The p42/p44 Mitogen-activated Protein Kinase Activation Triggers p27^Kip1 Degradation Independently of CDK2/Cyclin E in NIH 3T3 Cells*

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The p42/p44 mitogen-activated protein (MAP) kinase is stimulated by various mitogenic stimuli, and its sustained activation is necessary for cell cycle G1 progression and G1/S transition. G1 progression and G1/S transition also depend on sequential cyclin-dependent kinase (CDK) activation. Here, we demonstrate that MAP kinase inhibition leads to accumulation of the CDK inhibitor p27^Kip1 in NIH 3T3 cells. Blocking the proteasome-dependent degradation of p27^Kip1 impaired this accumulation, suggesting that MAP kinase does not act on p27^Kip1 protein synthesis. In the absence of extracellular signals (growth factors or cell adhesion), genetic activation of MAP kinase decreased the expression of p27^Kip1 as assessed by cotransfection experiments and by immunofluorescence detection. Importantly, MAP kinase activation also decreased the expression of a p27^Kip1 mutant, which cannot be phosphorylated by CDK2, suggesting that MAP kinase-dependent p27^Kip1 regulation is CDK2-independent. Accordingly, expression of dominant-negative CDK2 did not impair the down-regulation of p27^Kip1 induced by MAP kinase activation. These data demonstrate that the MAP kinase pathway regulates p27^Kip1 expression in fibroblasts essentially through a degradation mechanism, independently of p27^Kip1 phosphorylation by CDK2. This strengthens the role of this CDK inhibitor as a key effector of G1 growth arrest, whose expression can be controlled by extracellular stimuli-dependent signaling pathways.

Progression through the cell cycle involves the sequential activation and inhibition of cyclin-dependent kinases (CDKs).1 CDK activities are regulated by their association with cyclins and by phosphorylation/dephosphorylation events. More recently, small proteins (CDK inhibitors) that associate and inhibit the CDK-cyclin complexes were identified (1). Two gene families of CDK inhibitors are known, the CDK4- and CDK6-specific inhibitors of the INK4 family and the Kip/Cip inhibitors, which include p21^{Cip1} and p27^{Kip1}.

First identified in transforming growth factor-β-treated cells (2), p27^Kip1 accumulates in serum-starved and density-arrested cells as well as in response to anti-proliferative stimuli (3, 4). Its accumulation is associated to cell cycle arrest in G1 and/or to quiescence (5). Stimulation of quiescent cells by growth factors leads to p27^Kip1 down-regulation, this process being necessary for the G1/S transition (6). First considered as a CKI with broad specificity, p27^Kip1 now appears as an inhibitor of CDK2-cyclin E during the G1 phase. Its association with the CDK4-cyclin D1 complex does not inhibit this kinase and may even participate to its activation (see Ref. 1). Recently, a large number of clinical studies have linked a poor survival prognosis with low levels and reduced half-life of the p27^Kip1 protein in many tumor types (for reviews see Refs. 7 and 8). For these reasons, understanding the regulation of p27^Kip1 expression has received a peculiar attention during the last few years.

Various mechanisms of p27^Kip1 degradation have been described. The best characterized is the ubiquitin-dependent degradation by the proteasome. This involves Thr-187 phosphorylation by CDK2 (9–11), association with the F-box protein P65^Skp2 (12–14), ubiquitination (15, 16), and degradation by the proteasome in the cytoplasmic compartment. Translocation from the nucleus to the cytoplasmic compartment involves association with the protein Jab1 (17) and with the nuclear pore-associated protein mNPAP60 (18). Stable trimeric complex between CDK2, cyclin, and p27^Kip1 also appears necessary for further degradation (16). Although this pathway has been extensively dissected and is probably involved in various physiological process, other proteolytic mechanisms have been described. These include ubiquitin-independent degradation by the proteasome (19), caspase degradation in apoptotic (20, 21) and non-apoptotic (22) conditions, and degradation by calpain (23).

In addition, p27^Kip1 levels can be controlled by variations of protein synthesis. An increased rate of synthesis was described in HL-60 cells arrested in G0 by 12-O-tetradecanoylphorbol-13-acetate (24) or in fibroblasts arrested by contact inhibition (25), whereas a decrease of synthesis was observed upon stimulation of BALB/c 3T3 cells with platelet-derived growth factor (26). Transcriptional regulation of p27^Kip1 has been also reported (27–31).

Besides the level of p27^Kip1 regulation, the extracellular signal-regulated transduction pathways involved remain to be clarified. One of them is the phosphatidylinositol 3-kinase pathway; phosphatidylinositol 3-kinase pathway inhibitors increase the cellular level of p27^Kip1 in various cell types (32, 33), and the effects of the PTEN phosphatase, a negative regulator
of phosphatidylinositol 3-kinase pathway, occurrence through p27Kip1 (34). More recently, AFX-like forkhead transcription factors were found to regulate p27Kip1 gene transcription downstream of the Ras/phosphatidylinositol 3-kinase/protein kinase B pathway (28). In addition to the phosphatidylinositol 3-kinase pathway, the small G protein Rho was also described as a regulator of p27Kip1 expression (35–37).

Mitogenic stimulation by growth factors usually activates the p42/p44 MAP kinase through a pathway involving Ras, Raf, and MAP kinase kinase (MEK). Sustained activation of this pathway is necessary for G1 progression and DNA synthesis (38). Two well-characterized cell cycle targets of MAP kinase are cyclin D1 and p21Cip1 (for a review, see Ref. 39). This pathway has been also shown to affect p27Kip1 regulation in different conditions. In some studies, MAP kinase activation by constitutive Raf or MEK mutants led to p27Kip1 down-regulation (40–42). In other cases, similar activation did not modify p27Kip1 level but, rather, its association with CDKs (36, 43, 44).

For BrdUrd double-immunostaining, cells were treated with 50 μM BrdUrd (Sigma) for 4 h, rinsed with PBS, and fixed with 3.7% formaldehyde in 50 mM EDTA, 1% SDS, and 0.1% Tween 20 for 10 min. After washing, DNA was denatured by treatment with 1.5 N HCl for 10 min at room temperature. Cells were washed twice, incubated for 1 h at 37 °C with Alexa fluor antibodies, washed three times, and incubated with rhodamine-conjugated goat anti-mouse antibody. From that step, all the incubations were performed in the dark. Then the cells were washed twice, incubated for 1 h at 37 °C with Alexa fluor 488 goat anti-mouse. From that step, all the incubations were performed in the dark.

Regulation of p27Kip1 by MAP Kinase

Materials and Methods

Cell Culture—NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 5 g/liter glucose, 10% fetal calf serum, L-glutamine, and antibiotics. Serum deprivation experiments were performed in the presence of 0.5 or 0.2% serum in the medium depending on the time of the incubation. CCL64 cells were grown in RPMI 1640 medium containing 5 g/liter glucose, 10% fetal calf serum, L-glutamine, and 12 mM HCO3–, 10 mM KCl, and 5 mM Hepes (pH 7.05) for 20 min at room temperature.

Metabolic Labeling and Immunoprecipitation—In vivo labeling of proteins with [35S]methionine-cysteine was performed as follows. The cells were labeled for 1 or 2 h (150 μCi/ml [35S]Met-Cys in Dulbecco’s modified Eagle’s medium lacking methionine and cysteine) in the presence of 10 μM MG132. Denatured cell extracts were prepared before immunoprecipitation of p27Kip1 as described (48, 14) by resuspending cell pellets in 100 μl of denaturing buffer (50 mM Tris, pH 7.5, 0.5 mM EDTA, 1% SDS, 1 mM dithiothreitol). Samples were boiled for 10 min, and 800 μl of TNN buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.2 mM Na3VO4, 1 mM dithiothreitol, and 1 tablet of an anti-protease mixture) were added and incubated for 30 min on ice. Extracts were centrifuged, and appropriate volumes of supernatants were preincubated for 30 min at 4 °C with 25 μl of a 50% (w/v) slurry of protein G-Sepharose and protein A-Sepharose. The samples were centrifuged, and appropriate volumes of supernatants were added to primary antibody, monoclonal or polyclonal anti-p27Kip1 (5 μg/ml), for 1 h at 4 °C under shaking. After this incubation, 50 μl of protein A– or G-Sepharose were added to the sample for 2 additional hours. Immunoprecipitates were collected by centrifugation, washed 4 times in TNN buffer, twice in phosphate-buffered saline (PBS), and incubated in Laemmli sample buffer. Precipitated proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Western Blot Analysis—Cells were harvested in Laemmli sample buffer. Appropriate quantities of extracted proteins were resolved on a 10% SDS-polyacrylamide electrophoresis gel and transferred on a nitrocellulose membrane (20 V, 30 min). The membranes were then blocked by 5% nonfat milk and 1% bovine serum albumin in Tris-buffered saline with 0.05% Tween 20 (TBS-T) incubated for 1 h at 37 °C, washed three times in PBS, and blocked with 1% fetal bovine serum for 10 min at room temperature. After washing, DNA was denatured by treatment with 1.5 N HCl for 10 min at room temperature. Cells were rinsed twice and

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incubated with mouse monoclonal anti BrdUrd (1:50, DAKO) for 30 min at room temperature, rinsed, and incubated with fluorescein-conjugated goat anti-mouse (Alexa Fluor 488) for 30 min at room temperature. After washing, the coverslips were embedded with Moviol and mounted on slides. Fluorescence was recorded with a Zeiss axioskop microscope using a 40 × oil planapo lens.

Cell Cycle Analysis by Flow Cytometry—Cells were washed in PBS, harvested by trypsinization, and fixed in 70% ethanol. They were then treated with 0.1 mg/ml RNase A (Sigma). DNA was then stained with 200 μg/ml propidium iodide and quantified by flow cytometry with a FacsCalibur cytofluorometer (Beckton Dickinson).

Destruction Assays in Cell Extracts—NIH 3T3 cells were cultured for 24 h in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum or treated as described in Fig. 8. Extracts were prepared as follows. Cell pellets were washed twice with cold PBS, resuspended in cold hypotonic extraction buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol), and incubated on ice for 30 min. Next cells were disrupted with several strokes of a Dounce homogenizer, and the lysates were stored at −80 °C.

For destruction assays, extracts were supplemented with an energy-regenerating system (25 mM creatine and 10 mM dithiothreitol) and incubated on ice for 30 min. Next, cells were washed, and incubated with fluorescein-conjugated goat anti-MAP kinase antibody (Fig. 1). As expected, MAP kinase activity was detected by Western blot for the expression of p27kip1 (A) and for MAP kinase activity (in the case of NIH 3T3 cells) with an antibody against the phosphorylated active form of MAP kinase (P-MAPK) (B).

RESULTS

Inhibition of the MAP Kinase Pathway Leads to p27kip1 Accumulation in Asynchronous Cells

Chemical Inhibition of MEK Triggers p27kip1 Accumulation in Epithelial and Fibroblastic Cells—To investigate the role of MAP kinase on p27kip1 expression, we used the specific MEK inhibitor U0126. NIH 3T3 and CCL64 epithelial cells (CCL) were treated for different times with 10 μM MEK inhibitor U0126. Cells were harvested at different times (4, 8, and 12 h) and analyzed by Western blot for the expression of p27kip1 (A) and for MAP kinase activity (in the case of NIH 3T3 cells) with an antibody against the phosphorylated active form of MAP kinase (P-MAPK) (B).

As expected, treatment of cells with U0126 led to a strong decrease of MAP kinase activity. Western blot analysis of p27kip1 in these fractions (Fig. 1A) shows a 60% increase of the protein level in NIH 3T3 cells (130% increase after 24 h; not shown) and a 150% increase in CCL 64 cells after 12 h of treatment, suggesting that MAP kinase inhibition leads to p27kip1 accumulation in asynchronous epithelial and fibroblastic cells. After 12 h of treatment with U0126, 63% of NIH 3T3 accumulated in the G1 phase (versus 45% of the untreated control cells), as determined by flow cytometry (not shown).

A Dominant-negative Mutant of MEK Increases the Ectopic Expression of Human p27kip1 in NIH 3T3 Cells—To confirm this observation, a dominant negative mutant of the MAP kinase-activating kinase MEK1 (MEK(SA)) was overexpressed in NIH 3T3 cells. Since the transfection efficiency in these cells was not high enough to detect variations of the endogenous p27kip1, by Western blot, we performed co-transfections of human p27kip1 with the MEK mutant. Under these conditions, each cell transfected with the p27kip1 expression vector also received the MEKSA construct. Cells were harvested after 24 h of transfection and processed for Western blot analysis (Fig. 2). Overexpression of the dominant negative version of MEK induced a weak decrease of endogenous MAP kinase activity (Fig. 2B), as detected by Western blot with a phospho-MAP kinase antibody. This modest effect reflects the percentage of transfected cells (20–30%) obtained in our experiments. Nevertheless, the ectopic expression of human p27kip1 was significantly increased (2–3-fold increase) by cotransfection with the dominant-negative MEK mutant (Fig. 2A). This confirms the results obtained with the chemical inhibitor U0126. Note that in this and all subsequent experiments dealing with ectopic p27kip1 overexpression, the signal corresponding to endogenous p27kip1 is masked by the high overexpression of ectopic human p27kip1. Altogether these data suggest that inhibiting the MAP kinase pathway triggers p27kip1 accumulation in two different cell types.

MAP Kinase Activation Down-regulates p27kip1 in the Absence of Growth Factors or Cell Adhesion

A large panel of extracellular signals including growth factors and cell anchorage lead to MAP kinase activation. We tested the ability of the MAP kinase pathway to down-regulate p27kip1 expression in conditions where extracellular mitogenic signals were suppressed, in the absence of growth factors (serum starvation) and of cell-matrix interaction (cells grown in suspension).

Co-expression with Constitutively Active MEK Decreases Ectopic p27kip1 Expression in NIH 3T3 Cells—Cotransfections of p27kip1 with constitutively active mutants of MEK or B-Raf were performed in NIH 3T3 cells. Cells were then either serum-starved or placed in suspension for 24 h. p27kip1 expression and MAP kinase activity were detected by Western blot analysis. Serum starvation as well as anchorage disruption decreased the MAP kinase activity to basal levels in NIH 3T3 cells (not shown). This activity was restored by the expression of B-Raf.
CAAX or MEKD\textsuperscript{DD} constructs (Fig. 3, A and B, lower panels). By comparison, serum starvation and anchorage disruption also decreased the protein kinase B/Akt activity, but expression of the B-Raf-CAAX and MEKD\textsuperscript{DD} mutants did not restore this activity (not shown). In cells overexpressing the B-Raf and MEK mutants, p27\textsuperscript{kip1} expression was significantly reduced (a 3–4-fold decrease) both in serum-starved (Fig. 3A) and in suspended cells (Fig. 3B). In the absence of serum, the dominant-negative version of MEK had no effect on p27\textsuperscript{kip1} levels (Fig. 3A), likely due to the fact that MAP kinase activity was already low in serum-starved fibroblasts. This was also the case in anchorage-deprived cells (not shown). These data demonstrate that MAP kinase activation can lead to reduction of p27\textsuperscript{kip1} levels independently of other signaling pathways stimulated by either growth factors or cell adhesion.

**Down-regulation of Endogenous p27\textsuperscript{kip1} by Expression of Constitutively Active MEK** —To confirm these data, immunofluorescence labeling of endogenous p27\textsuperscript{kip1} was performed on serum-starved 3T3 cells transfected with MEKD\textsuperscript{DD}. First we verified that serum starvation-dependent accumulation of p27\textsuperscript{kip1} could be detected by this method. As shown in Fig. 4A, serum deprivation of nontransfected cells led to a clear increase of p27\textsuperscript{kip1} immunostaining. When transfection was performed with the constitutively active MEK mutant, an important ratio (50%) of cells expressing this mutant contained very low levels of p27\textsuperscript{kip1} by comparison with the neighboring nontransfected cells (Fig. 4B). As a control, we found no variation of p27\textsuperscript{kip1} expression in cells transfected with HA-tagged the regulatory B56 \textsuperscript{α} subunit of the PP2A phosphatase, a protein unrelated with p27 Kip1 regulation.

**MAP Kinase Modifies the Rate of p27\textsuperscript{kip1} Degradation**

To investigate how MAP kinase affects the level of p27\textsuperscript{kip1} protein expression, we first used a chemical inhibitor of the proteasome (MG132) to inhibit p27\textsuperscript{kip1} degradation. Western blot analysis (Fig. 5A) shows that treatment of cells (NIH 3T3 and CCL64) with MG132 induced p27\textsuperscript{kip1} accumulation. Similar results were obtained with the proteasome inhibitor LLLnL (not shown). When cells were treated with MG132 and the MEK inhibitor U0126 at the same time, no further increase of p27\textsuperscript{kip1} was observed, suggesting no additive effect of the two drugs. These data suggest that MAP kinase inhibition affects the degradation of p27\textsuperscript{kip1} rather than its synthesis. To assess more directly a possible effect of MAP kinase on p27\textsuperscript{kip1} synthesis, we carried out the following experiment. We performed \textsuperscript{[35S]}Met metabolic labeling of cells co-transfected with p27\textsuperscript{kip1} and the dominant-negative MEK mutant (MEK\textsuperscript{ΔN}). Ectopic p27\textsuperscript{kip1} neosynthesis was evaluated by autoradiography after immunoprecipitation (Fig. 5B). As mentioned for the experiments in Fig. 2, the expression level of ectopic p27\textsuperscript{kip1} is far higher that of the endogenous p27\textsuperscript{kip1} in these experiments. As a consequence, the protein detected by autoradiography corresponds to the ectopic p27\textsuperscript{kip1} exclusively. The endogenous radiolabeled protein would appear as a higher mobility band upon much longer exposure of the gels (not shown). As shown in Fig. 5B, we did not detect any change in the rate of synthesis of the protein. The same result was obtained in the presence of the proteasome inhibitor MG132 during labeling (not shown). Since the total amount of ectopic p27\textsuperscript{kip1} in these cells was increased by the coexpression of the dominant negative MEK (as described previously in Fig. 2), we conclude that p27\textsuperscript{kip1} accumulation induced by MAP kinase inhibition is due to a decreased degradation of the protein rather than an increased rate of synthesis.

To demonstrate the effect of MAP kinase inhibition on p27\textsuperscript{kip1} degradation, we investigated the stability of p27\textsuperscript{kip1} in the presence of the protein synthesis inhibitor cycloheximide. First, the half-life of the protein was estimated to 2 h in exponentially growing NIH 3T3 cells, as observed after Western blotting of asynchronous cells treated with cycloheximide for different times (Fig. 5C). To actually demonstrate an effect of
MAP kinase on p27Kip1 degradation, we proceeded as follows. Contact inhibited cells were rendered quiescent by serum starvation for 24 h. Cells were then reseeded at low density in the presence of serum for different times, and the level of p27Kip1 was detected by Western blot in the corresponding fractions. D, contact arrested cells were rendered quiescent by serum saturation (T0) and reseeded at low density in the presence of serum. After 4 h, cycloheximide was added to the medium with or without the MEK inhibitor U0126 (U0). After two additional hours (T6) cells were harvested, and p27 level was detected by Western blot.

MAP kinase inhibition affects the rate of p27Kip1 degradation. A, NIH 3T3 fibroblasts (NIH) and CCL64 epithelial cells (CCL) were treated for 12 h with either the protease inhibitor MG132 (MG) or with both this inhibitor and the MEK inhibitor U0126 (U0). Western blot analysis of p27Kip1 expression was then performed on the corresponding cell extracts. B, exponentially growing NIH 3T3 cells were cotransfected with p27Kip1 and either pcDNA empty vector (pcDNA) or dominant negative MEKΔA construct (MEKΔA). The day after the transfection, metabolic labeling was performed with [35S]Methionine. Cells were harvested, and immunoprecipitation of p27Kip1 was performed followed by SDS gel separation and autoradiography. Note that only the ectopic p27 (ect-p27) is seen in this picture, due to high overexpression of this protein. C, exponentially growing cells were treated for different times with cycloheximide, and p27Kip1 was detected by Western blot in the corresponding fractions. D, contact arrested cells were rendered quiescent by serum saturation (T0) and reseeded at low density in the presence of serum. After 4 h, cycloheximide was added to the medium with or without the MEK inhibitor U0126 (U0). After two additional hours (T6) cells were harvested, and p27 level was detected by Western blot.

MAP kinase-dependent Degradation of p27Kip1 in Cell-free Extracts—To go further into the mechanism of this down-regulation, we performed in vitro degradation experiments of p27Kip1 with cellular extracts from exponentially growing fibroblasts. [35S]Methionine-labeled p27Kip1 was produced by in vitro translation/translation and incubated for different times with NIH 3T3 cellular extracts. As can be seen in Fig. 5D, an almost complete degradation of p27Kip1 occurred after a 3-h incubation with extracts of untreated control cells. To test the influence of MAP kinase on this degradation, cells were transfected with MEKDD or with pcDNA as a control, placed in incubation with extracts of untreated control cells. To test the degradation activity of the extracts to catalyze p27Kip1 degradation was restored (Fig. 6B, right panel). These data confirm that MAP kinase activation induces a degradation of p27Kip1 in NIH 3T3 fibroblasts, and they suggest that this degradation is adhesion-dependent.

p27Kip1 Regulation by MAP Kinase Is Independent of Thr-187 Phosphorylation by CDK2

MAP kinase-dependent Degradation of Mutant T187A p27Kip1 in Cell-free Extracts—The ubiquitin-dependent proteasome degradation of p27Kip1 occurs after phosphorylation of the protein by CDK2 on Thr-187. To examine whether p27Kip1 regulation by MAP kinase was dependent on this phosphorylation, we used a mutant of p27Kip1 that cannot be phosphorylated by CDK2 due to the mutation of Thr-187 into alanine. As can be seen in Fig. 6C, in vitro degradation of the mutant form of p27Kip1 by extracts of untreated cells was similar to that observed with the wild type protein (Fig. 6A), suggesting that most of the in vitro degradation is independent of the Thr-187 phosphorylation by CDK2. We then tested the MAP kinase-dependent degradation with extracts from cells transfected with MEKDD and placed in suspension for 6 h. As can be seen in Fig. 6D, the MAP kinase-dependent degradation was equivalent for the wild type (Fig. 6B) and the mutant protein, demonstrating that MAP kinase activation induces a CDK2-independent degradation of p27Kip1 in NIH 3T3 fibroblasts.

MAP Kinase Activation by MEK Down-regulates Ectopic T187A-p27Kip1 Phosphorylation Mutant—We then performed coexpression experiments of this mutant with constitutively active version of MEK. Western blot analysis of these experiments indicates that the T187A mutant follows the same regulation as the wild type protein by the MAP kinase pathway (Fig. 7); ectopic p27Kip1 expression is reduced in cells transfected with MEKDD and placed in suspension for 6 h. As can be seen in Fig. 8A, treatment of cells with roscovitine did not impair the down-regulation of p27Kip1 by MAP kinase, further suggesting that CDK2 activity is not necessary for p27Kip1 down-regulation in this context. In fact, we found that in asynchronous NIH 3T3 cells treated for 24 h with roscovitine, no accumulation of p27Kip1 protein happened (not shown), suggesting that CDK2 activity is not a major regulator of p27Kip1 in cycling NIH 3T3 fibroblasts. Finally, when we coexpressed a dominant-negative version of CDK2 with MEKDD, the effect of MAP kinase activation on p27Kip1 expression and localization was not modified, confirming the data obtained with the chemical inhibitor roso-
itor of MEK1 (U0126). This inhibition induced a clear p27Kip1 accumulation after 8–12 h, although a weak effect was already detectable after 4 h of treatment. This relatively slow process may suggest either that p27Kip1 regulation by MAP kinase is not direct or that this signaling cascade represents only one from the multiple pathways involved. In fact different kinetics of p27Kip1 regulation by MAP kinase were described in the literature depending on the cell type, the expression system used, and eventually, the type of extracellular stimulus applied to the cells (see for example, Refs. 41 and 50). The discrepancy of these data further strengthens the need for a better understanding of p27Kip1 mechanism of regulation by MAP kinase.

**DISCUSSION**

In this work we provide evidence that MAP kinase signaling regulates p27Kip1 expression levels in fibroblasts as well as in epithelial cells. Similar observations had been reported previously, but controversial data had been also published describing an absence of regulation of p27Kip1 levels in cells expressing constitutively active forms of Raf or MEK (36, 43). Our first approach was to inhibit the MAP kinase with a specific inhibitor of MEK1 (U0126). This inhibition induced a clear p27Kip1 accumulation after 8–12 h, although a weak effect was already detectable after 4 h of treatment. This relatively slow process may suggest either that p27Kip1 regulation by MAP kinase is not direct or that this signaling cascade represents only one from the multiple pathways involved. In fact different kinetics of p27Kip1 regulation by MAP kinase were described in the literature depending on the cell type, the expression system used, and eventually, the type of extracellular stimulus applied to the cells (see for example, Refs. 41 and 50). The discrepancy of these data further strengthens the need for a better understanding of p27Kip1 mechanism of regulation by MAP kinase.

In contrast with a recent work by Rivard et al. (40), we could not detect variations of p27Kip1 synthesis linked to MAP kinase activation or inhibition. One reason for this discrepancy could be the genetic background of the cell types used, although in both studies a fibroblastic and an epithelial cell line were compared. As an example of variations due to cell type specificity, the amplitude of p27Kip1 accumulation induced by MEK inhibition was clearly higher in epithelial CCL 64 cells than in NIH 3T3 fibroblasts in our experiments (see Fig. 1). Cell type-dependent regulation of p27Kip1 by MAP kinase was recently strengthened by the observation that in A-431 cells inhibition of the MAP kinase pathway does not induce p27Kip1 accumulation, whereas inhibition of phosphatidylinositol 3-kinase does (51), suggesting a cell type-dependent predominance of one or the other of these signaling pathways for p27Kip1 regulation. Another difference comes from the proliferative status of the cells in these two studies. We addressed the question of MAP kinase inhibition and of the subsequent p27Kip1 increase in asynchronous cells, whereas Rivard et al. (40) investigated the down-regulation of p27Kip1 induced by MAP kinase activation or growth factors when quiescent cells reenter the cycle. Finally, we cannot rule out the existence of weak variations of p27Kip1 synthesis that we could not detect under our experimental conditions. Such variations would not account for the variations of p27Kip1 levels observed in our conditions of MAP kinase inhibition but would be more pronounced during serum or MAP kinase-induced exit from a quiescent state. At least in asynchronous cells, we can conclude that inhibiting MAP kinase signaling modifies the degradation rate of p27Kip1 rather than its synthesis. In fact there are not many reports describing clear variations of p27Kip1 protein synthesis in the literature. Such variations were described in quiescent cells and upon quiescent cell stimulation by platelet-derived growth factor. Post-transcriptional mechanisms were suggested in all these studies based on the observation that the mRNA levels remained unchanged. In addition, there are recent reports of transcriptional regulations of p27Kip1, although some of these data may be controversial with others (26, 27).

To define the specific role of MAP kinase in p27Kip1 regulation, we investigated the capacity of this pathway to down-regulate p27Kip1 expression in the absence of extracellular...
stimuli in cotransfection experiments. Our observation that p27Kip1 was down-regulated by MAP kinase activation in the absence of growth factors (serum deprivation) is in good agreement with other studies. Interestingly, we also found that MAP kinase activation (by MEK3D or B-Raf-CAAX expression) potently down-regulates p27Kip1 in the absence of cell matrix adhesion. Increased p27Kip1 expression due to impairment of cell matrix interaction was already described (for a review, see Ref. 52), and the importance of cell adhesion for growth factor-dependent MAP kinase activation was demonstrated, although the target(s) of this regulation (Ras, Raf, or MEK) was controversial (53, 54). In a recent work, it was found that ectopic activation of MAP kinase was not sufficient to restore the expression of cyclin D1 in suspended cells, in contrast to serum-activation of MAP kinase was not sufficient to restore the MAP kinase-dependent activation was demonstrated, although the importance of cell adhesion for growth factor-cell matrix interaction was already described (for a review, see Ref. 52), and the absence of additive effects of the MAP kinase inhibitor and of the proteasome inhibitors on p27Kip1 accumulation would argue for the implication of the proteasome machinery in the MAP kinase-dependent regulation of p27Kip1. However, although we used two different proteasome inhibitors (MG132 and LLeu), we cannot completely rule out an inhibitory effect of these compounds on other proteolytic machinery, which would explain these observations. In fact, other pathways of p27Kip1 degradation have been already described. Caspase-dependent cleavage of p27Kip1 into 22- and 14-kDa fragments was reported by different laboratories, but the eventual phosphorylation by CDK2 was not investigated in these studies. Similarly, ubiquitin-independent degradation by the proteasome as well as calpain proteolysis during mitotic clonal expansion of preadipocytes was reported, but again, the dependence of these processes on CDK2 phosphorylation was not established. Interestingly, phosphorylation of p27Kip1 on Ser-10 by an unknown kinase was recently found to stabilize the protein (58), suggesting that the action of phosphatase(s) could be involved in the down-regulation of the protein in response to mitogenic signals. Whether such a molecular event is involved in the MAP kinase-dependent regulation would be important to establish. Experiments are in progress in our lab to identify the proteolytic pathway involved in the MAP kinase-dependent degradation of p27Kip1.

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