Cyclin D1 Expression Is Regulated Positively by the p42/p44MAPK and Negatively by the p38/HOGMAPK Pathway*

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We have previously shown that the persistent activation of p42/p44MAPK is required to pass the G1 restriction point in fibroblasts (Pages, G., Lenormand, P., L’Allemain, G., Chambard, J. C., Meloche, S., and Pouyssegur, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8319–8323) and postulated that MAPKs control the activation of G1 cyclin-dependent complexes. We examined the mitogen-dependent induction of cyclin D1 expression, one of the earliest cell cycle-related events to occur during the G1/S transition, as a potential target of MAPK regulation. Effects exerted either by the p42/p44MAPK or the p38/HOGMAPK cascade on the regulation of cyclin D1 promoter activity or cyclin D1 expression were compared in CCL39 cells, using a co-transfection procedure. We found that inhibition of the p42/p44MAPK signaling by expression of dominant-negative forms of either mitogen-activated protein kinase kinase 1 (MKK1) or p44MAPK, or by expression of the MAP kinase phosphatase, MKP-1, strongly inhibited expression of a reporter gene driven by the human cyclin D1 promoter as well as the endogenous cyclin D1 protein. Conversely, activation of this signaling pathway by expression of a constitutively active MKK1 mutant dramatically increased cyclin D1 promoter activity and cyclin D1 protein expression, in a growth factor-independent manner. Moreover, the use of a CCL39-derived cell line that stably expresses an inducible chimera of the estrogen receptor fused to a constitutively active Raf-1 mutant (ΔRaf-1E1R) revealed that in absence of growth factors, activation of the Raf > MKK1 > p42/p44MAPK cascade is sufficient to fully induce cyclin D1. In marked contrast, the p38MAPK cascade showed an opposite effect on the regulation of cyclin D1 expression. In cells co-expressing high levels of the p38MAPK kinase (MKK3) together with the p38MAPK, a significant inhibition of mitogen-induced cyclin D1 expression was observed. Furthermore, inhibition of p38MAPK activity with the specific inhibitor, SB203580, enhanced cyclin D1 transcription and protein level. Altogether, these results support the notion that MAPK cascades drive specific cell cycle responses to extracellular stimuli, at least in part, through the modulation of cyclin D1 expression and associated cyclin-dependent kinase activities.

Mammalian cells express multiple mitogen-activated protein (MAP)3 kinases that mediate the effects of extracellular signals on a wide array of biological processes. In eukaryotic cells, three distinct MAPK cascades have been described, which appear to be linked to separate signal transduction pathways resulting in the final activation of either p42/p44MAPK, p38/HOGMAPK, or stress-activated protein kinases (SAPKs) also called J un kinases (J N K s) (2). Depending on the cellular context, extracellular signals are thought to elicit a specific cellular response (proliferation/differentiation/apoptosis) through the preferential activation of one of the MAPK cascades, which have distinct spectra of substrates (3).

In most cell types, the mitogenic signal is relayed from the cytoplasm into the nucleus by the nuclear translocation of the ubiquitously expressed p42/p44MAPK isoforms (also called ERK2 and ERK1 for extracellular regulated kinase) (4, 5), resulting in activation of a range of transcription factors such as Elk1 (6–8), c-Ets-1, and c-Ets-2 (9, 10). In fibroblasts, agents that elicit a short term MAP kinase activation are not mitogenic, whereas potent mitogens that induce DNA synthesis drive long term MAP kinase activation (11). Previous studies have shown that a sustained activation of the p42/p44MAPK is required for fibroblasts to pass the G1 restriction point and enter S-phase (1, 12). Moreover, activation of the p42/p44MAPK module is sufficient to stimulate early gene transcription and to reduce growth factor requirement for DNA synthesis (13, 14). Therefore, the p42/p44MAPK cascade is likely to regulate some mid-late changes in gene expression that are rate-limiting events for S-phase entry, during the G1 progression of the cell cycle.

In contrast to p42/p44MAPK, which are strongly activated by growth factors and growth-promoting hormones, J N K s and p38MAPK are poorly sensitive to growth signals, and their activation is preferentially triggered by pro-inflammatory cytokines and environmental stresses (3). The J N K cascade has been implicated in the modulation of AP-1-regulated gene expression through the phosphorylation of the proto-oncogene c-j un (3, 15, 16). The two stress-activated signaling pathways can mediate the phosphorylation of the transcription factor ATF2 on residues that increase its transcriptional activity in vivo, suggesting that these two cascades may participate in the

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regulation of ATF-2-dependent gene expression (17–20). Initial reports suggested that the JNK cascade could be required for mitogenesis in fibroblasts (21). However, a more recent study indicated that activation of JNK and p38MAPK and concurrent inhibition of p42/p44MAPK promote the ability of nerve growth factor to induce apoptosis in PC12 cells (22). Nonetheless, JNK and p38MAPK cascades lie on separate signaling pathways and have, in addition to their common substrates, specific cellular targets, i.e. c-jun and MAPKAPK-2, respectively (23, 24). These two cascades are thus likely to be involved in the regulation of distinct cellular functions; however, it is not clear what specific role can be attributed to one or the other stress signaling pathways at the level of cell division mechanisms.

Transduction of extracellular signals culminates in the expression and assembly of different kinase holoenzymes, the cyclin-cdk (cyclin-dependent kinase) complexes, which are formed and activated at specific stages of the cell division cycle. Although the kinase-associated activity of these complexes is modulated by specific phosphorylation-dephosphorylation events on the catalytic subunits, the temporal activation of the holoenzymes is primarily dependent on the synthesis and accumulation of specific regulatory subunits, the cyclins (25). Cyclin-cdk complexes have, in addition to their common substrates, specific cellular targets, i.e. c-jun and MAPKAPK-2, respectively (23, 24). These two cascades are thus likely to be involved in the regulation of distinct cellular functions; however, it is not clear what specific role can be attributed to one or the other stress signaling pathways at the level of cell division mechanisms.

Because of the strict requirement for a persistent p42/p44MAPK activation to successfully pass the G1 restriction point (27, 30, 31), however, cyclin D1 requirement becomes dispensable in a cell background deficient for the tumor suppressor (pRB) function (32). This observation further emphasizes the functional link between pRB and cyclin D1-associated kinase activity, which has been shown to be responsible in part for hyperphosphorylating pRB (33–37). Therefore, early appearance of cyclin D1 upon growth factor stimulation of resting fibroblasts plays a central role in regulating the G0-G1 transition of the cell cycle.

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**RESULTS**

**Potent Mitogens Promote Long-term p42/p44 MAPK Activation, Cyclin D1 Expression, and pRb Phosphorylation**—D-type cyclin expression profile was analyzed in whole cell extracts of G0-arrested Chinese hamster fibroblasts (CCL39), by Western blot technique using specific antibodies. In G0-arrested CCL39 cells, cyclin D1 protein expression was barely detectable (Fig. 1A). However, serum stimulation of cells led to a dramatic accumulation of cyclin D1 protein that became easily detectable at 6 h poststimulation and increased until cells entered S-phase (Fig. 1A). As previously shown in other fibroblastic cell lines (30, 49), the protein expression level of cyclin D1 in CCL39 cells was not cell cycle-modulated and only a modest peak of accumulation was observed in late G1 (12–16 h poststimulation). Cyclin D2 and D3 expression were also analyzed in CCL39 cells using specific monoclonal antibodies. In contrast to cyclin D1, for that expression was strictly dependent on the presence of mitogens, cyclin D3 was present in resting cells and its level was poorly increased by growth factors (Fig. 1A). Cyclin D2 was not detectable in either quiescent or growth factor-stimulated CCL39 cells. The hyperphosphorylation of pRb was analyzed for each time point following serum stimulation of resting cells. A specific antibody able to detect the active hypophosphorylated form of pRb (lower band) as well as the inactive hyperphosphorylated form of the protein (upper band) was used. In G0-arrested cells, pRb was exclusively found in its active hypophosphorylated state, suggesting that cyclin D3, although expressed does not contribute to pRb phosphorylation in this cell system and thus, is not a limiting factor for progression through G1-phase of the cell cycle. However, pRb inactivation (hyperphosphorylated form) became apparent at 10–12 h poststimulation, when cyclin D1 expression was maximal (Fig. 1A, lower panel) and when the first cells enter S-phase. The results suggest that cyclin D1 is the major mitogen-regulated D-type cyclin identifiable in this cell system and, therefore, that its associated kinase activity is likely to participate in pRb inactivation by phosphorylation.

Previous studies demonstrated that in fibroblasts only potent mitogens that drive the G0 to S-phase transition can maintain a long term activation of the p42/p44MAPK cascade (11). Initial experiments to link this signal transduction pathway to mitogen regulation of cyclin D1 expression revealed a tight correlation between the extent of cyclin D1 accumulation and the ability of various agents to induce sustained p42MAPK activation. Cells were serum-starved for 24 h and stimulated for 9 h in the presence of various agonists, and whole cell extracts were analyzed for cyclin D1 protein levels, pRb phosphorylation, and p42MAPK activity. The polyclonal anti-p42MAPK antibody used detected three electrophoretically distinct forms of the kinase: a fast-migrating band representing the inactive unphosphorylated form of the endogenous p42MAPK, an intermediate band that corresponds to the phosphorylated and active enzyme, and an upper, slow-migrating form that corresponds to cross-reactivity of the antibody with the p44MAPK isoform (Fig. 1B, lower panel). Cyclin D1-associated kinase activity that is mainly dependent on the level of cyclin D1 was also evaluated in each sample by analyzing pRb phosphorylation. The ability of the various mitogens to induce cyclin D1 expression and pRb hyperphosphorylation was closely related to their ability to maintain p42MAPK activities throughout G0 progression of the cell cycle (Fig. 1B). Only potent mitogens, such as whole serum and thrombin, which potently initiate DNA synthesis in arrested CCL39 cells, were able to promote high levels of cyclin D1 accumulation and allow pRb phosphorylation (appearance of a minor slow-migrating band at 9 h post-stimulation) (Fig. 1B, middle panel). Whereas a slight effect was observed with a weakly mitogenic combination of epidermal growth factor plus insulin, neither factor alone was able to promote long term p42MAPK activation, cyclin D1 expression, and pRb phosphorylation, suggesting an interdependent relationship between the long lasting activation phase of p42/p44MAPKs, cyclin D1 expression, and pRb inactivation in CCL39 fibroblasts.

The p42/p44MAPK Cascade Positively Regulates Cyclin D1 Expression in Fibroblasts—To investigate the role of the p42/p44MAPK signaling pathway in the regulation of cyclin D1 expression more directly, we used previously characterized expression constructs to modulate either positively or negatively the endogenous p42/p44MAPK activity (see “Experimental Procedures”). Cyclin D1 transcription was monitored by transfecting CCL39 cells with a previously cloned fragment of the human cyclin D1 promoter fused to the luciferase reporter gene (D1Δ-944) (39), together with the relevant constructs. The results showed that the p42/p44MAPK activity strongly affects cyclin D1 transcription in CCL39 cells. When a constitutively activated form of MAPK kinase (MKK1-SS/DD) was expressed, a large increase in luciferase expression (6–10-fold) could be detected in exponentially growing cells, when compared with the luciferase expression in control cells transfected with the empty vector (Fig. 2A). This dramatic up-regulation of the cyclin D1 reporter expression in cells overexpressing the constitutively active MKK1 mutant likely resulted from a higher p42/p44MAPK activity in these cells, since co-expression of the MAPK phosphatase (MKP-1), a dual specificity phosphatase shown to be able to inactivate p42/p44MAPKs (42), totally abolished the MKK1-SS/DD-dependent increase in cyclin D1-lucif-

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5 G. L’Allemain, J. N. Lavoie, and J. Pouyssegur, unpublished data.
erase expression (Fig. 2A). No significant effects of these plasmids were observed in the same experiments on the β-galactosidase internal control reporter expression. A positive effect on the cyclin D1-luciferase expression was also measured in G0-arrested cells expressing the constitutively active form of MKK1. In these cells, activation of the p42/p44MAPK cascade in the absence of mitogen led to an increase of cyclin D1-luciferase expression that was even higher than the level measured in serum-starved cells: 1.15 ± 0.11. Results are the means ± S.E. of three independent experiments. Endogenous MKK1 expression (lower band) is shown as an internal control.

MKK1 activity in cells expressing this mutated form of MKK1 (40). Moreover, the serum-stimulated luciferase expression was strongly inhibited (80%) in cells expressing the negative regulatory constructs, as compared with control cells expressing the reporter gene alone (Fig. 2B). These results thus demonstrate that the p42/p44MAPK signal transduction pathway positively regulates cyclin D1 transcription in fibroblasts.

Previous studies have shown that an increase in the level of cyclin D1 messenger RNA does not always lead to an increase in cyclin D1 protein levels in transfected cells, suggesting that post-transcriptional processes might also play an important role in the regulation of cyclin D1 expression (50–52). We thus examined whether the modulation of cyclin D1 transcription by the p42/p44MAPK pathway also resulted in modification of cyclin D1 protein levels in CCL39 fibroblasts. The effects of the same constructs (MKK1, MKK1-SS/DD, or MKP-1) on the level of endogenous cyclin D1 protein were analyzed in CCL39 cells. To do so, the expression plasmids were co-transfected with a selection vector that encodes an amiloride-resistant Na⁺/H⁺ exchanger isoform, NHE3. Nontransfected cells were selec-
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Cells expressing the constitutively active MKK1 mutant, a higher level of cyclin D1 could be detected as compared with cyclin D1 expression in control cells transfected with either the empty vector or the wild type MKK1 (Fig. 2C). This increase of cyclin D1 protein level in MKK1-expressing cells kept in serum-supplemented medium was associated with an overall increase in the amount of the hyperphosphorylated form of pRb; however, the relative total amount of pRb expression was also increased, keeping constant the ratio between the hyper- and hypophosphorylated forms. Conversely, inactivation of p42/p44MAPks by expression of the MKP-1 markedly reduced cyclin D1 protein expression below the level detected in control cells (Fig. 2C). The significant inhibition of cyclin D1 protein expression in MKP1-overexpressing cells also correlated with a 45% decrease in the amount of the hyperphosphorylated form of pRb, suggesting that the resulting low level of cyclin D1 in these cells was limiting for cyclin D1-associated kinase activity. When similar experiments were performed in resting cells, a strong positive effect on cyclin D1 expression could be detected upon activation of the p42/p44MAPK pathway. As shown in Fig. 2D, cyclin D1 was barely detectable in serum-starved control cells, whereas in serum-starved MKK1-SS/DD expressing cells, a growth factor-independent expression of cyclin D1 was detected. Furthermore, this increase of cyclin D1 protein level mediated by expression of the constitutively active MKK1 mutant was comparable with the level measured in thymbin-stimulated control cells (see quantitations in the legend of Fig. 2D). Conversely, when the p42/p44MAPK activity was blocked by the presence of an elevated level of MKP-1, thymbin-induced accumulation of cyclin D1 was severely impaired, being inhibited by as much as 51%, as compared with the level measured in cells transfected with the corresponding empty vector (Fig. 2D). Altogether, these results strongly suggest an important contribution of the p42/p44MAPK signal transduction pathway in the regulation of cyclin D1 expression in response to growth signals, both at the level of transcription and protein synthesis.

Activation of the p42/p44MAPK Cascade Is Sufficient to Promote Cyclin D1 Accumulation in CCL39-ΔRaf-1:ER Cells—The contribution of the p42/p44MAPK cascade on mitogen-induced cyclin D1 protein synthesis was next examined, using a CCL39-derived cell line (CCL39-ΔRaf-1:ER) expressing an estradiol-dependent human Raf-1 protein kinase (44). In this cell line, the ΔRaf-1:ER chimera is activated in response to estradiol, thereby activating MKK1 and then p42/44MAPks. Previous characterization of CCL39-ΔRaf-1:ER cells has shown that addition of estradiol to serum-starved cells stimulates p42/44MAPks within minutes. The p42/44MAPK activity increases for up to 1 h, thus reaching a level comparable to the maximal p42/44MAPK activation measured in serum-stimulated CCL39 control cells, and remains elevated in the presence of estradiol. We used this cell system to directly measure the specific contribution of Raf > MKK1 > p42/44MAPK cascade on cyclin D1 protein expression. CCL39-ΔRaf-1:ER cells were serum-starved for 24 h and then stimulated with either 10 μM estradiol or 10% FCS for varying periods. Whole cell extracts were analyzed for cyclin D1 protein expression and the pRb phosphorylation state by Western blots using specific antibodies. The results showed that estradiol-treated CCL39-ΔRaf-1:ER cells accumulated cyclin D1 protein to a level comparable with that stimulated in the same cells by 10% serum (Fig. 3). Moreover, cyclin D1 accumulation followed a similar time course in estradiol-treated cells when compared with serum-stimulated cells, indicating that activation of the Raf pathway alone (leading to p42/44MAPK activation) initiated positive regulatory signals responsible for cyclin D1 protein synthesis. Nevertheless, the Raf pathway alone was not sufficient to promote activation of the D1-associated kinase activity, since neither pRb phosphorylation (Fig. 3, lower panels) nor in vitro cdK4 kinase activity (data not shown) could be detected in estradiol-stimulated CCL39-ΔRaf-1:ER cells.

The p38HOGMAPK Cascade Antagonizes Mitogen-induced Expression of Cyclin D1 in Fibroblasts—In addition to the p42/p44MAPK pathway, at least two other MAPK cascades are implicated in the transduction of external stimuli in mammalian cells; the p38HOGMAPK and the JNKs (2). We next examined whether other MAPK pathways could have some regulatory effect on cyclin D1 expression. Previous studies indicated that the proto-oncogene c-jun can increase expression of a cyclin D1 promoter-controlled luciferase reporter in co-transfection experiments, suggesting a positive regulatory effect of the JNK pathway on cyclin D1 transcription (39, 53). However, the effect on protein expression has not been shown. The role of the p38MAPK cascade in gene expression is less clear. We thus focused on the putative effect of the p38MAPK cascade on cyclin D1 transcription and protein expression in CCL39 cells. Co-transfection experiments were performed, using plasmids encoding different members of this cascade: the p38MAPK kinase (MKK3), the constitutively active MKK3 mutant (MKK3-S189G/T193G called MKK3-Glu), the p38MAPK, and the dominant-negative p38MAPK (p38MAPK-TY/AF), together with the cyclin D1-luciferase reporter construct. Surprisingly, overexpression of MKK3, which resulted in a higher basal level of p38MAPK activity in CCL39 cells, did not activate cyclin D1 promoter; rather cyclin D1-luciferase expression was reduced by 33% in exponentially growing cells, when compared with cells transfected with the empty vector (Fig. 4A). A stronger inhibitory effect was detected when a wild type form of the p38MAPK was expressed together with MKK3, as if endogenous p38MAPK was limiting. In contrast, expression of dominant-negative p38MAPK-TY/AF together with MKK3 totally abolished the MKK3-mediated inhibition of cyclin D1 luciferase expression, indicating that the negative effect on cyclin D1 transcription results from increased MKK3 activity. Expression of the constitutively active MKK3 mutant (MKK3-Glu) elicited a more pronounced inhibition of cyclin D1-luciferase expression (62%) than the wild type MKK3, a result further indicating that the inhibitory effect was dependent on MKK3 activity. When assays were performed in G0-arrested cells, basal luciferase expression of the cyclin D1 promoter-controlled
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Reported was also reduced (Fig. 4B). Furthermore, increasing p38MAPK activity by expression of MKK3 resulted in a marked inhibition of the serum-induced cyclin D1-luciferase expression in resting cells. This result suggests that the p38MAPK cascade could be involved in the negative regulation of cyclin D1 transcription and thus antagonize the mitogen-dependent stimulation of cyclin D1 transcription mediated, at least in part, by the p42/p44MAPK cascade.

This opposing effect of the p38MAPK cascade, as compared with the positive regulatory effect of the p42/p44MAPK pathway, was also detected on endogenous cyclin D1 protein expression in CCL39. The effects of MKK3 and p38MAPK were monitored following co-transfection of the relevant constructs together with the selection vector encoding an amiloride-resistant Na⁺/H⁺ exchanger isoform, which allowed us to monitor cyclin D1 protein levels in a population of cells enriched for the transfected genes. Cyclin D1 expression was measured in whole cell lysates of resistant cells by immunoblotting using a specific antibody. The results showed that cyclin D1 protein levels were reproducibly decreased by 40–50% in cells expressing the MKK3 construct when compared with control cells transfected with the corresponding empty vector (Fig. 4C). This inhibition of cyclin D1 expression was associated with a slight decrease in the amount of the slow-migrating hyperphosphorylated form of pRb in the same cell extracts, together with a corresponding increase in the amount of the hypophosphorylated form, suggesting that the MKK3-mediated interfering effect on cyclin D1 expression was affecting cyclin D1/cdk4–6 activity (Fig. 4C). Even though this modification of the pRb phosphorylation state was not pronounced, we could reproducibly quantitate 25% decrease of the slow-migrating form. Similarly, the thrombin-induced accumulation of endogenous cyclin D1 was inhibited by 55% upon co-expression of the p38MAPK together with its activator, MKK3, when compared with the level of thrombin-induced cyclin D1 in cells transfected with the empty vectors (Fig. 4D).

As an independent approach to probe the role of the p38MAPK cascade, we used a chemical compound, the SB203580, which has been described recently as a specific inhibitor for p38MAPK, but without inhibitory action on p42/p44MAPKs and JNKs (54, 55). When the endogenous p38MAPK activity was inhibited by pretreatment of CCL39 with SB203580, the cyclin D1-luciferase expression was reproducibly enhanced by 2–3-fold (Fig. 5A). Furthermore, treatment of MKK3-expressing cells with SB203580 reversed the inhibition of cyclin D1-luciferase expression, a result that indicates that the MKK3-mediated inhibition of cyclin D1 transcription results from an increased p38MAPK activity. A 2–3-fold enhancing effect of SB203580 on the level of endogenous cyclin D1 protein expression was also observed in exponentially growing cells as well as in resting cells in absence of mitogens (Fig. 5B). Altogether, these results strongly suggest that the p38MAPK cascade may exert a nega-
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A single external stimulus can lead to the simultaneous activation of multiple MAPK signal transduction pathways (2). However, the magnitude and time course of the response observed for each pathway is greatly dependent upon the nature of the stimulus. In order to determine if the opposing effects of p42/p44MAPK and p38MAPK on cyclin D1 expression were physiologically relevant, we designed experiments to measure the differential effects on cyclin D1 in response to appropriate stimuli. α-Thrombin mainly activates p42/p44MAPK, whereas IL-1β drives a strong p38MAPK activation with little effect on p42/p44MAPK. When these two MAPK pathways were simultaneously activated by the co-addition of α-thrombin and IL-1β to resting CCL39 cells, the cyclin D1 protein level obtained after a 9-h stimulation was attenuated by 25–30% as compared with the level measured in cells exposed to thrombin alone (Fig. 6). Based on co-transfection experiments shown above, the reduced expression of cyclin D1 obtained upon simultaneous activation of p42/p44MAPKs and p38MAPK likely resulted from opposite regulatory effects of these two signaling pathways. Therefore, this result strongly indicates that the differential regulation of cyclin D1 by the p42/p44MAPK and the p38MAPK cascades could have some physiological relevance.

DISCUSSION

Entry into the cell cycle upon growth factor stimulation requires fine coordination of events from the membrane to the nucleus. To pass the restriction point, mammalian cells must be continuously “fired” by signals during the first 6–8 h that precede the onset of DNA replication (56). At least two types of Ser/Thr protein kinases play a determinant role during this critical period of G0/G1 progression: (i) the MAP kinase isoforms p42/p44MAPKs (ERK2/ERK1) and (ii) the G1 cyclin-cdk complexes, particularly cyclin D1/cdk4. Specific inactivation of either of these two protein kinase signaling systems results in specific growth arrest in G1 (1, 12, 27, 30, 31).

We previously demonstrated that persistent activation of p42/p44MAPKs is critical for the commitment of cell cycle entry in fibroblasts (5, 11). Only potent mitogens are capable of sustaining a stimulated kinase activation of p42/p44MAPKs and the concomitant nuclear translocation of these enzymes (5). The long-term activation (that represents 10–30% of the peak activity measured 5 min following mitogen addition) usually persists for several hours before declining to barely detectable levels when cells enter and progress through S-phase (5, 11). In marked contrast to p42/p44MAPKs, cyclin D1/cdk4 activity in CCL39 fibroblasts emerges rather late in G1-phase and increases progressively as the cells approach and pass through S-phase (5, 11). Based on this temporal difference in activation and on the mitogenic regulatory roles of these two protein kinase signaling systems, we postulated that the p42/p44MAPKs control the activation of cyclin D1/cdk4-cdk6, the first complex to be activated in early G1. However, mechanisms that govern G1 cyclin/cdk activation are complex. First, de novo synthesis of the regulatory cyclin subunit is required, and this step is certainly the most limiting step in G0-arrested cells. Second, appropriate sites on the catalytic subunit must be phosphorylated by the activating kinase Cdk4 and diphosphorylated by the specific cdk25 phosphatase (57). Third, multiple G1-cdk inhibitors (CKIs) must be blunted for the kinase activity to emerge (58). It is possible that p42/p44MAPKs control many of these steps acting on the cyclin D1/cdk4-cdk6 activation. The present study focused only on the first level of regulation that concerns the growth factor-sensitive cyclin D1 induction.

We found that among the D-type cyclins, cyclin D1 is the only detectable early G1-cyclin to be regulated by growth factors in

6 A. Brunet, J.-M. Brondello, and J. Pouységur, unpublished results.
CCL39 cells. Furthermore, we demonstrated that the p42/p44MAPK cascade controls cyclin D1 expression in response to growth signals thus contributing to the regulation of S-phase entry. Such a modulation of cyclin D1-associated kinase activity will participate in pRb inactivation (pRb hyperphosphorylation). As a result, E2F-regulated gene expression is expected to be suppressed in cells where p42/p44MAPK activity is inhibited. In fact, not only cyclin D1 and its associated kinase activity were blocked in cells expressing either the dominant-negative p44MAPK or the MKP-1, but also the cdk2 activity was inhibited (data not shown). This result is consistent with the fact that cdk2 activation in late G1/early S-phase is dependent upon transcription and synthesis of its regulatory subunits, cyclin E and cyclin A, which in turn require E2F release for their expression, as many other inducible genes at the G1/S boundary (59–62). Although preliminary data suggest that MKP-1 could have the ability to inactivate other members of the MAPK family, in particular the JNKs,9 a similar inhibition of cyclin D1 expression, pRb hyperphosphorylation and cdk2 activity were observed using the dominant-negative p44MAPK mutant, a more specific interfering construct. Considering previous studies that have shown that inhibition of the p42/p44MAPK cascade blocks DNA synthesis and cell proliferation (1), we conclude that the requirement of this signaling pathway for S-phase entry may thus rely in part on its essential function as a positive regulator of cyclin D1 expression.10

This study revealed that not only the p42/p44MAPK cascade is required for cyclin D1 transcription and protein synthesis, but it is sufficient by itself. Expression of a constitutively active MAP kinase kinase produced an increase in cyclin D1 protein level in absence of any other growth signal, which was equivalent to the expression level measured in cells stimulated with a strong mitogen. More importantly, the use of a CCL39-derived cell line expressing an estrogen-dependent human Raf-1 protein kinase (CCL39ΔRaf-1:ER) demonstrated that the exclusive activation of the Raf > MKK1 > p42/p44MAPK cascade was able to induce cyclin D1 protein expression to the same magnitude and with an identical time course as that induced by serum in these cells. Even though stimulation of the Raf pathway was sufficient to induce cyclin D1 accumulation, it could not promote pRb phosphorylation or cdk2 kinase activation (data not shown). As a result, no significant estradiol-induced DNA synthesis could be detected in this system.8 The apparent increase in the overall pRb hyperphosphorylation in cells expressing the constitutively active MKK1 mutant in the presence of serum may thus likely result from a cooperative effect between the p42/p44MAPK cascade and other signaling pathways. Interestingly, we could detect a similar increase in the overall amount of pRb expression (and thus in the pRb hyperphosphorylated form), associated with a marked increase in the level of cyclin D1 expression in stable CCL39 transfectants expressing a high constitutive level of the MKK1-SS/DD mutant.9 The use of CCL39ΔRaf-1:ER-derived cell line allowed us to discriminate between secondary versus immediate effects of p42/p44MAPK activation in a synchronized population of cells. We thus conclude that although activation of the p42/p44MAPK cascade is necessary and sufficient for cyclin D1 expression, other signals are required to promote the activation of cyclin D1/cdk4–6 complex and thus the hyperphosphorylation of pRb to ensure passage through S-phase. However, activation of the Raf > MKK1 > p42/p44MAPK by estradiol treatment led to a decrease in growth factor requirement for DNA synthesis, a result in agreement with our previous finding in cells expressing the constitutively active MKK1 mutant (13). Interestingly, a CCL39-derived cell line expressing high levels of human cyclin D1 exhibited a similar higher sensitivity to growth factors.9

In marked contrast to the positive action of p42/p44MAPK activation, the p38MAPK signaling pathway exerted a negative effect on cyclin D1 expression. Activation of the p38MAPK cascade led to a significant decrease in cyclin D1 transcription and, conversely, inhibition of this signaling pathway by the specific inhibitor SB203580 had an opposite enhancing effect. Although less pronounced, this inhibitory effect of the p38MAPK on cyclin D1 expression was also visualized at the level of protein synthesis and reproducibly detected using different strategies (either expression of MKK3, p38MAPK, or pretreatment with SB203580). In addition, IL-1β, a physiological inducer of p38MAPK activation, showed a similar antagonizing effect on thrombin-induced cyclin D1 accumulation in CCL39 cells. However, treatment of CCL39 fibroblasts with IL-1β also increases the JNK activity.10 The fact that the JNK cascade has been shown to promote cyclin D1 transcription and cell proliferation in fibroblasts (21, 53) may explain the poor inhibitory effect of IL-1β on DNA synthesis in CCL39 cells.11 However, the results presented here strongly suggest that the p38MAPK-mediated inhibition of cyclin D1 expression may have more dramatic effects in cell systems where IL-1β negatively regulates cell division. Interestingly, it has been recently shown that hypophosphorylation of pRb could mediate the G0/G1 growth arrest induced by IL-1β in human A375-C6 melanoma cells (63).

The molecular mechanisms underlying the negative regulation of cyclin D1 expression by p38MAPK is not clear. The transcription factor ATF2 is substrate for p38MAPK and, therefore, may account for the effect of this signaling pathway. The cyclin D1 promoter contains multiple regulatory elements (TRE, E2F, Oct, SP1, CRE) and some uncharacterized elements that may also play a role in transcription of the gene (39). Thus cyclin D1 expression may be responsive to a large set of transcription factors. In addition, multiple MAPK cascades appears to be implicated in the regulation of the promoter activity (this study) (39, 53), some of that modulating the activity of a common substrate. This is the case for Elk-1, a substrate for the three distinct mammalian MAPKs, which can therefore integrate signals from multiple MAPK cascades in response to extracellular stimuli (64). The transcription factor ATF2 is also a common substrate for p38MAPK and JNKs (17, 18, 20). However, only the JNK cascade can activate c-jun (16, 65–67). Therefore, activation of a specific MAPK cascade is likely to produce a differential effect on gene expression. Finally, there are many mechanisms for the regulation of the respective MAPK signal transduction pathways and both positive and negative cross-talk between these MAPK cascades probably exists, which could explain the interfering effect of the p38MAPK cascade on cyclin D1 expression.9

Although the data obtained here strongly suggest that the p42/p44MAPK and the p38MAPK exert their regulatory action on cyclin D1 expression at the level of the transcriptional machinery, they do not exclude a possible additional post-transcriptional regulation. It has been suggested that the p42/p44MAPK cascade could up-regulate translation initiation of specific genes in response to insulin (68). A recent study has shown that overexpression of eIF-4E, which is released upon phosphorylation of PHAS-1 protein by p42/p44MAPKs, increased both cyclin D1 mRNA and protein in resting fibroblasts (52). There is also

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1. J. -M. Brondello, F. McKenzie, and J. Pouyssegur, unpublished results.
2. P. Lenormand and J. Pouyssegur, unpublished results.
3. A. Brunet, J. N. Lavoie, and J. Pouyssegur, unpublished results.
4. J. N. Lavoie, A. Brunet, and J. Pouyssegur, unpublished results.
Regulation of Cyclin D1 Expression by MAPKs

Evidence suggests a role for p38 MAPK in the control of gene expression by post-transcriptional regulation of specific gene transcripts (54). Modulation of cyclin D1 expression by the MAPK cascades may thus result from a combination of both transcriptional and post-transcriptional events.

We thus conclude that MAPK signal transduction pathways play essential and differential roles in the regulation of cyclin D1 expression, thus establishing a link between receptor-coupled intracellular signaling and cell cycle machinery. The positive regulatory role of the p42/p44 MAPK cascade further emphasizes its essential function as a positive regulator of cell proliferation in response to growth signals. In contrast, the negative effect of the p38 MAPK cascade on cyclin D1 expression suggests that this signaling pathway may be detrimental to cell growth. A recent study has reported that activation of p38 MAPK together with the JNK cascade is critical for the induction of apoptosis in PC12 cells (22). This is in agreement with a possible negative function of the p38 MAPK signal transduction pathway in cell division mechanisms. Interestingly, in CCL39 cells, growth factor removal leads to rapid inactivation of the p42/p44 MAPK (11, 69, 70), whereas the p38 MAPK activity seems to increase with the duration of starvation,12 correlating with the abrogation of cyclin D1 expression. It thus seems that in contrast to the p42/p44 MAPK cascade that controls the G0 to G1 phase transition in fibroblasts and enhances cell differentiation in PC12 cells (14), the p38 MAPK cascade could be implicated in the maintenance of cell quiescence in fibroblasts and in promotion of programmed cell death in differentiated cellular systems.

While this manuscript was in preparation for submission, data were presented showing, in agreement with the results presented here, a positive modulation of cyclin D1 transcription by the p42/p44 MAPK. (53). This positive effect has been shown to be mediated by the transcription factor c-Ets-2 and dependent on the presence of a putative Ets-like binding domain on the proximal region of the human cyclin D1 promoter.

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