Regulation of p21WAF1/CIP1 Expression through Mitogen-activated Protein Kinase Signaling Pathway

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Abstract

p21WAF1/CIP1 is a cyclin-dependent kinase inhibitor whose expression in mammalian tissues is highly induced in response to stress as well as due to normal development and differentiation. Induction of p21WAF1/CIP1 in response to DNA damage occurs through a transcriptional mechanism that is dependent on the activation of the tumor suppressor protein p53. Recent evidence indicates that p21WAF1/CIP1 can also be induced independently of p53, but the signal transduction mechanisms involved in regulating p21WAF1/CIP1 expression in these situations have not been elucidated. In this study, we have addressed the role of the mitogen-activated protein kinase signaling pathway in the induction of p21WAF1/CIP1 in response to growth factor treatment. Using an experimental approach involving cotransfection of a p21WAF1/CIP1 promoter-luciferase construct with a variety of plasmids expressing dominant positive or dominant negative mutant proteins involved in this signaling pathway, we provide evidence to support a role for mitogen-activated protein kinase in the transcriptional activation of p21WAF1/CIP1 by growth factor stimulation.

Introduction

The cdk2 inhibitor p21WAF1/CIP1 (also referred to as SDII and CAP20) plays a critical role in regulating cell cycle progression (1). It has been implicated in mediating growth arrest in response to a variety of conditions, including DNA damage and terminal differentiation (2–4), but may also serve to promote proliferation following serum stimulation of quiescent cells (5, 6). Induction of p21WAF1/CIP1 in response to DNA damage occurs primarily through a transcriptional mechanism that requires interaction of the tumor suppressor protein p53 with a p53-binding site in the p21WAF1/CIP1 promoter (2, 3). However, induction of p21WAF1/CIP1 in other situations, such as those following growth factor stimulation or during cellular differentiation, appears not to require p53 (6–8). The mechanisms serving to regulate p21WAF1/CIP1 expression in these p53-independent circumstances have not been elucidated, although recent studies suggest that both transcriptional and posttranscriptional events contribute to the level of expression (3, 8).

In this study, we have addressed the role of the MAPK signaling pathway in mediating transcriptional activation of p21WAF1/CIP1 in response to growth factor stimulation. MAPK (also referred to as extracellular signal-regulated kinase) is activated following growth factor stimulation through a signaling cascade involving Ras, Raf, and MAPKK (9). Activated MAPK, in turn, plays a critical role in the activation of transcription factors involved in regulating proliferation-related genes (10, 11). Herein, we provide evidence to support the involvement of the MAPK pathway in the transcriptional activation of the p21WAF1/CIP1 promoter in response to growth factor stimulation.

Materials and Methods

Cell Culture, Treatments, and Transfections. HeLa and PC12 cells were maintained in a 37°C humidified atmosphere containing 5% CO2 in DMEM supplemented with 10% FBS. PC12 cells were also supplemented with 5% horse serum. Cells were serum starved by placement in medium containing 0.5% FBS for at least 24 h prior to treatment. Serum stimulation was achieved by direct addition of FBS to a final concentration of 10% in the medium. EGF (Life Technologies, Gaithersburg, MD), NGF (Boehringer Mannheim, Indianapolis, IN), and phorbol ester TPA (60 ng/ml; Sigma, St. Louis, MO) were added directly to the medium and left on the cells until the time of harvest.

Transient transfection was accomplished using the calcium phosphate procedure as previously described (12). In experiments involving cotransfection of two or more plasmids, the total amount of DNA was kept constant through the addition of empty vector DNA. After transfection, the cells were placed in medium containing 0.5% FBS for 16 h prior to treatment with the various agents. Control or treated cells were harvested 18 h later to analyze luciferase activity.

Expression Plasmids and Reporter Constructs. The wild-type and p53 site-deleted p21WAF1/CIP1 promoter-luciferase reporters (WPP-Luc and DM-Luc, respectively) were provided by Bert Vogelstein (2). The WPP-Luc reporter contains a 2.4-kb fragment of the human p21WAF1/CIP1 promoter, and the DM-Luc was constructed by deleting the p53 site in the WPP-Luc construct. The construct-expressing dominant negative mutant Ha-Ras (RasAsn17) as well as the PC12 cell lines harboring the MMTV-Ras-Asn17 construct were from Geoffrey Cooper (13). The dominant positive mutant Ha-Ras (RasVal12) plasmid was from Michael Wigler (14); the dominant positive mutant Raf (Raf-BBX) plasmid was from Ulf Rapp (15); and the plasmid expressing dominant positive MAPKK (MEK-1-S218E-S222E, hereafter referred to as MEKEE) was obtained from Christopher Marshall (16). The MKP-1 expression construct has previously been described (12). The PC12 cell line overexpressing the NGF receptor trk was provided by David Kaplan (17).

Immunoprecipitation and Kinase Assays. Endogenous MAPK was immunoprecipitated from cells using a rabbit polyclonal antibody (anti-p42MAPK; Santa Cruz Biotechnology, Santa Cruz, CA) and assayed for kinase activity using a MBP substrate as previously described (12).

Northern Blot Analysis. Cells were harvested directly into STAT-60 (Tel-Test "B", Inc., Friendswood, TX), and total RNA was extracted according to the manufacturer's recommendations. Northern blot analysis was performed as previously described (12). A human p21WAF1/CIP1 cDNA was used to detect p21WAF1/CIP1 expression in HeLa cells, while a p21WAF1/CIP1 oligonucleotide (5'-CTCCGGCAGCAGGTCAAAATCGTCCCGGCCTCTCAGAGCAGC3') corresponding to a mouse gene sequence was used for hybridizations with RNA from PC12 cells.

Results and Discussion

p53-Independent Induction of p21WAF1/CIP1 following Mitogenic Stimulation. p21WAF1/CIP1 expression was examined in serum-starved HeLa cells at various times following the readdition of serum (Fig. 1A). In keeping with previous reports obtained using other cell types (6), serum stimulation resulted in a rapid, transient elevation in...
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- The responsiveness of the p21\textsuperscript{WAF1/CIP1} promoter to mitogenic stimulation is not limited to HeLa cells but also occurs in other cell types, as shown in Fig. 1C for rat pheochromocytoma PC12 cells.

- Similar to that observed in HeLa cells, the p21\textsuperscript{WAF1/CIP1} promoter activity was increased following treatment of PC12 cells with either serum or EGF. In addition, treatment of these cells with NGF, which results in their differentiation to a neuronal phenotype, also resulted in a dose-dependent increase in p21\textsuperscript{WAF1/CIP1} promoter activity.

- The role of Ras and Ras effectors on p21\textsuperscript{WAF1/CIP1} promoter activity. Since MAPK is an important mediator of gene activation in response to extracellular signals (9–11), we explored the possibility that it is also involved in regulating p21\textsuperscript{WAF1/CIP1} expression in response to mitogens. We first examined the contribution of Ras, an important mediator of the MAPK pathway, to the response. For this purpose, we utilized a PC12 cell line stably transfected with a glucocorticoid-inducible dominant negative Ras construct (MMTV-Ras-Asn17; Ref. 13). Treatment of these cells with dexamethasone renders them Ras deficient. Control and dexamethasone-pretreated MMTV-Ras-Asn17 cells were subsequently treated with EGF, NGF, serum, or TPA and assayed for MAPK activity using an immunocomplex kinase assay (Fig. 2A). Consistent with previous reports (9), EGF, NGF, serum, and TPA all potently activated MAPK in control cells. In contrast, this activation was significantly reduced in cells treated with dexamethasone.

- Activation of the p21\textsuperscript{WAF1/CIP1} promoter by these mitogenic treatments was likewise greatly reduced in the dexamethasone-pretreated cells (Fig. 2B).

- A cotransfection assay, in which the Ras-Asn17-expressing plasmid was cotransfected into cells with the p21\textsuperscript{WAF1/CIP1} promoter-luciferase reporter, was used to determine the role of Ras in mediating serum-induced p21\textsuperscript{WAF1/CIP1} promoter activation in HeLa cells. Although forced expression of the Ras-Asn17-negative mutant had little effect on the basal activity of the p21\textsuperscript{WAF1/CIP1} promoter, it abolished the serum-induced p21\textsuperscript{WAF1/CIP1} promoter activation (Fig. 2C), confirming the requirement for Ras in the activation of p21\textsuperscript{WAF1/CIP1} transcription by growth factors.

Next, we examined the positive influence of Ras and other downstream components of MAPK signaling pathway on p21\textsuperscript{WAF1/CIP1} promoter activity. First, p21\textsuperscript{WAF1/CIP1} promoter-luciferase reporter was cotransfected into HeLa and PC12 cells with a construct which expresses a dominant positive mutant form of Ras (Ras-Val12; Ref. 14). This results in high levels of constitutively activated Ras. As shown in Fig. 3A, this mutant potently increased p21\textsuperscript{WAF1/CIP1} promoter activity. Since Raf and MAPKK act downstream of Ras to
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Fig. 2. Analysis of the Ras involvement in the mitogenic activation of MAPK and the p21WAF1/CIP1 promoter. A, effect of the dominant negative Ras-Asn17 allele on MAPK activation in PC12 cells. PC12-MMTV Ras-Asn17 cells were either left untreated or pretreated for 24 h with 1 μM dexamethasone, then treated with the indicated mitogenic agents for 15 min (EGF, 100 ng/ml; NGF, 50 ng/ml; serum, 10% FBS; TPA, 60 ng/ml). MAPK was immunoprecipitated using rabbit polyclonal anti-p42/44MAPK antibody and assayed for activity via phosphorylation of MBP. Samples were electrophoresed through 15% SDS-polyacrylamide gels, dried, and analyzed using autoradiography. B, effect of dominant negative Ras on p21WAF1/CIP1 promoter activity in PC12 cells. PC12-MMTV Ras-Asn17 cells were either left untreated or treated with dexamethasone (1 μM) immediately following transfection with WWP-Luc. Luciferase activity was measured 48 h posttransfection following the 18 h treatment with the indicated doses of NGF. The relative fold induction refers to the ratio of luciferase activity measured in treated cells relative to the activity observed in the untreated control cells. C, effect of dominant negative Ras on the serum induction of p21WAF1/CIP1 promoter activity in HeLa cells. The WWP-Luc reporter (1 μg) was cotransfected with 10 μg of either empty vector or the Ras-Asn17 expression plasmid into HeLa cells. The transfected cells were subsequently left untreated or serum stimulated (10% FBS) for 18 h prior to harvest. Luciferase activity was measured, normalized for cellular protein concentration, and reported as the ratio of normalized activity from treated cells to that from untreated cells transfected with WWP-Luc alone.

activate MAPK, we also examined the effect of introducing vectors constitutively expressing activated Raf (Raf-BXB; Ref. 15) and activated MAPKK (MEKEE; Ref. 16) along with the p21WAF1/CIP1 promoter-luciferase reporter plasmid. Both Raf-BXB and MEKEE strongly enhanced p21WAF1/CIP1 promoter activity in HeLa and PC12 cells (Fig. 3A).

As additional evidence to support the role of MAPK in regulating p21WAF1/CIP1 expression, plasmids constitutively expressing high lev-
Fig. 4. Comparison of MAPK activation and p21\textsuperscript{WAF1/\textsuperscript{CIP1}} mRNA levels in wild-type- versus trk-overexpressing PC12 cells in response to NGF treatment. Wild-type PC12 (wt PC12) and trk-overexpressing PC12 (trk PC12) cells were treated with 50 ng/ml NGF for the indicated times prior to analysis. A, following NGF treatment, cells were harvested, and MAPK was immunoprecipitated using rabbit polyclonal anti-p42MAPK antibody and assayed for activity via phosphorylation of MBP. B, Northern blot analysis of p21\textsuperscript{WAF1/\textsuperscript{CIP1}} mRNA levels in NGF-treated wild-type PC12 and trk PC12 cells. In the diagram showing quantitation of p21\textsuperscript{WAF1/\textsuperscript{CIP1}} mRNA levels, the values have been normalized for 18S rRNA and are reported as the relative fold induction of normalized p21 mRNA levels over the untreated (0 h) time point.

Role of Growth Factor Receptors in Mediating p21\textsuperscript{WAF1/\textsuperscript{CIP1}} Induction. It has been shown that growth factor receptors play a crucial role in mediating the signal transduction pathways, leading to MAPK activation by growth factors (9). NGF binds to the trk receptor, triggering its autophosphorylation and subsequent signaling events involved in the activation of MAPK (17). A cell line overexpressing the trk receptor has been developed and used to study the signal transduction pathways involved in NGF-induced differentiation. These cells have been shown to display sustained tyrosine phosphorylation of MAPK following NGF treatment, although kinase activity has not been directly measured (17). Here, we examined the relationship between trk levels, MAPK activity, and p21\textsuperscript{WAF1/\textsuperscript{CIP1}} expression in NGF-treated cells. As indicated in Fig. 4A, NGF caused a rapid activation of MAPK as assessed by the immunocomplex kinase assay. In wild-type PC12 cells, optimum activation was detected as early as 5 min after NGF addition and thereafter declined, returning to basal levels within 3 h after treatment. Cells overexpressing trk showed similar basal levels of kinase activity. However, they showed a higher magnitude of kinase activation following NGF treatment that was sustained for a longer period of time. Comparison of p21\textsuperscript{WAF1/\textsuperscript{CIP1}} induction in control and trk-overexpressing cells revealed a significantly higher level of p21\textsuperscript{WAF1/\textsuperscript{CIP1}} mRNA expression in the trk mutants following NGF treatment (Fig. 4B), further supporting the participation of the trk receptor in the MAPK signaling pathway leading to p21\textsuperscript{WAF1/\textsuperscript{CIP1}} activation by NGF.

General Discussion. We have shown that the induction of p21\textsuperscript{WAF1/\textsuperscript{CIP1}} in response to growth factor stimulation occurs, at least in part, through a transcriptional mechanism that involves the phosphorylation cascade leading to MAPK activation. Although the enhancement in the activity of the human p21\textsuperscript{WAF1/\textsuperscript{CIP1}} promoter by growth factor stimulation is modest (2–5-fold increase, dependent on
the particular treatment), it is consistent with recent studies utilizing a mouse p21\textit{WAF1/CIP1} promoter-luciferase construct (3). The precise cis elements responsible for mediating the induction remain to be identified. However, it is worth noting that a 710-bp fragment from the mouse p21\textit{WAF1/CIP1} promoter that appears to control the response to serum contains a putative Ets-binding site (3). Ets-related transcription factors have been linked to the serum responsiveness of other promoters (10, 11), and in some instances have been found to be activated by MAPK-dependent phosphorylation (20). Our finding that p21\textit{WAF1/CIP1} expression is transcriptionally regulated through the MAPK pathway is also consistent with recent findings of Canman et al. (21), who demonstrated that forced expression of either v-Src or activated c-Raf in murine hematopoietic Baf-3 cells prevents downregulation of p21 expression that normally occurs in these cells in response to growth factor withdrawal.

Given the general importance of the MAPK signaling pathway in mediating cellular responses to a wide range of extracellular cues, the relevance of our findings is likely to extend far beyond that shown here for growth factors. For instance, it is well established that c-fos activation by genotoxic agents is mediated by the MAPK signaling pathway (22). Since p21\textit{WAF1/CIP1} is also highly induced by such treatments, even in the absence of p53, it is likely that MAPK plays a role in this response.

The induction of p21\textit{WAF1/CIP1} by growth factors appears to be somewhat contradictory to the purported role of p21\textit{WAF1/CIP1} in negatively regulating cell growth through its ability to act as a cdk inhibitor. However, this paradox has been resolved by the finding that p21\textit{WAF1/CIP1} also promotes the assembly of cyclin-cdk-proliferating cell nuclear antigen complexes (5), and therefore in low concentrations exerts a positive influence on cell cycle progression. It is only when p21\textit{WAF1/CIP1} levels are high (such as following a severe stress like DNA damage) that it becomes inhibitory for growth (1, 23). This also emphasizes the need for independent mechanisms to regulate p21\textit{WAF1/CIP1} expression during normal growth and during stress. Indeed, in addition to the transcriptional mechanisms discussed in this article, there is also evidence to indicate that p21\textit{WAF1/CIP1} is regulated by posttranscriptional events that alter the stability of p21\textit{WAF1/CIP1} mRNA (8). Further understanding of the mechanisms contributing to control p21 expression could improve our understanding of how this gene product is affected in tumorigenesis and perhaps lead to the development of chemotherapeutic strategies to alter its expression.

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