cruz Biotech) at 4°C. A 200-μg sample of precleared lysates was added to protein G-Sepharose beads precoated with anti-HA MAb (12CA5) and precipitated for 2 h at 4°C. Following six washes with PBS-TDS, eluted proteins were separated by SDS–13% PAGE, fixed, and dried and the signal from radiolabeled HA-$p21^{CIP1}$ was quantified with a PhosphorImager (Cyclone Phosphor System; Packard Instrument Co., Meriden, Conn.) with OptiQuant Image Analysis Software.

Ubiquitination assay. The in vivo $p21^{CIP1}$ ubiquitination assay was carried out as described previously (48). ECV 304 cells were transfected with $p21^{CIP1}$, His-ubiquitin, or mock cDNA alone or cotransfected with $p21^{CIP1}$ and His-ubiquitin cDNA. After 24 h, cells were starved in serum-free medium for 12 h. These transfected cells were pretreated with or without β-lactone for 2 h at the end of the starvation period. Cells cotransfected with $p21^{CIP1}$ and His-ubiquitin were then allowed to attach to FN- or P-L-L-coated dishes for 30 min. Cells transfected with $p21^{CIP1}$, His-ubiquitin, or mock cDNA were used as controls.

For the ubiquitination assay, cells were lysed in 6 M guanidinium hydrochloride (GuaCl), 5 mM imidazole, and 100 mM sodium phosphate (NaP, pH 8.0) per 100-mm dish. Following sonication of lysates by three bursts, 50 μl of lysates was removed and used as a whole-cell lysate input control. His-ubiquitin-conjugated proteins were pulled down by the Ni^{2+}-agarose beads (Qiagen) for 2 h at 4°C in accordance with the manufacturer's instructions. The beads were loaded onto columns and sequentially washed with lysis buffer, 6 M GuaCl in NaP, 6 M GuaCl in protein buffer (50 mM NaP [pH 8.0], 20% glycerol, 0.2% Igepal, 100 mM KCl) containing 50% NaP, 6 M GuaCl in protein buffer containing 25% NaP, 10 mM imidazole in protein buffer, and 100 mM imidazole in protein buffer. Finally, bound proteins were eluted with 200 mM imidazole in protein buffer and precipitated with trichloroacetic acid as described by the manufacturer (Qiagen). Eluted proteins were separated by SDS-PAGE (upper part of gel,7%; lower part, 13%) and then transferred onto a PVDF membrane that was immunoblotted with anti-$p21^{CIP1}$ MAb SX118.

Isolation of total RNA and Northern blotting. Total cellular RNA was isolated with a Qiagen RNeasy kit in accordance with the manufacturer's protocol. For each sample, 10 μg of total RNA was denatured and separated in a formaldehyde-containing 1% agarose gel and then transferred to a BrightStar-Plus positively charged nylon membrane (Ambion, Austin, Tex.). After fixation and prehybridization with Prehyb/Hyb buffer (Ambion) at 65°C for 1 h, the membrane was hybridized with ^{32}P-labeled probes including human $p21^{CIP1}$ cDNA (provided by Steven I. Reed) and chicken actin cDNA in Prehyb/Hyb buffer at 42°C overnight. The membrane was washed twice for 15 min each time at room temperature in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA)–0.5% SDS, twice for 15 min each time at 37°C in 1× SSPE–0.5% SDS, and three times for 15 min each at 65°C in 0.1× SSPE–1% SDS. The extent of hybridization was analyzed with a PhosphorImager (Cyclone Phosphor System; Packard Instrument Co.) with OptiQuant Image Analysis Software.

RESULTS

Down-regulation of Cdk2 inhibitors by attachment to ECM. Previous studies have suggested that regulation of the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ by anchorage may function during cell cycle progression by controlling cyclin E-Cdk2 activity (11). To elucidate whether integrin-mediated cell adhesion, by itself, regulates the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$, ECV 304 bladder carcinoma cells were allowed to attach to various ECM proteins, including FN, VN, ColI, and LM, with P-L-L as a control. The cells were plated in the absence of serum to avoid any contribution from growth factor signaling in order to analyze exclusively the influence of anchorage-induced signaling. To this end, cells were also starved for 12 h before plating to eliminate any sustained growth factor signaling. Firstly, the morphology of ECV 304 cells attached to distinct ECM proteins and control P-L-L was observed (Fig. 1A). ECV 304 cells spread well on FN, VN, ColI, and LM within 30 min. However, cells plated on P-L-L attached but did not spread. This can be explained by the fact that attachment to P-L-L is mediated by charge in an unspecific manner and does not involve integrins. The effect by cell attachment to various ECM components on $p21^{CIP1}$ and $p27^{KIP1}$ protein levels was then determined by Western blotting (Fig. 1B and C). We found the $p21^{CIP1}$ and $p27^{KIP1}$ protein levels to be markedly reduced within 30 min when ECV 304 cells were attached to FN, VN, or ColI, while attachment to P-L-L did not influence $p21^{CIP1}$ or $p27^{KIP1}$ levels. In addition, we observed a similar down-regulation of $p21^{CIP1}$ and $p27^{KIP1}$ by attachment to ECM upon serum-free replating of regularly cultured, nonstarved cells (data not shown). The levels of $p21^{CIP1}$ were also reduced by attachment to LM but with somewhat slower kinetics compared to those seen after adhesion to the other ECM proteins (Fig. 1B and C). To test whether integrin-mediated regulation of $p21^{CIP1}$ is similar in different cell types, $p21^{CIP1}$ levels were also analyzed in primary HUVECs and in murine NIH 3T3 fibroblasts. The levels of $p21^{CIP1}$ protein were rapidly decreased in HUVECs by attachment to FN, VN, or ColI within 30 min and were kept low for at least 4 h on FN (Fig. 1D). Attachment to LM also decreased $p21^{CIP1}$ levels in HUVECs but within 60 min, while attachment to P-L-L did not influence the levels of $p21^{CIP1}$ (Fig. 1D). Furthermore, a similar down-regulation of $p21^{CIP1}$ was observed in NIH 3T3 fibroblasts plated on FN (data not shown). Taken together, our results demonstrate that cell attachment to ECM initiates down-regulation of $p21^{CIP1}$ in various cell types, both transformed cells and primary cells of different origin, suggesting that this down-regulation constitutes a general phenomenon regardless of the cell type.

Integrin ligation induces down-regulation of Cdk2 inhibitors. To investigate whether cell attachment-induced down-regulation of $p21^{CIP1}$ and $p27^{KIP1}$ is mediated specifically by integrin ligation, ECV 304 cells were plated on immobilized anti-α5β1, anti-β1, or anti-αvβ3 MAbs. Under these conditions, these anti-integrin MAbs act as agonistic integrin-ligating proteins, as shown previously (43). Cells plated on anti-β1 or anti-αvβ3 MAb both attached and spread within 30 min, whereas cells plated on anti-α5β1 MAb attached but did not spread within 30 min (Fig. 2A). Interestingly, the $p21^{CIP1}$ and $p27^{KIP1}$ proteins were almost eliminated by specific ligation to all three of these anti-integrin MAbs within 30 min (Fig. 2B), suggesting that specific-integrin ligation results in rapid down-regulation of the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$. Because attachment to P-L-L did not induce any change in the levels of the Cdk2 inhibitors (Fig. 2B), we can exclude the action from any additional factors coinciding with integrin ligation. Also, given that attachment to the anti-α5β1 MAb did not cause cell spreading but still down-regulated $p21^{CIP1}$ and $p27^{KIP1}$, this down-regulation appears to be specific for integrin ligation regardless of cell spreading.

Attachment to FN decreases $p21^{CIP1}$ protein stability. Because down-regulation of $p21^{CIP1}$ by anchorage occurred as a general phenomenon in cells of various origins, we aimed to elucidate the underlying molecular mechanisms. To this end, we utilized one of the cell lines, ECV304, that is readily transfectable with high efficiency. To examine whether the rapid down-regulation of $p21^{CIP1}$ by cell attachment occurs at the mRNA level, Northern blotting was performed (Fig. 3A). We found that the $p21^{CIP1}$ mRNA levels were not influenced by attachment to FN within 60 min, indicating that the rapid anchorage-induced down-regulation of $p21^{CIP1}$ could not be explained by modulation of $p21^{CIP1}$ transcription or mRNA

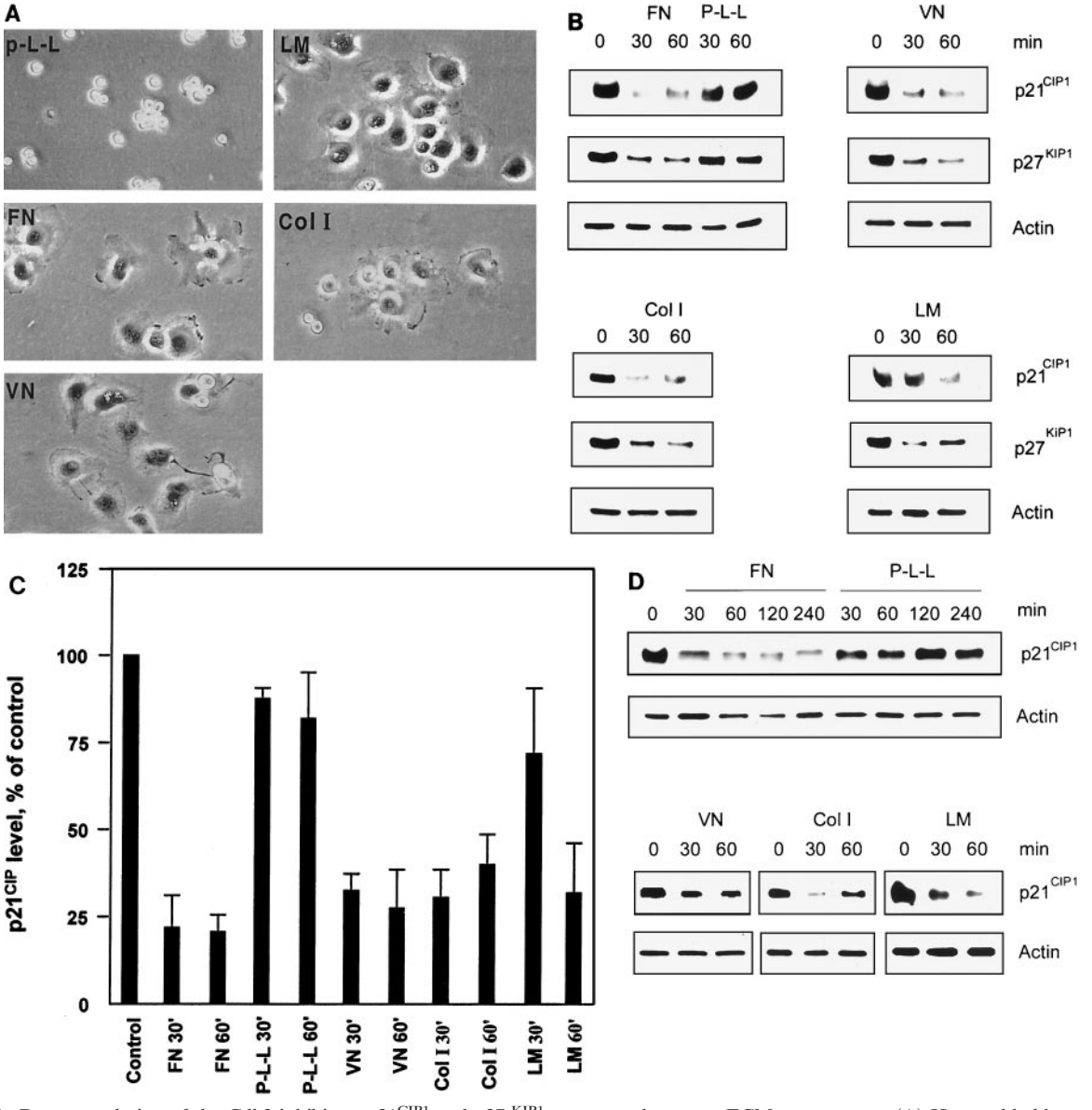

FIG. 1. Down-regulation of the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ upon attachment to ECM components. (A) Human bladder carcinoma ECV 304 cells were allowed to attach to dishes precoated with FN, VN, LM, ColI, or P-L-L under serum-free conditions. Photographs (20× objective) show the representative morphology of ECV 304 cells after attachment to the distinct ECM proteins for 30 min. (B) $p21^{CIP1}$, $p27^{KIP1}$, and actin protein levels in ECV 304 cell lysates were analyzed by Western blotting after attachment for the indicated times to different ECM components or P-L-L under serum-free conditions in order to analyze the exclusive influence of integrin ligation without intervening growth factor signaling. Actin levels were detected as a loading control. (C) Estimation of $p21^{CIP1}$ levels in ECV 304 cells by densitometry of Western blots. Bars represent mean values of three independent experiments ± the standard error of the mean. The level at time zero is defined as 100% (control). (D) HUVECs were allowed to attach to FN, P-L-L, VN, ColI, or LM for various times. Cell lysates were analyzed for $p21^{CIP1}$, $p27^{KIP1}$, and actin protein levels by Western blotting.

stability. However, another possibility is that $p21^{CIP1}$ might be controlled at the level of protein stability. To test this, the stability of the $p21^{CIP1}$ protein was analyzed by ^{35}S incorporation-based pulse-chase analysis. To successfully immunoprecipitate $p21^{CIP1}$, we transiently overexpressed HA-tagged $p21^{CIP1}$ in ECV 304 cells. We first examined the levels of HA-$p21^{CIP1}$ protein after attachment to FN and compared it to that in cells kept in suspension. By Western blotting, we found that the levels of HA-$p21^{CIP1}$ were down-regulated on FN within 30 min, similar to the regulation of endogenous $p21^{CIP1}$ (Fig. 3B). We then examined the stability of HA-$p21^{CIP1}$ by examining levels of ^{35}S-HA-$p21^{CIP1}$. We found that the stability of the HA-$p21^{CIP1}$ protein was markedly decreased in cells attached to FN compared to that of HA-$p21^{CIP1}$ protein in cells kept in suspension (Fig. 3C). These results suggest that integrin-mediated cell adhesion stimulates $p21^{CIP1}$ protein degradation.

Integrin-mediated cell attachment induces proteasomal degradation of $p21^{CIP1}$. Considering that the integrin-induced rapid down-regulation of $p21^{CIP1}$ coincided with changed pro-

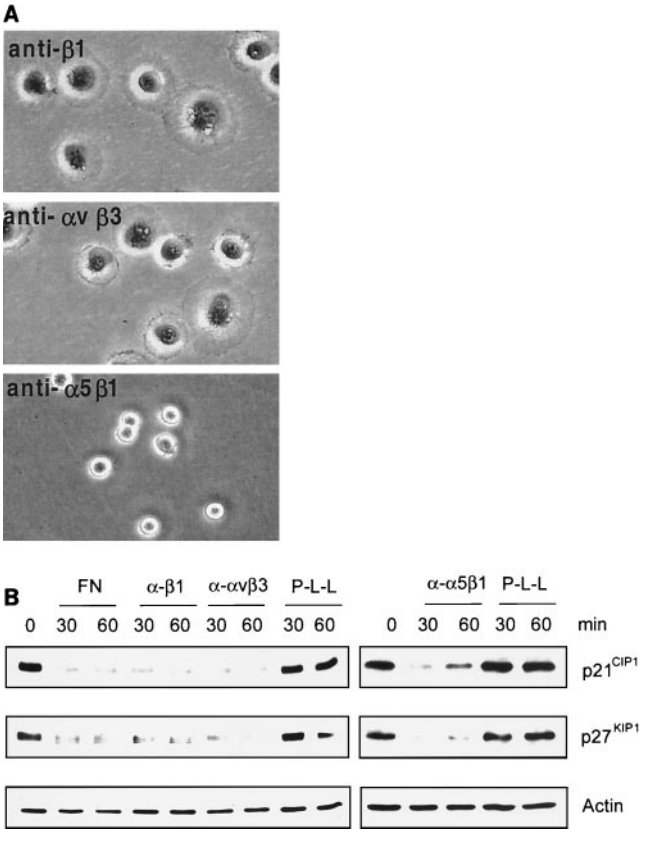

FIG. 2. Integrin ligation specifically down-regulates $p21^{CIP1}$ and $p27^{KIP1}$. (A) ECV 304 cells were plated on immobilized anti-β1 (P4C10), anti-αvβ3 (LM609), or anti-α5β1 (JBS5) for 30 min and photographed with a 20× objective. (B) ECV 304 cells attached to immobilized anti-β1, anti-αvβ3, or anti-α5β1 MAb were analyzed for $p21^{CIP1}$, $p27^{KIP1}$, and actin protein levels by Western blotting.

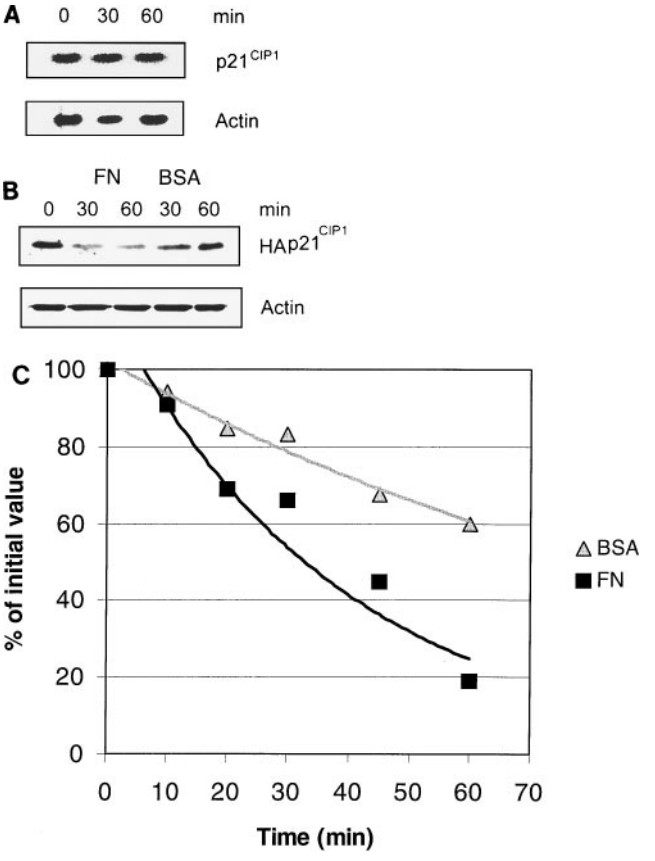

FIG. 3. Cell attachment to FN affects $p21^{CIP1}$ protein stability. (A) Total mRNA isolated from ECV 304 cells previously plated on FN for the indicated times was analyzed for $p21^{CIP1}$ mRNA levels by Northern blotting. β-Actin mRNA levels were measured as a loading control. (B) ECV 304 cells transiently transfected with HA-tagged $p21^{CIP1}$ were allowed to attach to FN or suspended upon BSA, and then HA-$p21^{CIP1}$ levels were analyzed by Western blotting. Actin levels were used as a loading control. (C) ECV 304 cells transfected with HA-$p21^{CIP1}$ were plated on FN or kept in suspension (BSA), and then the stability of HA-$p21^{CIP1}$ was detected by pulse-chase analysis with incorporated [^{35}S]methionine. The values shown are the means of two or three independent experiments.

tein stability, we hypothesized that Cdk2 inhibitors might be proteolyzed as a response to integrin ligation. In fact, both $p21^{CIP1}$ and $p27^{KIP1}$ have previously been found to be targeted by proteasomal degradation (1, 31, 40, 45). Therefore, we analyzed whether $p21^{CIP1}$ and $p27^{KIP1}$ are subjected to proteasomal degradation upon attachment to FN. For this, we utilized three specific proteasome inhibitors, lactacystin, β-lactone, and LLnL. Interestingly, the reduction of $p21^{CIP1}$ and $p27^{KIP1}$ by adhesion to FN was completely blocked by each of these inhibitors (Fig. 4A) while cells did not display any obvious defects and spread normally (data not shown). This indicates that integrin-mediated attachment to FN activates proteasomal proteolysis of $p21^{CIP1}$ and $p27^{KIP1}$.

To assess whether other cell cycle regulators known to be targeted by proteasomes are also regulated by integrin-mediated cell attachment, the levels of cyclin E and p53 were examined in the same experiments as the Cdk2 inhibitors (Fig. 4B). Interestingly, cyclin E and p53 protein levels were not changed by attachment to FN within 60 min, suggesting that integrin ligation selectively induces degradation of $p21^{CIP1}$ and $p27^{KIP1}$.

Integrin-mediated proteasomal degradation of $p21^{CIP1}$ is independent of ubiquitination. Targeted proteolysis by proteasomes preceded by ubiquitination regulates diverse biological systems (36). To analyze whether integrin-mediated cell attachment regulates ubiquitination of $p21^{CIP1}$, potential ubiquinated-$p21^{CIP1}$ conjugates were detected by Western blotting (Fig. 5A). We found that native $p21^{CIP1}$ was degraded by attachment to FN and that this degradation was blocked by either lactacystin or LLnL. In addition, inhibition of proteolysis by these inhibitors led to an increase in high-molecular-weight bands of $p21^{CIP1}$. The rapid appearance in the presence of proteasome inhibitors and the pattern of these bands, which appear similar to previously described polyubiquitinated $p21^{CIP1}$ (42), indicate that these bands may represent ubiquitinated $p21^{CIP1}$. However, we did not detect any differences in the degree of $p21^{CIP1}$-associated high-molecular-weight bands after specific attachment to FN compared to nonspecific adhesion to P-L-L. To further clarify if the degree of $p21^{CIP1}$ ubiquitination was affected by cell attachment, a more specific ubiquitination assay was performed to detect ubiquitinated $p21^{CIP1}$ conjugates. $p21^{CIP1}$ and His-ubiquitin were cotrans-

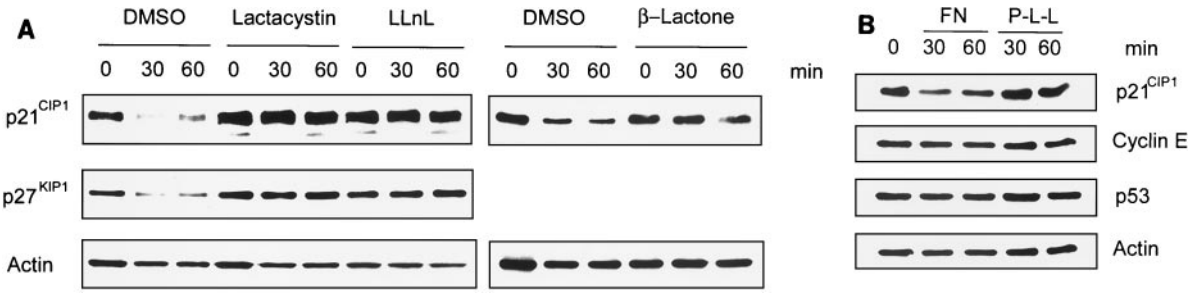

FIG. 4. Proteasome inhibitors block FN-induced down-regulation of $p21^{CIP1}$ and $p27^{KIP1}$. (A) ECV 304 cells were pretreated with lactacystin, β-lactone, or LLnL or with the dimethyl sulfoxide (DMSO) vehicle as a control and then plated on FN for the indicated times. $p21^{CIP1}$, $p27^{KIP1}$, and actin protein levels were analyzed by Western blotting. (B) ECV 304 cells were allowed to attach to FN or P-L-L, and then the levels of cyclin E and p53 were examined by Western blotting. The levels of $p21^{CIP1}$ were used as a comparison and determined with anti-$p21^{CIP1}$ MAb SX118.

fected into ECV 304 cells, and His-ubiquitin was then trapped by an Ni^{2+} column, followed by Western blotting for $p21^{CIP1}$, specifically detecting ubiquitinated $p21^{CIP1}$ (Fig. 5B). No ubiquitinated bands appeared in cells transfected with $p21^{CIP1}$ or mock transfected, even in the presence of β-lactone. However, intensive bands representing ubiquitinated $p21^{CIP1}$ were found when ECV 304 cells were cotransfected with $p21^{CIP1}$ and His-ubiquitin in the presence of β-lactone compared to non-inhibitor treatment. These bands appeared similar in size and pattern to the $p21^{CIP1}$-associated bands in Fig. 5A, suggesting that the high-molecular-weight bands in both of these experiments represent ubiquitinated $p21^{CIP1}$. Interestingly, we found no difference in the levels of accumulated ubiquitin-$p21^{CIP1}$ bands between cells attached to FN and those attached to P-L-L, suggesting that proteasomal proteolysis of $p21^{CIP1}$ induced by specific attachment to FN is not preceded by increased ubiquitination. Importantly, the levels of a ubiquitination-deficient $p21^{CIP1}$ mutant (p21K6R) (42, 47) were also reduced upon attachment to FN in a manner similar to that of wild-type p21 (Fig. 5C). Furthermore, this reduction of p21K6R was completely blocked by lactacystin (Fig. 5C), demonstrating that integrin-activated proteasomal proteolysis of $p21^{CIP1}$ is independent of ubiquitination.

Cell attachment to FN activates Cdc42 and Rac1. The small GTPases Cdc42 and Rac1 can be activated in response to integrin ligation to FN and collagen (7, 19, 33). To investigate potential activation of Cdc42 and Rac1 in ECV 304 cells by adhesion to FN, we used a GST-PAK1-CRIB domain fusion protein pull-down assay to quantify activated Cdc42 and Rac1. As shown in Fig. 6A, both Cdc42 and Rac1 were activated by adhesion to FN within 20 min, indicating that both of these signaling components are activated by an integrin-dependent mechanism in ECV 304 cells. However, both Rac1 and Cdc42 activities (GTP-Rac1 and GTP-Cdc42) induced by cell attachment were completely inhibited by transient expression of a dn N17Cdc42 mutant, which indicates that blocking of Cdc42 might lead to inhibition of Rac1 activation by cell attachment. Meanwhile, expression of a dn N17 Rac1 mutant blocked Rac1 activity and led to somewhat decreased endogenous levels of active Cdc42. However, Cdc42 was still activated by cell attachment to FN in the presence of dn N17Rac1 (Fig. 6A). Taken together, this could be interpreted as induction of a pathway by cell attachment in which Cdc42 is upstream of Rac1. Alternatively, dn Cdc42 and dn Rac1 might bind to and sequester the same GTP exchange factor(s) needed for activation of both Cdc42 and Rac1 but bind to these exchange factors with different affinities. In any case, our results demonstrate that attachment to FN leads to rapid activation of both Cdc42 and Rac1.

To examine whether dn Cdc42 or dn Rac1 may also block the capacity of cells to activate other integrin-dependent signaling components, we analyzed the degree of activated FAK and ERK1/2 in ECV 304 cells transfected with dn Cdc42 or dn Rac1 mutants upon attachment to FN (Fig. 6B). Both phospho-FAK^{Y397} and phospho-ERK1/2 appeared upon attachment to FN within 60 min. Importantly, induction of phospho-FAK^{Y397} and phospho-ERK1/2 was also detected in the presence of dn Cdc42 or dn Rac1 after attachment to FN, indicating that these integrin-induced signaling components are still activated by attachment when Cdc42 and Rac1 are blocked within our system.

An anchorage-activated Cdc42/Rac1 signaling pathway mediates down-regulation of $p21^{CIP1}$. It has been suggested that both Cdc42 and Rac1 play critical roles in progression through the G_1 phase of the cell cycle (23, 25, 29). However, it is not entirely clear how the effects on the cell cycle of endogenous Cdc42 and Rac signaling could be executed in terms of regulation of specific cell cycle components. To explore this, we investigated if Cdc42 and/or Rac1 might regulate $p21^{CIP1}$. Transient overexpression of ca Cdc42 or ca L61Rac1 promoted reduction of $p21^{CIP1}$ levels compared to those in a mock-transfected control (Fig. 7A), indicating that both of these GTPases are able to down-regulate $p21^{CIP1}$. To elucidate the role of endogenous Cdc42 and Rac1 in the integrin-dependent regulation of $p21^{CIP1}$, the dn N17Cdc42 and dn N17Rac1 mutants were utilized. Transient overexpression of N17Cdc42 or N17Rac1 blocked the reduction of $p21^{CIP1}$ caused by adhesion to FN (Fig. 7B). Moreover, overexpression of N17Cdc42 increased the stability of $p21^{CIP1}$ in cells attached to FN (Fig. 7C), indicating that integrin-induced signaling through Cdc42 increases the proteolysis rate of $p21^{CIP1}$. Taken together, these results indicate that Cdc42/Rac1 signaling is required for integrin-mediated $p21^{CIP1}$ degradation through proteasomal proteolysis.

On the basis of analysis of integrin-induced Cdc42 and Rac1 signaling (Fig. 6A), we hypothesized that Cdc42 might act upstream of Rac1 in the integrin-induced signaling pathway, leading to degradation of $p21^{CIP1}$. To examine whether Cdc42

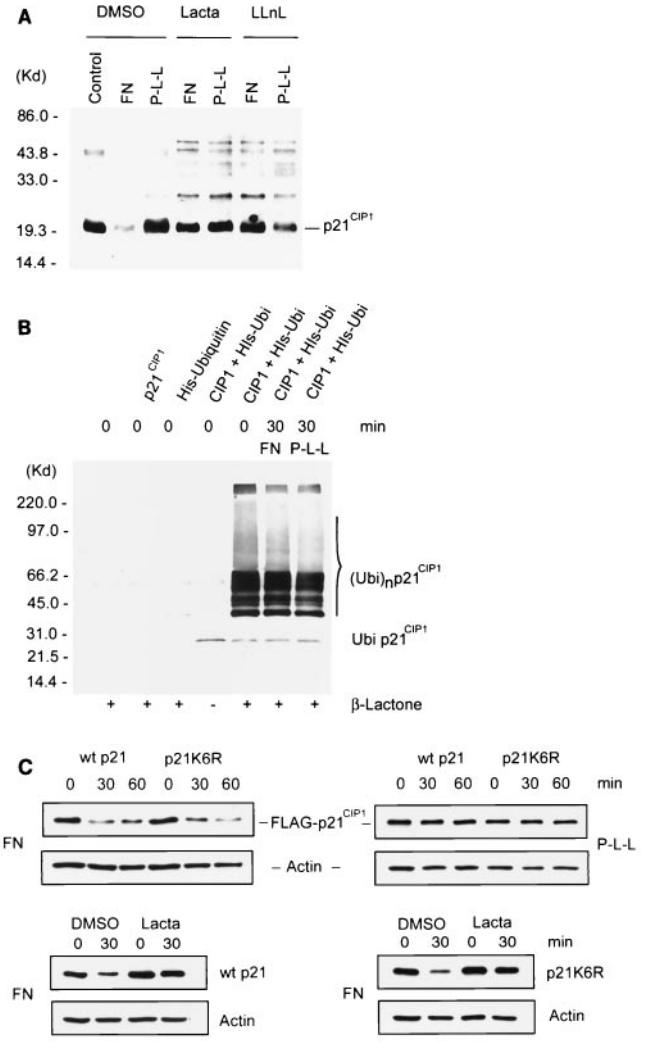

FIG. 5. Ubiquitination of $p21^{CIP1}$ upon cell attachment. (A) ECV 304 cells were treated with the proteasome inhibitor lactacystin (Lacta) or LLnL and then plated on FN or P-L-L for 30 min. Cell lysates were analyzed by Western blotting with anti-$p21^{CIP1}$ PAb (Ab5; Oncogene). DMSO, dimethyl sulfoxide; MW, molecular mass; Kd, kilodaltons. (B) ECV 304 cells transiently mock transfected or transfected with $p21^{CIP1}$ or His-ubiquitin or cotransfected with $p21^{CIP1}$ and His-ubiquitin were treated with or without the proteasome inhibitor β-lactone, as indicated. Cells cotransfected with $p21^{CIP1}$ and His-ubiquitin were allowed to attach to FN or P-L-L, and then His-ubiquitin was trapped by an Ni^{2+} column and ubiquitinated $p21^{CIP1}$ was detected with anti-$p21^{CIP1}$ MAb SX118 by Western blotting. (C, upper portion) ECV 304 cells transiently transfected with FLAG-tagged wild-type (wt) p21 or with the ubiquitination-deficient FLAG-tagged p21K6R mutant were plated on FN or P-L-L for the times indicated. The levels of overexpressed $p21^{CIP1}$ were detected by Western blotting with anti-FLAG MAb (M2; Sigma). Actin levels were analyzed as a loading control. (C, lower portion) ECV 304 cells transiently transfected with FLAG-tagged wild-type p21 or the FLAG-tagged p21K6R mutant were treated with the specific proteasome inhibitor lactacystin or with the dimethyl sulfoxide vehicle and plated on FN for 30 min. The levels of wild-type p21 and p21K6R were determined by Western blotting with anti-FLAG MAb (M2).

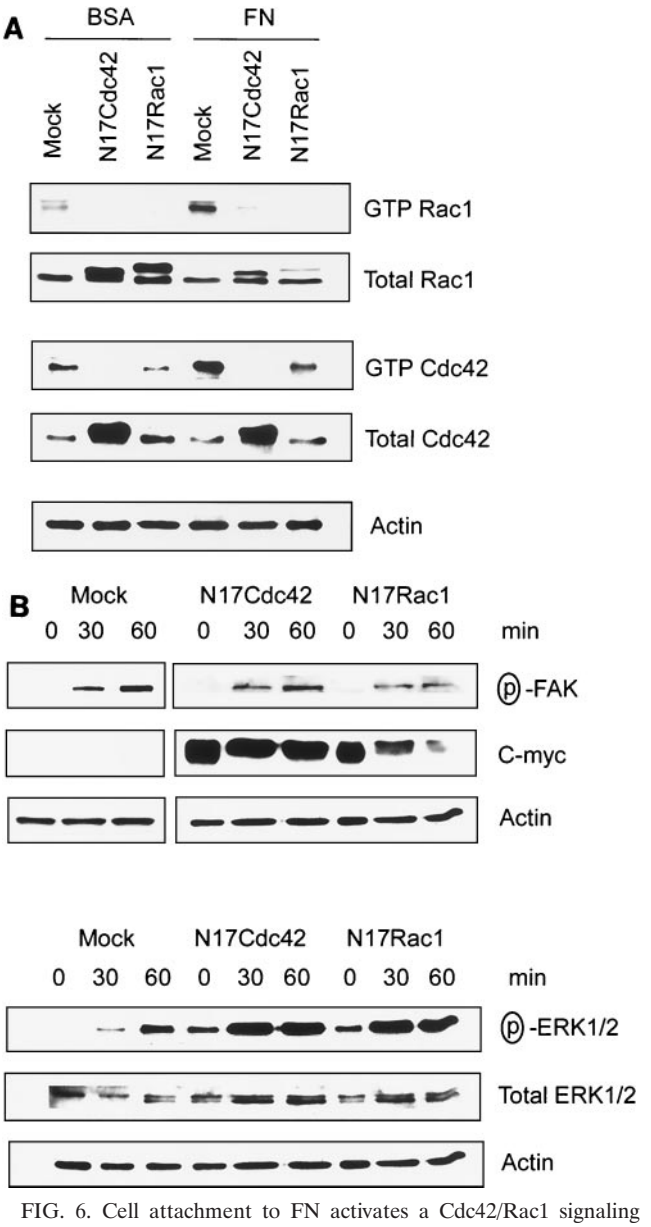

FIG. 6. Cell attachment to FN activates a Cdc42/Rac1 signaling pathway. (A) ECV 304 cells transiently transfected with dn N17Cdc42 or dn N17Rac1 cDNA or mock transfected were plated on FN for 20 min. Cell lysates were analyzed for active GTPases by binding to a GST-PAK-CRIB domain fusion protein, followed by Western blotting with anti-Rac1 or anti-Cdc42 antibodies. Total levels of Rac1, Cdc42, and actin were analyzed by Western blotting of original cell lysates. (B) ECV 304 cells transfected with Cdc42 or dn Rac1 cDNA or mock transfected were allowed to attach to FN for the indicated times, and then activated FAK and ERK1/2 were analyzed by Western blotting with anti-phospho-FAK^{Y397} PAb or anti-phospho-ERK1/2 PAb. The levels of c-myc were detected with anti-c-Myc MAb 9E10. Actin levels were analyzed as a loading control.

and Rac1 are linked into the same pathway for the regulation of $p21^{CIP1}$ and to elucidate their internal order, we transiently transfected cells with combinations of wild-type Cdc42 and dn Rac1 or ca Rac1 and dn Cdc42, respectively (Fig. 7D). Overexpression of wild-type Cdc42 was used to enhance the response of Cdc42 signaling after cell attachment. This was performed in order to test if blocking of Rac1 would block Cdc42-induced down-regulation of $p21^{CIP1}$, i.e., if Cdc42 and Rac1 are ordered into the same integrin-induced signaling pathway and if Rac1 acts downstream of Cdc42 in this pathway. The

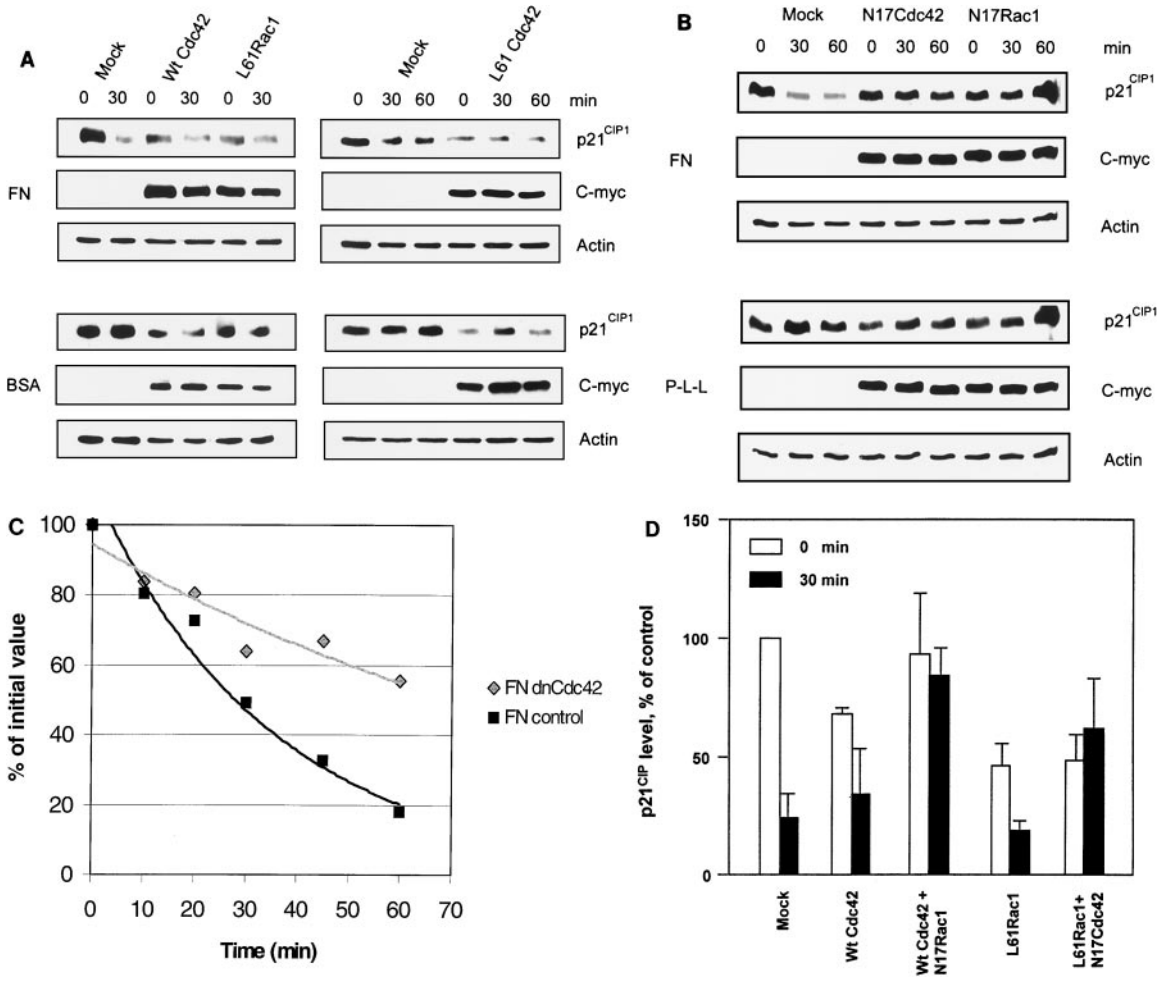

FIG. 7. Anchorage-dependent Cdc42/Rac1 signaling regulates proteolysis of $p21^{CIP1}$. (A) ECV 304 cells transiently transfected with c-myc-tagged wild-type (Wt) Cdc42 or c-myc-tagged mutant ca Cdc42 or ca L61Rac1 were plated on FN- or BSA-coated plates for the indicated times. Levels of $p21^{CIP1}$, c-myc, and actin were analyzed by Western blotting. Levels of c-myc were analyzed for transfection efficiency with anti-c Myc MAb 9E10, and actin was used as a loading control. (B) ECV 304 cells transiently overexpressing c-myc-tagged mutant dn N17Cdc42 or dn N17Rac1 were plated on FN or P-L-L. $p21^{CIP1}$, c-myc, and actin levels were analyzed by Western blotting. (C) ECV 304 cells transiently transfected with HA-$p21^{CIP1}$ or cotransfected with HA-$p21^{CIP1}$ and dn Cdc42 were allowed to attach to FN for the times indicated, and then the stability of HA-$p21^{CIP1}$ was examined by pulse-chase analysis. (D) Quantitative estimations of $p21^{CIP1}$ levels in ECV 304 cells by densitometry of Western blots from single mock transfections or transfections with L61Rac1 or wild-type Cdc42 or cotransfections with ca L61Rac1 and dn N17Cdc42 or wild-type Cdc42 and dn N17Rac1, respectively, before and 30 min after plating onto FN. Bars represent mean values of three independent experiments ± the standard error of the mean.

combination of ca L61Rac1 and dn N17Cdc42 was used to test if blocking of Cdc42 could block the effects of L61Rac1, which would reveal if Rac1 acts upstream of Cdc42 in the pathway. Coexpression of ca L61Rac1 and dn N17Cdc42 resulted in a low initial level of $p21^{CIP1}$ that was comparable to levels in cells transfected with L61Rac1 alone. However, in the cotransfected cells, $p21^{CIP1}$ levels were not markedly influenced by cell attachment to FN. This is consistent with the notion that L61Rac1 induces down-regulation of $p21^{CIP1}$ independently of Cdc42 but with N17Cdc42 blocking any additional decrease in $p21^{CIP1}$ caused by attachment to FN. Because N17Cdc42 did not block the initial down-regulation of $p21^{CIP1}$ induced by ca L61Rac1, Rac1 must act independently or downstream of Cdc42. Furthermore, transient transfection of dn N17Rac1 blocked the down-regulation of $p21^{CIP1}$ by attachment to FN even when wild-type Cdc42 was used to enhance the Cdc42 signaling response (Fig. 7D). Because N17Rac1 was not able to block the attachment-induced activation of endogenous Cdc42 (Fig. 6A), its capacity to block down-regulation of $p21^{CIP1}$ by attachment to FN in the presence of overexpressed wild-type Cdc42 indicates that Rac1 acts downstream of Cdc42 in regulating $p21^{CIP1}$ degradation. Taken together, our results point to a novel mechanism linking integrins, Cdc42, and Rac1 into the same signaling pathway, leading to $p21^{CIP1}$ proteolysis, with Cdc42 ordered upstream of Rac1 within the pathway.

DISCUSSION

Integrin ligation to ECM components leads to the activation of a variety of signaling pathways. These signals are essential for cell proliferation in all tissue cells, suggesting that integrin-activated signaling events regulate key cell cycle components that are important for the control of cell cycle progression (13).

To this end, we found that cell attachment to FN induced proteasomal proteolysis of the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$.

Cell adhesion to FN, in some cases, engages both integrins and additional receptors, including syndecans (40). However, we used a purified 120-kDa RGD-containing cell-binding fragment of FN for which integrins are the only identified cellular receptors and that does not contain the heparin-binding domain involved in syndecan engagement (37). Furthermore, attachment to three specific immobilized agonistic anti-integrin MAbs gave the same response on the regulation of Cdk2 inhibitor levels as adhesion to FN. This occurred while attachment to P-L-L under the same conditions in the presence of all other potential regulators did not influence $p21^{CIP1}$ or $p27^{KIP1}$ levels, showing that integrin ligation is specifically responsible for the down-regulation of $p21^{CIP1}$ and $p27^{KIP1}$. Besides cell adhesion, cell spreading is also an important factor in regulation of cellular functions, including cell proliferation and survival (5). Although cell spreading could induce down-regulation of $p27^{KIP1}$ (18), our results showed that both $p21^{CIP1}$ and $p21^{KIP1}$ levels were strongly reduced by attachment to an immobilized anti-$\alpha5\beta1$ MAb to which the cells adhered but did not spread. This suggests that the degradation of $p21^{CIP1}$ and $p27^{KIP1}$ is influenced primarily by specific integrin-induced signaling and not by the degree of cell spreading.

Integrin ligation to the ECM also plays an important role in the control of anchorage-dependent cell proliferation by facilitating progression through the G_1 phase of the cell cycle (31). To this end, cell anchorage regulates the levels and/or activity states of various cell cycle components in the G_1 phase of the cell cycle, including cyclins D and E, Rb, p53, and the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ (11, 25, 45, 49, 52). Importantly, cell anchorage promotes the activity of cyclin E-Cdk2 kinase by controlling Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ (11), suggesting that suppression of Cdk2 inhibitor levels by cell attachment is required during the cyclin E-Cdk2 point in late G_1 phase and is needed until this point is passed. In fact, down-regulation of $p27^{KIP1}$ in late G_1 phase has been identified as central for S-phase entry (43). Taken together, this suggests that regulation of the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ by cell attachment is important for progression through late G_1 phase. In addition to binding and blocking of cyclin E-Cdk2 activity in G_1 phase, $p21^{CIP1}$ also associates with proliferating-cell nuclear antigen, thereby inhibiting DNA replication (12). Furthermore, $p21^{CIP1}$ can assert an inhibitory function in the G_2/M transition (27). However, it is unclear if the regulation of $p21^{CIP1}$ by cell anchorage may influence these functions of $p21^{CIP1}$ in the S and G_2/M phases of the cell cycle.

Our finding that cell attachment regulates proteasomal proteolysis of $p27^{KIP}$ might be predicted, since proteasomal degradation is considered the major $p27^{KIP1}$ level regulation pathway (32, 44). However, regulation of $p21^{CIP1}$ has been associated mostly with transcriptional control, including the induction of $p21^{CIP1}$ by p53 that causes cell cycle arrest after UV irradiation, and the regulation of $p21^{CIP1}$ in fibroblasts by cell anchorage in the presence of serum factors, where no changes in protein stability were detected (2, 10, 11, 52). However, our experiments that were performed without serum in order to examine the exclusive effect of integrin-mediated signaling showed that integrin ligation to ECM per se did not rapidly influence mRNA levels. Instead, we found that $p21^{CIP1}$ is regulated by ECM at the level of proteolysis since the stability of $p21^{CIP1}$ was decreased by attachment to ECM and the down-regulation of $p21^{CIP1}$ could be blocked by three distinct specific proteasome inhibitors. Although we cannot exclude the possibility of an additional contribution by regulation of the $p21^{CIP1}$ translational rate, our results that demonstrate that cell attachment to the ECM induces rapid proteasomal degradation of $p21^{CIP1}$ is the first example of regulation of proteasomal proteolysis by integrin signaling. The difference in the regulation of $p21^{CIP1}$ stability observed by us compared to the study by Bottazzi et al. (2) in a more complex setup may depend on the different methods and/or cell types used, where we examined the isolated effects of integrin signaling. Our setup, analyzing the exclusive effects of cell anchorage, may limit our conclusions to situations with very active integrin signaling, such as after replating of cells onto the ECM. However, the effects of such signals are likely to represent a contribution by integrins also in more complex situations.

In most studied cases, proteasomal proteolysis is preceded by ubiquitination, where targeted proteins covalently link to multiple ubiquitin molecules and form ubiquitinated protein conjugates, leading to rapid degradation by 26S proteasomes (34). However, certain proteins, including ornithine decarboxylase and $p21^{CIP1}$, do not require ubiquitination for proteasomal processing (26, 42). In the case of $p21^{CIP1}$, the lack of a need for ubiquitination may be explained by the binding of the C terminus of $p21^{CIP1}$ directly to the C8-α subunit of the 20S proteasome complex, leading to degradation of $p21^{CIP1}$ (47). To this end, our findings indicate that the activation of proteasomal degradation of $p21^{CIP1}$ by specific integrin-mediated cell anchorage is not preceded by increased ubiquitination. Importantly, the $p21^{CIP1}$ mutant p21K6R, lacking all of the potential ubiquitination lysine residues, was still degraded upon attachment to FN, a degradation that was blocked by the specific proteasome inhibitor lactacystin. This suggests that integrin-induced proteolysis of $p21^{CIP1}$ may represent a physiological proteasomal pathway that is independent of ubiquitination.

Several integrin-activated signaling pathways promote cell proliferation, including FAK, mitogen-activated protein kinase (ERK1/2), PI-3K, and integrin-linked kinase (13). However, a FAK, MEK1/ERK1/2, or PI-3K block in our system did not reverse the integrin-induced down-regulation of $p21^{CIP1}$ (W. Bao and S. Strömblad, unpublished data). Instead, we linked this down-regulation of $p21^{CIP1}$ to signaling by the small GTPases Cdc42 and Rac. One feature of these GTPases is that Cdc42 activation can induce subsequent activation of Rac (22, 28). This may be brought about by the exchange factor PIX, which is enriched in Cdc42- and Rac1-driven focal complexes and has been suggested to link Cdc42 to Rac activation by coupling of the Cdc42 effector PAK (22, 24, 34). Furthermore, it has been indicated that an attachment-activated Cdc42 block inhibits cell spreading, a function that could be restored by transfection with ca Rac, indicating that Rac may act downstream of Cdc42 in integrin signaling (33). However, activation of the Cdc42 and Rac1 signaling components in this putative integrin signaling pathway and their potential interdependence have not been thoroughly examined. To this end, by analysis of the interdependence of Cdc42 and Rac1 activation upon at-

tachment, and by cotransfecting various combinations of Cdc42 and Rac1 mutants for analysis of $p21^{CIP1}$ regulation, we found that integrin ligation coordinates Cdc42 and Rac1 signaling in the same pathway that regulates $p21^{CIP1}$, with Cdc42 ordered upstream of Rac1.

It is known that small GTPases influence the cell cycle in terms of regulation of specific cell cycle components since overexpression of ca Cdc42 and Rac mutants promotes cyclin D transcription and pRB hyperphosphorylation, induces E2F transcriptional activity, and contributes to S-phase entry (14). Integrin-activated Rac controls progression through the G_1 phase of the cell cycle by regulating cyclin D1 synthesis, while Rho affects the timing of cyclin D (25, 49). In addition, Rho and Ras signaling stimulates $p27^{KIP1}$ degradation and regulates $p21^{CIP1}$ expression in response to growth factors (15, 30, 46). However, although the small GTPases Rho, Rac, and Cdc42 may be functionally involved in G_1-phase progression (15, 25, 30, 49), it was not previously clear if small GTPases could affect Cdk2 inhibitors as a response to cell anchorage. Thus, our finding that integrin activation of endogenous Cdc42/Rac1 signaling induces proteasomal degradation of the Cdk2 inhibitor $p21^{CIP1}$ might contribute to clarification of the complex function of small GTPases in cell cycle progression.

Rho, Rac, and Cdc42 also regulate the assembly of multimolecular focal adhesion complexes that are associated with the formation of actin stress fibers, lamellipodia, and filopodia, respectively (28, 34). In addition, integrin-activated Cdc42 and Rac1 are involved in the regulation of cell spreading (7, 33). However, our results indicate that inhibition of Cdc42 or Rac1 did not block activation of FAK or ERK1/2 induced by cell attachment to FN. Considering also that down-regulation of $p21^{CIP1}$ and $p27^{KIP1}$ also occurred in the absence of cell spreading, we conclude that an integrin-induced signaling pathway through Cdc42/Rac1 specifically causes proteasomal proteolysis of $p21^{CIP1}$.

In conclusion, our results demonstrate that integrin-mediated cell attachment to the ECM induces proteasomal proteolysis of the Cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ and that degradation of $p21^{CIP1}$ is independent of ubiquitination. The integrin-induced Cdc42/Rac1 signaling pathway activates proteasomal degradation of $p21^{CIP1}$. Integrin regulation of proteasomal proteolysis might contribute to the control of anchorage-dependent cell proliferation.

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