The Stress-activated Protein Kinases p38α and JNK1 Stabilize p21\textsuperscript{Cip1} by Phosphorylation*

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Cell cycle progression is mediated by the sequential activation of members of the cyclin-dependent kinase (CDK)\textsuperscript{1} family. Cell cycling is blocked when CDK-cyclin complexes cannot form or when the catalytic activity of these complexes is blocked through binding of a CDK inhibitor molecule. One of these inhibitors is p21\textsuperscript{Cip1}, which was originally identified as a PCNA-binding protein (2), and p21 inhibits DNA replication by preventing PCNA from contributing to DNA polymerase δ activity, which results in the translocation of p21 from the nucleus to the cytoplasm in Her-2/neu-transformed NIH3T3 cells (11), at Thr\textsuperscript{146} and Ser\textsuperscript{146} in glioblastoma cells (13). Akt also phosphorylates p21 at Thr145 in Her-2/neu-transformed NIH3T3 cells (11), at Thr145 and Ser146 in glioblastoma cells (13). Akt activates GSK3β, which phosphorylates p21 at Thr\textsuperscript{57} (14). Phosphorylation at Thr145 does not affect p21 stability, but results in the transition of p21 from the nucleus to the cytoplasm in Her-2/neu-transformed NIH3T3 cells (11). Phosphorylation of p21 at Ser\textsuperscript{146} has been shown to mediate phosphorylation of p21 at Ser\textsuperscript{146} in vivo, and mutation of Ser\textsuperscript{146} to alanine rendered p21 less stable than wild-type p21. TGF-β1 increased the stability of wild-type p21, but not the p21-S130A mutant. These findings demonstrate that SAPKs can mediate cell cycle arrest through post-translational modification of p21.

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‡ The abbreviations used are: CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; GSK3β, glycogen synthase kinase-3β; SAPK/JNK, p38 MAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; TGF-β1, transforming growth factor-β1; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; DN, dominant-negative; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; M KK, MAPK kinase.

EXPERIMENTAL PROCEDURES

Materials—TGF-β1 was purchased from R&D Systems. Antibodies to p21 and to the FLAG epitope were from Transduction Laboratories and Sigma, respectively, and antibodies to the HA epitope and JNK1/2 were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to Akt, phospho-Akt, phospho-ERK1/2, ERK1/2, activated p38 MAPK, and p38 MAPK were from Cell Signaling and New England Biolabs Inc.
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**RESULTS**

**Post-transcriptional Up-regulation of p21 by TGF-β1—** Up-regulation of protein levels of the CDK inhibitor p21<sup>Cip1</sup> is known to mediate the growth arrest of normal cells. The HD3 human colon carcinoma cell line has many characteristics of normal colon epithelial cells and was cloned from its parental cell line because of its unusual “flat” morphology. At high density, the line spontaneously differentiates into one of the lineages in the normal colon, the fluid-transporting enterocyte. Differentiated cells exhibit a brush border and are joined by functional junctional complexes of tight junctions, gap junctions, and desmosomes (18). HD3 cells exhibit the normal growth control, as described in Ref. 22; they can be synchronized in G<sub>1</sub> by density arrest and are arrested in G<sub>1</sub> by TGF-β1. Arrest occurs through inhibition of the kinases that phosphorylate the retinoblastoma protein (22). TGF-β1 increased p21 abundance up to 9-fold in HD3 cells as shown by Western blotting (Fig. 1A). These elevated levels of p21 bind to and block CDK2 kinase activity as shown by immu-
munneprecipitation of CDK2 and kinase assay of histone H1 (23). All these data are consistent with the known growth-arresting properties of TGF-β1.

The unexpected finding was that the TGF-β1-induced increase in p21 protein levels in HD3 cells was mediated at the post-transcriptional level. Most studies have concentrated on transcriptional activation of p21. The increase in abundance of p21 in HD3 cells occurred rapidly and was detectable within 1–3 h (Fig. 1B; mean of two experiments). HD3 cells exhibit both mutant p53 (22) and the truncation mutation SMAD4Q311st (23), so transcription of p21 is p53-independent and should be independent of TGF-β1-activated SMAD proteins. TGF-β1 did not increase p21 mRNA levels in HD3 cells (Fig. 1D; mean of two experiments, p21 mRNA normalized to GAPDH mRNA) or activate a p21 promoter reporter (Fig. 1C) in these cells. Taken together, these data confirm that the increase in p21 protein abundance by TGF-β1 is mediated by post-transcriptional, SMAD-independent mechanisms.

**TGF-β1 Signaling Pathways in HD3 Cells**—We next wished to determine which signaling pathways mediate the rapid post-transcriptional increase in p21 protein levels. TGF-β1 can initiate both SMAD-dependent and SMAD-independent signaling pathways. Because HD3 cells exhibit the truncation mutation SMAD4Q311st (23), TGF-β1 signaling should be SMAD-independent. To resolve this issue, we cotransfected pSBE4-BV/luc, a reporter containing four repeats of an 8-bp palindromic SMAD-binding element that is known to be activated only by a SMAD3-SMAD4 complex (24), into HD3 cells together with SMAD4 expression plasmids. The pSBE4-BV/luc reporter was activated only by TGF-β1 when wild-type SMAD4 (but not DN-SMAD4ΔC) was cotransfected (Fig. 2A). Parallel experiments demonstrated that TGF-β1 could not activate a mutant form of this reporter construct (p6MBE) when cotransfected with either wild-type or mutant SMAD4 (data not shown).

Therefore, endogenous SMAD4 is nonfunctional, and TGF-β1 signaling is SMAD-independent in HD3 cells.

TGF-β1 initiates several SMAD-independent signaling pathways, including Ras/ERK (25), Rho/JNK (26), TAK1/p38 MAPK (27, 28), protein phosphatase 2A/p70 S6 kinase (29), phosphatidylinositol 3-kinase 3-kinase/Akt (30), and possibly others. Because phosphorylation of p21 at Ser146 has been reported to stabilize p21 (12), so p70 S6 kinase has been reported to be unable to phosphorylate p21 in vivo. p70 S6 kinase has been reported to be unable to phosphorylate p21 in HD3 cells. PD98059 caused a strong activation of TGF-β1 signaling in HD3 cells, implying that activated ERKs block TGF-β1 signal transduction. Activated p38 is known to complex with ERK1/2 (31), so it is possible that blocking activation of ERKs leads to further up-regulation of p38 activity. These transient transfection data suggest that TGF-β1 activates p38 (but not Akt, ERK1/2, or SMAD) signaling in HD3 cells. These data were confirmed by biochemical analysis. The increase in p21 abundance in HD3 cells was detectable within 1–3 h (Fig. 1B), so any TGF-β1 signaling that affects p21 must occur within this time frame.

TGF-β1 activates p38 MAPK and JNK within 30 min, so both may play a role in p21 modulation. The activities of p38α and JNK1 were determined by immune complex kinase reaction using exogenous purified p21 protein cleaved from GST as
substrate (Fig. 3A). Cells were treated with 6 ng/ml TGF-β1 for 0–300 min, and p38α or JNK1 was immunoprecipitated at various times as indicated. TGF-β1 activated both p38α and JNK1 by 15–30 min, and both kinases phosphorylated p21. The activity returned to background levels by 300 min. Controls include preimmune rabbit serum. Equal amounts of IgG were detected in the immunoprecipitates, and equal amounts of exogenous p21 were analyzed (Fig. 3A, lower panel). GST was cleaved from recombinant GST-p21 before assay and migrated as a non-phosphorylated band at ~29 kDa (data not shown).A similar time course for p38 and JNK activation was shown. A similar time course for p38 and JNK activation was shown. A similar time course for p38 and JNK activation was shown.

**TGF-β1 Increases the Half-life of p21 by an SB203580-sensitive Mechanism**—Because TGF-β1 was shown to activate p38 in HD3 cells (Figs. 2 and 3), the effect of p38 inhibition on accumulation of the p21 protein was assessed. Treatment with the p38αβ inhibitor SB203580 blocked the TGF-β1-mediated increase in p21 abundance in a dose-dependent manner, with the amount of p21 halved by treatment with 5–10 μM (Fig. 4A, upper panel). As a control for the specificity of the inhibitor, p21 mRNA levels were assayed by Northern analysis, and no modulation by SB203580 was seen (Fig. 4A, lower panel), indicating that the effects on p21 are post-transcriptional and probably post-translational. The p21 protein is unstable, with a short half-life of ~20–30 min in most cells. To determine whether the elevated levels of p21 in TGF-β1-treated HD3 cells (Fig. 1) were due to protein stabilization, the turnover of the p21 protein was assayed. The half-life of p21 was determined by Western blotting after blocking translation with cycloheximide and was normalized to actin abundance determined on the same Western blot (Fig. 4, C and E; means ± S.E. of four separate experiments). The half-life of p21 was increased to ~90–120
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FIG. 4. TGF-β1 increases the half-life of p21 by an SB203580-sensitive mechanism. A, Western blot showing the abundance of p21<sub>cip1</sub> and actin in HD3 cells treated with increasing concentrations of SB203580. Cells were serum-starved overnight and then treated with 6 ng/ml TGF-β1 for 24 h and either SB203580 (SB) or Me<sub>2</sub>SO (DMSO) vehicle as indicated, with SB203580 added 30 min before TGF-β1. Northern blots of parallel cultures for p21<sub>cip1</sub> and GAPDH (G3PDH) mRNAs are shown in the lower panel. B, autoradiogram showing the abundance of [35S]methionine-prelabeled p21<sub>cip1</sub> in immunoprecipitates from TGF-β1-treated cells 0, 30, and 90 min after chase with unlabeled methionine ± 10 μM SB203580 as indicated. The lower panel shows Coomassie Blue-stained IgG light chain to demonstrate equal addition of antibody. C, Western blot showing turnover of p21<sub>cip1</sub> in HD3 cells treated with TGF-β1, no TGF-β1, or TGF-β1 plus 10 μM SB203580. Cells were serum-starved overnight and then treated with 6 ng/ml TGF-β1 or no additions for 24 h. 10 μM SB203580 was added to half of the TGF-β1-treated cultures; and then 30 min later, 20 μg/ml cycloheximide was added to all cultures, and p21<sub>cip1</sub> and actin abundance was determined at the indicated time points. D, plot of p21<sub>cip1</sub> turnover by the pulse-chase experiment in B, E, plot of p21<sub>cip1</sub> turnover following addition of cycloheximide from C, with results from four separate experiments summarized (means ± S.E.).
can phosphorylate p21 in vitro by purified recombinant JNK1 and by purified recombinant p38α or purified recombinant GST-p21. The lower panel is Coomassie Blue (CB) staining of the blotted autoradiogram showing the amount of GST-p21 or GST in each reaction. B, means ± S.E. of three in vitro kinase assays, with data normalized to the wild-type (WT) p21 values for each experiment.

**FIG. 5.** In vitro kinase assay demonstrates that p38α and JNK1 can phosphorylate p21 in vitro, whereas mutation to T57A or S130A or the double mutation T57A/S130A decreases the amount of phosphorylation. A, autoradiogram showing 32P-labeled p21 constructs. Equal amounts of purified GST-p21 constructs were added to each kinase reaction with either purified recombinant p38α or purified recombinant JNK1. The lower panel is Coomassie Blue (CB) staining of the blotted autoradiogram showing the amount of GST-p21 or GST in each reaction. B, means ± S.E. of three in vitro kinase assays, with data normalized to the wild-type (WT) p21 values for each experiment.

*p38α and JNK1 (but Not p38β) Phosphorylate p21 in Vivo—*We next determined whether JNK1 or either isoform of p38 (α or β), which are inhibited by SB203580, phosphorylates p21 in vivo by coexpression experiments. Constructs for p38α and DN-p38α as a control were transfected into HD3 cells, allowed to express overnight, and then pulse-labeled with [32P]orthophosphate in the presence of 10 μg/ml anisomycin for 3 h to strongly activate exogenous p38α. The 32P-labeled p21 constructs and the p38 constructs were immunoprecipitated by their FLAG epitopes and analyzed by autoradiography after SDS-PAGE. The p21 protein coexpressed with wild-type p38α incorporated 3.5 times as much [32P]orthophosphate as the p21 protein coexpressed with DN-p38α (Fig. 6A, upper panel). Equivalent amounts of FLAG-p21 were immunoprecipitated as determined by Western blotting for the FLAG epitope (Fig. 6A, lower panel). Therefore, p21 is phosphorylated in vivo in a p38α-sensitive manner. Both p38 constructs demonstrated similar expression as shown by examination of their abundance by Western blotting for their FLAG epitopes (Fig. 6A, lower panel). The wild-type p38α construct exhibited much more autophosphorylation than the DN-p38α construct, as expected. Therefore, p38α phosphorylates p21 in vivo. A similar experiment was performed with JNK1, DN-JNK, p38β, and DN-p38β (Fig. 6B). p38β and DN-p38β were expressed in vivo at similar levels, but the phosphorylation of p21 was not modulated by either form (Fig. 6B), indicating that p38β does not phosphorylate p21 in vivo. However, DN-JNK1 reduced the phosphorylation of p21 by ~10-fold when protein expression levels were normalized. In addition, coexpression of JNK1 clearly increased the phosphorylation of p21 over control levels. Therefore, both JNK1 and p38α (but not p38β) initiate the phosphorylation of p21 in vivo.

**Coexpression of JNK1 Increases the Abundance of p21 in Vivo—**Coexpression of DN-JNK inhibited p21 expression in a number of experiments in addition to Fig. 6B, so we turned to another experimental design. We assayed for the effect of JNK1 on p21 protein levels directly, hypothesizing that if JNK1 phos-
phosphorylated p21 in vivo, p21 would be stabilized, and more protein would be detected. The effect of JNK1 on p21 protein levels in vivo was determined by transiently transfecting a FLAG epitope-tagged expression plasmid for p21 together with HA-tagged JNK1. An expression plasmid for GFP driven by the same promoter as that used in the p21 construct was used as an internal transcription rate control. Increasing amounts (1, 3, and 5 μg) of wild-type JNK1 were transfected with the total amount of DNA kept constant using vector DNA. After 24 h of expression, the amounts of FLAG-p21 protein, GFP protein, and HA-JNK1 were determined by Western blotting. The two proteins were identified by their electrophoretic mobility.

**FIG. 7.** Stability of mutant p21 constructs in vivo. **A,** HD3 cells were cotransfected with either wild-type (WT) or mutant FLAG-p21 constructs together with an expression construct for FLAG-p38a. After 24 h, the p21 and p38 proteins were immunoprecipitated (IP) by their FLAG epitopes and Western blotted for FLAG. The two proteins were identified by their electrophoretic mobility. **B,** HD3 cells were cotransfected with either wild-type or mutant FLAG-p21 constructs together with an expression construct for FLAG-p38a. After 24 h to allow expression of each construct, 20 μg/ml cycloheximide (CH) was added to all cultures, and p21S146A and β-tubulin abundance was determined at the indicated time points. **C,** the Western blot shows that equivalent amounts of exogenous p38a were expressed in each experiment shown in **B, D–F,** data from **B** and a duplicate experiment are plotted (means ± S.E. (bars)).

**Mutant p21 Constructs Are Less Abundant in Vivo—**Phosphorylation of p21 at Ser146 by Akt has been reported to stabilize p21; and as a result, mutation of Ser146 to a non-phosphorylatable alanine prevents a stabilizing phosphorylation. The p21-S146A mutant was present at about half the level of wild-type p21 (Fig. 7A), consistent with the known capability of S146A to destabilize p21 (13). The p21-T57A/S130A double mutant was expressed at steady-state levels comparable to the unstable S146A construct, whereas the T57A and S130A mutant constructs were intermediate between the double mutant and wild-type p21 (Fig. 7A). These data are consistent with the hypothesis that phosphorylation at Ser130 and Thr57 stabilizes p21, whereas mutation of these sites allows more rapid proteolysis. S130A or T57A Mutation Destabilizes p21—After transient transfection and expression of p21 constructs in HD3 cells, the half-life of each construct was determined by translational arrest by cycloheximide treatment (Fig. 7, B–F). The abundance of p21 proteins was determined by Western blotting at intervals 0–6 h after addition of cycloheximide. Cycloheximide is known to activate JNK (33, 34), and our hypothesis is that JNK, as well as p38, phosphorylates and stabilizes p21. Supporting this hypothesis, the initial 30-min time point showed an increase in the protein level for the wild-type p21 and p21-S130A constructs, and the p21-T57A construct exhibited no loss in protein level (Fig. 7B). However, the S130A, T57A, and double mutant constructs were more rapidly proteolyzed than the wild-type control (Fig. 7, B and D; mean of two experiments). Therefore, mutation of either Ser146 or Thr57 to a non-phosphorylatable alanine prevents a stabilizing phosphorylation.

The effect of p38α on the stability of each p21 construct was next determined by cotransfection studies. Exogenous p38α was coexpressed at similar levels with each construct, and elevated levels were maintained at each time point (Fig. 7C). Cotransfection of wild-type p21 with an expression construct for p38α caused a 2–5-fold increase in p21 protein levels, which
was maintained during the cycloheximide treatment (Fig. 7, B and E). In contrast, coexpression of p38α together with the p21-T57A construct or the double mutant did not decrease their rapid proteolysis, which was clearly seen 1.5–3 h after addition of the drug (Fig. 7, B and E). These data suggest that phosphorylation at Thr57 is necessary for stabilization of p21. The p21-S130A mutant displays a different phenotype. Coexpression of p38α together with the p21-S130A construct decreased its rapid proteolysis (Fig. 7, B and F). There is evidently a site (or sites) in p21-S130A that can be phosphorylated by p38α or another kinase activated by p38α and that stabilizes p21. Note that the stabilizing effect of p38α on p21-S130A was much less than its effect on wild-type p21. Phosphorylation at Ser130 by either p38α or another kinase, perhaps JNK, is necessary to optimally stabilize p21.

**FIG. 8. Peptide mapping of p21.** A, in vitro phosphorylation of p21 constructs by purified recombinant p38α. Wild-type (WT) p21, p21-T57A, p21-S130A, and p21-T57A/S130A were phosphorylated in vitro with p38α, digested to completion with chymotrypsin; and subjected to two-dimensional peptide mapping. The positions of the peptide containing Thr57 and the peptide containing Ser130 are indicated. B, in vivo phosphorylation of wild-type (WT) p38α-p21 by 6 ng/ml TGF-β1 treatment, by cotransfected p38α, by both TGF-β1 treatment and coexpression of p38α, or by no treatment (Control) was determined by two-dimensional peptide mapping following digestion with chymotrypsin (autoradiograms shown in the **upper panels**). In vivo phosphorylation of mutant FLAG-p21-T57A by treatment with 6 ng/ml TGF-β1 or by no treatment was determined by two-dimensional peptide mapping following digestion with chymotrypsin (autoradiograms shown in the **lower panels**). The positions of the Ser130-containing and Thr57-containing peptides are indicated by arrows, and the two novel peptides phosphorylated following treatment are indicated by arrowheads.

TGF-β1 and p38α Stabilize p21 in Vivo by Phosphorylation at Ser130—Although coexpressed p38α effectively stabilized p21 in vivo, it was necessary to define the sites of phosphorylation in vivo. Two-dimensional phosphopeptide mapping of p21 was performed. To facilitate the identification of the specific peptides, we initially performed mapping using wild-type and mutant p21 constructs phosphorylated in vitro by purified recombinant p38α using reaction mixtures containing [γ-32P]ATP. These p38α-treated p21 constructs were digested to constituent peptides with chymotrypsin, and the 11 peptides were separated by electrophoresis, followed by thin-layer chromatography. As shown in Fig. 8A, peptide fragments including Thr57 and Ser130 were readily identified by comparison with mutant p21 constructs. The peptide containing Thr57 migrated rapidly upon chromatography, but showed little electrophoretic mobility, whereas the peptide containing Ser130 exhibited little migration upon chromatography, but efficient electrophoretic mobility (Fig. 8A). HD3 cells were then transfected with wild-type FLAG-p21 alone or cotransfected with an expression plasmid for p38α. After 24 h of expression, cells were switched to low phosphate medium; [32P]orthophosphate was added; and cells were either treated for 6 h with 6 ng/ml TGF-β1 or left untreated. Wild-type FLAG-p21 was then immunoprecipitated and digested with chymotrypsin, and peptide mapping was performed exactly as described above. Treatment with TGF-β1 or coexpression with p38α yielded six [32P]-labeled peptides (Fig. 8B), three of which were also seen in cells not treated with TGF-β1. The remaining three peptides were identical in wild-type p21 isolated from TGF-β1-treated cells and in wild-type p21 isolated from p38α-expressing cells. In multiple experiments, both TGF-β1 and p38α induced phosphorylation of the Ser130-containing peptide in vivo, but not the Thr57-containing peptide (Fig. 8B, **upper panels**, arrows). TGF-β1 treatment of cells transfected with p38α amplified the phosphorylation of the Ser130-containing peptide, confirming its designation as the site of in vivo phosphorylation.

The lack of phosphorylation at Thr57 in vivo was surprising because this site was strongly phosphorylated in vitro (Fig. 5). Two novel peptides were phosphorylated following TGF-β1 treatment or p38α expression (Fig. 8B, arrowheads). The hypothesis was considered that p21 is phosphorylated in vitro at a site atypical for MAPKs, Thr57, as well as at Thr57. This would add an additional phosphorylation site within peptide 54–63 derived from p21 by chymotrypsin digestion. The doubly phosphorylated peptide would not have the same mobility as the Thr57-phosphorylated peptide derived following in vitro phosphorylation and could be one of the two novel peptides. To test this hypothesis, a FLAG-p21-T57A construct was expressed in HD3 cells, which were then treated with TGF-β1 or left untreated as described above. The immunoprecipitated FLAG-p21-T57A construct was digested with chymotrypsin, and peptide mapping was performed exactly as described above. In duplicate experiments, TGF-β1 treatment led to the phosphorylation of...
the Ser130-containing peptide and the same two novel peptides as had been seen in digests of wild-type p21 (Fig. 8B, lower panels). The same pattern of phosphorylated peptides was also seen when FLAG-p21-T57A was cotransfected with an expression plasmid for p38/H9251 (data not shown). Neither novel peptide could be sequenced because of their low abundance (data not shown). However, because the same novel phosphopeptides were detected in digests from wild-type p21 and the p21-T57A mutant, these peptides could not have been derived from p21 doubly phosphorylated at Thr55 and Thr57 in vivo. These data confirm the observation that Thr 57 is not phosphorylated in vivo in HD3 cells following treatment with TGF-β/H9252 or by activated p38. Possibly, phosphorylation of p21 at Ser130 alters the conformation of p21, allowing phosphorylation by other kinases in vivo, or TGF-β1-activated p38 in turn activates other downstream kinases that phosphorylate p21. There are several serines at the N and C termini of p21 that could be phosphorylated in vivo and give rise to the novel phosphopeptides. These data demonstrate that both TGF-β1 and p38α induce phosphorylation of p21 at Ser130 in vivo (Fig. 8) and that phosphorylation mediates p21 stabilization (Fig. 7).

We then determined whether the stabilization of p21 mediated by TGF-β1 in vivo (Figs. 1 and 4) could be prevented by mutation of p21 at Ser130. HD3 cells were cotransfected with either wild-type FLAG-p21 or FLAG-p21-S130A and, after 5 h of expression, treated with 6 ng/ml TGF-β1 for 24 h. Cycloheximide was then added to block translation, and parallel lysates were examined for abundance of p21 and tubulin as a control for periods up to 6 h (Fig. 9). TGF-β1-treated p21 was more stable than untreated p21 at each point in the cycloheximide chase (Fig. 9, A and B). Mutation of p21 to S130A (Fig. 9, A and C) or to S130A/T57A (data not shown) prevented TGF-β1 from stabilizing the mutant p21 construct. Taken together, these experiments demonstrate that p38α mediates the stabilization of the p21 protein in vivo by phosphorylation at Ser130.

**DISCUSSION**

MAPK (ERK, SAPK/JNK, and p38) pathways are composed of three-tiered core signaling modules in which MAPKs are activated by concomitant phosphorylation on TXY in the activation loop of kinase subdomain VIII. MAPK phosphorylation and activation are catalyzed by MAPK kinases (MEKs or MKKs). MEKs in turn are activated by Ser/Thr phosphorylation catalyzed by a divergent family of MAPK kinase kinases. These in turn are regulated by a wide variety of upstream activators and inhibitors (reviewed in Ref. 34). TGF-β1 has been shown to activate both SAPKs/JNKs and p38 proteins through SEK1, MKK3, and MKK6 (27), and JNKs through activation of Rho (35). The present study has implicated both p38α and JNK1, activated by TGF-β1, in cell cycle control through the stabilization of p21. The increased levels of p21 induced by TGF-β1 in HD3 cells, the system used in this study, have been shown to mediate cell cycle arrest in G1 through a block in retinoblastoma protein phosphorylation (23). Using peptide mapping following in vivo phosphorylation, we have demonstrated that TGF-β1-activated p38α mediates the stabilization of the p21 protein in vivo by phosphorylation at Ser130. We have also shown that the stabilization of p21 by TGF-β1 in vivo is prevented by mutation of p21 Ser130 to a non-phosphorylatable alanine.
Both p38 and JNK are protein kinases that are activated by environmental stresses such as inflammatory cytokines, vaso-active peptides, heat shock, nutrient withdrawal, hypertonic stress, and genotoxic chemotherapy agents (reviewed in Ref. 34). These kinases function by transmitting stress signals to a bank of downstream transcription factors that alter cellular response. Responses to stress signals can include induction of apoptosis or cell differentiation. G1 arrest has been detected in G1-synchronized NIH3T3 cells in response to p38, with greater arresting activity induced by the p38 upstream activator Cdc42Hs (36). SAPK/JNK do not cause G1 arrest, whereas expression of ERK induces a small increase in entry into S phase. Our observations in this study suggest that p38 mediates G1 arrest through stabilizing p21 and delaying its proteosomal breakdown. G1 arrest in response to stress signals would enable cells to repair stress-related damage. Alternatively, G1 arrest in response to stress signals in some cells could be mediated by down-regulation of G1 cyclins (37). In the present study, coexpressed p38α and JNK1 (but not p38β) increased the phosphorylation of p21 in vitro, and purified recombinant p38α and JNK1 phosphorylated p21 in vitro at Thr57 and Ser130. In vivo stabilization of p21 was blocked by the p38α/β inhibitor SB203580, providing strong corroboration for a role for p38α in p21 stabilization. The evidence for a role for JNK1 in p21 stabilization is less strong, and the lack of effect of SAPK/JNK in inducing G1 arrest in NIH3T3 fibroblasts (36) argues against its involvement, at least in fibroblasts. However, both JNK and p38 are activated by TGF-β in HD3 cells within the time frame necessary for phosphorylation of p21, and JNK1 phosphorylates p21 in vivo; so JNK may play a role in p21 stabilization and G1 arrest in colon epithelial cells. Although the observations in this study that p21 is stabilized by p38α demonstrate a novel role for p38α in G1 arrest, p38α has been shown to mediate rapid activation of the G2/M checkpoint after UV irradiation of dermal fibroblasts or HeLa cells (38) or after hypertonic stress in renal inner medullary epithelial cells (39). Therefore, p38α functions to mediate checkpoints at both G1 and G2/M.

The effect of phosphorylation of p21 at Thr57 is controversial and may be cell type-specific. We have shown that mutation of this residue to alanine renders the mutant p21 construct less stable than wild-type p21 in HD3 colon epithelial cells (Fig. 7). However, Rösing et al. (14) have presented clear evidence that GSK3β phosphorylates p21 at Thr57 in vitro; that treatment of normal endothelial cells with LiCl, a GSK3β inhibitor, stabilizes p21; and that the p21-T57A mutant is more stable than wild-type p21 in endothelial cells. Although this difference in stability of p21-T57A was observed between HD3 colon carcinoma cells and endothelial cells, a second p21 mutation gave similar results in two cell types. Mutation of p21 at Ser446 to alanine, which cannot be phosphorylated, destabilized p21 in glioblastoma cells (13) and also destabilized p21 in HD3 colon epithelial cells in the current study (Fig. 7 and data not shown).

When confluent, the HD3 cells used in this study spontaneously differentiate to fluid-transporting enterocytes, which elaborate functional junctional complexes between adjacent cells that render them impermeable to transported fluids (18). The transport of fluids from the apical through the basal side of this HD3 cell sheet causes a fluid bubble or “dome” to form in the differentiated monolayers. The normal colon is routinely exposed to hyperosmotic fluid (40), and this exposure immediately triggers a response to control cell volume through activation of p38 MAPK (41). The mechanism of this control of cell volume has been delineated in Saccharomyces cerevisiae. Hyperosmotic conditions activate the p38 MAPK homolog Hog1, which stimulates accumulation of glycerol to increase intracellular osmolarity (42). In mammalian cells, nutritional starvation, heat shock, and UV as well as osmotic shock activate p38 MAPK. The tumor suppressor protein p53 is also targeted by p38 by osmotic shock. p38 activates p53 transcriptional activity by phosphorylating p53 at Ser15 (43). Thus, p38 mediates responses to osmotic shock by phosphorylating the p53 tumor suppressor. The results of this study indicate that p38α can also respond to cellular stress by phosphorylating the p21Cip1 cell cycle regulator, which also can be considered a tumor suppressor.

p38 MAPK has been shown to mediate differentiation in intestinal epithelium. Phosphorylated active forms of p38 were detected in the nuclei of differentiated villus cells (44). Moreover, p38 levels increase when Caco-2/15 cells become confluent and differentiated, and p38-dependent phosphorylation of the homeoprotein transcription factor CDX3 occurs in differentiated Caco-2/15 cells (44). Therefore, p38 MAPK modulates intestinal cell differentiation through regulation of CDX3 function. The stabilization of the p21 protein by p38 MAPK shown in this study could initiate intestinal cell differentiation by arresting cells in G1 as well as by enabling cells to arrest and repair in G1 after environmental stresses.

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