p38α Antagonizes p38γ Activity through c-Jun-dependent Ubiquitin-proteasome Pathways in Regulating Ras Transformation and Stress Response*

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Mitogen-activated protein kinase (MAPK)3 pathways consist of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 cascades (1, 2). The ERK activity is generally required for cell proliferation and transformation (3, 4), whereas JNK and p38 pathways are involved in stress response (5, 6). Both antagonistic and cooperative activities among these three MAPK pathways have been reported in mediating various biological responses (5, 7–9), but mechanisms involved in these signaling integrations are mostly unknown. Moreover, each of MAPK pathways consists of several family members (1, 2, 10), and how these isoform proteins coordinate for a biological response is unclear. Demonstrating both specific and integrated activities of MAPK family proteins as well as underlying mechanisms is essential for understanding MAPK functions in regulating proliferation, transformation, and stress response.

The p38 family consists of four isoforms, α, β, γ, and δ (10). The p38 upstream activators include MAPK kinase 6 (MKK6) and MKK3. Downstream effectors consist of kinases such as MAPK-activating protein kinase 2 and PRAK (p38-related/activated protein kinase) as well as transcription factors including activating transcription factor-2 (ATF2), myocyte enhancement factor 2, and c-Jun (10, 11). p38α (also called p38 (10)) is the most abundant and ubiquitously expressed family protein and has a well established role in stress response and inflammation (10–12). Another important function of p38α is to inhibit Ras oncogene activity (7, 13–16). p38γ, on the other hand, is also expressed in many cancer cell lines, and its phosphorylation has also been involved in stress response (17–19). Our recent studies showed that p38γ expression is induced by Ras oncogene, which in turn is required for Ras transforming and/or invasive activity independent of phosphorylation (20, 21). These results together suggest that p38α and p38γ may antagonize each other, but the direct evidence for this antagonism has been lacking. This information is critical for understanding how these two p38 family proteins coordinate for an orchestrated biological response in Ras transformation and stress response.

One major obstacle for demonstrating specific effects of p38α versus p38γ phosphorylation is the lack of constitutively active kinases. All MAPKs are activated by dual phosphorylation on the Thr and Tyr residues of the catalytic domains. Activity of p38γ and p38α is mainly regulated by cellular redox status, protein kinase C, and protein kinase A, respectively. Regulation of both kinases is critical for their biological functions, but how these two kinases respond to various stimuli is still unclear. Here we show a feed-forward mechanism by which p38α may regulate Ras transformation and stress response through depleting its family member p38γ protein via c-Jun-dependent ubiquitin-proteasome pathways. Analyses of MAPK kinase 6 (MKK6)-p38 fusion proteins showed that constitutively active p38α (MKK6-p38α) and p38γ (MKK6-p38γ) stimulates and inhibits c-Jun phosphorylation respectively, leading to a distinct AP-1 regulation. Depending on cell type and/or stimuli, p38α phosphorylation results in either Ras-transformation inhibition or a cell-death cascade that invariably couples with a decrease in p38γ protein expression. p38γ, on the other hand, increases Ras-dependent growth or inhibits stress induced cell-death independent of phosphorylation. In cells expressing both proteins, p38α phosphorylation decreases p38γ protein expression, whereas its inhibition increases cellular p38γ concentrations, indicating an active role of p38α phosphorylation in negatively regulating p38γ protein expression. Mechanistic analyses show that p38α requires c-Jun activation to deplete p38γ proteins by ubiquitin-proteasome pathways. These results suggest that p38α may, upon phosphorylation, act as a gatekeeper of the p38 MAPK family to yield a coordinative biological response through disrupting its antagonistic p38γ family protein.
tion on threonine and tyrosine residues within a conservative Thr-Xaa-Tyr motif (22–24). Although fusing enzyme-substrate approaches have been used for studying JNK (25, 26) and ERK (27) activation, there have been so far no similar studies for p38 MAPKs. In this report constitutively active p38α and p38α fusion constructs (MKK6-p38) were first utilized to demonstrate their opposite and/or distinct localizations and activities in regulating c-Jun phosphorylation, Ras transformation, and/or cell death. Further experiments showed that p38α phosphorylation triggers p38γ protein depletion by c-Jun-dependent ubiquitin-proteasome pathways in inhibiting Ras-dependent growth and/or increasing stress-induced cell death. These results suggest a feed-forward mechanism by which p38α phosphorylation augments with resultant p38γ protein depletion in regulating Ras transformation and stress response.

MATERIALS AND METHODS

Reagents, Cell Culture, and cDNA Constructs—Cell culture materials were supplied by Invitrogen, and all other chemicals were purchased from Sigma. Fetal bovine serum was obtained from BioWhittaker. Protein-Sepharose G and protein A-Sepharose 4B beads were purchased from Zymed Laboratories Inc. p38 isoform-specific antibodies were purchased from RD Systems or Santa Cruz Biotechnology, Inc. ERK1/2, JNK, MKK6, c-Jun, and ATF2 antibodies were from Santa Cruz. Phospho-p38 (p-p38), p-ERK, p-c-Jun (Ser-63), and p-ATF2 antibodies were from Cell Signaling. Mouse monoclonal antibodies against FLAG (M2) and HA (clone 12CA5) were purchased from Sigma and Roche Applied Science, respectively. Anti-mouse-Cy3 and fluorescein isothiocyanate antibodies were from Jackson Laboratories. Rat intestinal epithelial 6 cells (IEC-6) the Ras transformed sub-line (IEC-6/K-Ras), and mouse NIH3T3 fibroblasts were previously described (20, 21). For protein expression and localization, cells were transiently transfected and analyzed 48–72 h after infection (pSUPER) to deplete p38γ protein were performed as previously described (20, 21). For protein expression and localization, cells were transiently transfected and analyzed 48–72 h after transfection. To assess the effects of fusion protein expression in IEC-6/K-Ras cells, MKK6-p38 and MKK6-p38α constructs (and their AGF mutants) were stably transfected through G418 selection (32), and early passages of these cells were used for analyses.

For soft-agar assays, the vector and fusion construct stably transfected cells were plated in growth medium containing 0.33% Seaplaque-agarose. Formation of multicellular colonies was visualized and quantitated about 2 weeks later as previously described (20). To assess cell death, cells were treated with and without 20 μM arsenite (ARS) or infected with adenoviruses expressing MKK6 or β-galactosidase as a control, and cell death induced was estimated by viability assays (trypan blue staining) and/or flow cytometry as previously described (13, 39). All experiments were repeated at least three times and analyzed by Student’s t test and/or analysis of variance for statistically significant differences.

Immunostaining, Immunoprecipitation, and Western Blot Assays—For immunostaining, cells were plated on coverslips and fixed in 3.7% formaldehyde. After permeabilized in a buffer containing 0.5% Triton X-100 and 0.5% Nonidet P-40, cells were blocked in 3% bovine serum albumin in phosphate-buffered saline. Cells were then double-stained for HA-tagged proteins using a mouse monoclonal anti-HA/anti-mouse fluorescein isothiocyanate and for phospho-p38 using a rabbit phospho-p38 anti-rabbit Cy3, as previously described (20, 35). For in vivo ubiquitination and protein degradation assays, 293T cells were transiently transfected for 24 h followed by a 2-h pulse treatment with and without 20 μM ARS 1 day later. After an additional 24 h incubation (+10 μM MG132 or 15 μM lactacystin for the last 6 h), cells were lysed in modified radiomimetic precipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 10 mM NaF, 1 mM Na3VO4, 1 mM phenethylsulfonyl fluoride).
fluoride, and 1 μg/ml aprotinin, leupeptin, and pepstatin). Lysates were then subjected to immunoprecipitation with a HA antibody or FLAG antibody with a portion analyzed directly by Western blot as an input control. All the following procedures for Western blotting were the same as previously described (31, 32).

RESULTS

Constitutively Active p38α Increases, Whereas Constitutively Active p38γ Decreases c-Jun Phosphorylation That Couples with Their Opposite Localizations—To study the specific effects of p38γ versus p38α phosphorylation, p38γ/α cDNA (and the non-phosphorylatable mutant through changing the conservative phosphorylation motif TGY to AGF) were fused in-frame with its activator MKK6 through a decapeptide linker (Gly-Glu)₅ (Fig. 1A) as previously described (25–27). In addition, a HA tag was incorporated at the N-terminal end to facilitate detection of the fusion protein. Western analysis of transiently transfected 293T cells showed expression of the MKK6-p38γ and MKK6-p38γ/AGF fusion proteins at about 80 kDa, but only MKK6-p38γ is recognized by a p-p38 antibody (constitutively active) (Fig. 1B). The integrity and specificity of the fusion proteins were further demonstrated by immunoprecipitation with a HA antibody and Western blotting with MKK6, p38α, or p-p38 antibody (Fig. 1C). Similar results were also obtained with analyses of the p38α fusion proteins (Fig. 2C and data not shown).

p38α is known to be translocated into the cytoplasm upon phosphorylation by stress signaling (40). p38γ, on the other hand, was previously shown to be both in the nucleus and cytoplasm (21, 28), but the relationship between p38γ phosphorylation and its localization has not been studied. To determine whether p-p38γ is localized differently than p-p38α, fusion proteins were transiently expressed, and their localizations were examined by double-immunostaining against HA and p-p38 (or Myc) as indicated. DAPI, 4’,6-diamidino-2-phenylindole.

**FIGURE 1.** Constitutively active p38α is cytosolic, whereas constitutively active p38γ is both in nucleus and cytoplasm. A, a diagram for MKK6-p38γ and MKK6-p38α fusion proteins (and their AGF mutants). B and C, expression and phosphorylation of p38 fusion proteins. 293T cells were transfected with indicated constructs, and the protein expression was examined by direct Western (WB; B) and HA immunoprecipitations (C). Similar results were also obtained for MKK6-p38α fusion proteins (data not shown). D and E, localizations of p38α (MKK6-p38α), p38γ (MKK6-p38γ), and ERK2 (ERK2-MEK1) fusion proteins. IEC-6/K-Ras cells were transiently transfected with different constructs and double immuno-stained against HA and p-p38 (or Myc) as indicated. DAPI, 4’,6-diamidino-2-phenylindole.
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FIGURE 2. Phospho-p38α stimulates c-Jun phosphorylation and AP-1–dependent transcription, whereas the active phospho-p38γ inhibits c-Jun phosphorylation without significant effects on AP-1 activity. A and B, constitutively active p38α but not p38γ increases AP-1–dependent transcription. Human 293T cells were transfected with different constructs together with an AP-1 reporter (A) or a mouse VDR promoter (B) and analyzed for luciferase activity 48 h later. Results shown are mean of three to four experiments (±S.D.) with the asterisk indicating a statistically significant difference as compared with the vector control (p < 0.05, Student’s t test). C, constitutively active p38α stimulates and constitutively active p38γ inhibits c-Jun phosphorylations. Cells were transiently transfected with plasmids as indicated together with either a c-Jun or ATF2 expression construct, and protein expression/phosphorylations were analyzed by direct Western.

contrary, MKK6-p38α is both in the cytoplasm and nucleus, whereas its non-phosphorylatable AGF is predominantly in the cytoplasm (Fig. 1D). These opposite and phosphorylation-dependent localizations of p38α and p38γ fusion proteins are not because of the integrated MKK6, as every fusion protein contains the same molecule that was visible both in nucleus and cytoplasm (Fig. 1D, bottom). The cellular distributions of the active p38γ are similar to those described for the active ERK2 fusion proteins (ERK2- MEK1-LA), as previously described (Fig. 1E; (27). These results indicate that cellular p38γ opposes p38α in cellular localizations by phosphorylation-dependent mechanisms.

Signaling through the p38 pathway is known to stimulate AP-1–dependent transcription (10). To demonstrate if p38 fusion proteins are functionally active in regulating AP-1, 293T cells were transiently transfected with these constructs together with an AP-1 reporter (AP-1-Luc) or an AP-1–dependent VDR genomic promoter (VDR-Luc) (32, 33), and luciferase activity was assayed. Results in Fig. 2A showed that MKK6-p38α significantly increases AP-1 activity, an effect more than that achieved by expressing MKK6 alone, whereas all other fusion proteins have no substantial effects. A similar activation was also observed with the VDR promoter, albeit both AGF mutants are suppressive in this case (Fig. 2B). These results suggest that one specific function of the constitutively active p38α may be stimulating AP-1–dependent transcription, whereas the p38γ, regardless of its phosphorylation status, may not play a significant role in this regulation.

MAPKs regulate AP-1 activity by activating/phosphorylating its components such as c-Jun, c-Fos, and ATF2 (41). To explore whether MKK6-p38α stimulation of AP-1 couples with its activity to phosphorylate c-Jun and/or ATF2, fusion constructs were co-transfected with either a c-Jun- or ATF2-expressing plasmid, and their effects on c-Jun/ATF2 phosphorylation were examined by direct Western. Results in Fig. 2C showed that the constitutively active p38α increases, whereas its AGF mutant decreases c-Jun phosphorylation. These effects are opposite to p38γ fusion proteins, as c-Jun phosphorylation was decreased by MKK6-p38γ but increased by MKK6-p38γ/AGF. Although ATF2 is a well established substrate of all p38 family proteins in vitro and in vivo (10, 31), both p38α and p38γ fusion proteins increase ATF2 phosphorylation independent of phosphorylation. These results indicate that these p38 fusion proteins are functionally active in regulating c-Jun/AP-1 activity and that only MKK6-p38α, but not MKK6-p38γ, induced c-Jun (not ATF2) phosphorylation contributes positively to the AP-1–transcriptional activity.

**p38γ Increases Ras Soft-agar Growth and Inhibits Stress-induced Cell Death Independent of Phosphorylation—**Our previous studies have shown that Ras increases p38γ protein expression, and induced p38γ in turn promotes Ras transformation independent of phosphorylation (20). Specific effects of p38γ phosphorylation on Ras transformation, however, remain unknown. To address this question, we examined whether the constitutively active p38γ has a distinct activity in regulating Ras-dependent growth as compared with its non-phosphorylatable mutant as well as p38α fusion proteins. In this case, these fusion proteins were stably expressed in K-Ras transformed rat intestinal epithelial cells (IEC-6/K-Ras), and their effects on Ras transformation were examined by colony formation assays (20). Expression of both MKK6-p38γ and MKK6-p38γ/AGF proteins increases the soft-agar growth as compared with the vector control with a more substantial effect observed with the mutant protein, whereas neither MKK6-p38α nor its AGF mutant showed a substantial effect (Fig. 3, A–D). These results indicate that both the active and mutant p38γ can increase Ras transformation, and the non-phosphorylatable
fusion protein has a greater potency in this regulation. This effect is consistent with the effector role of p38γ in Ras transformation through Ras-induced expression/dephosphorylation as we previously proposed (20). The inability of the constitutively active MKK6-p38α in regulating Ras soft-agar growth, on the other hand, differs from the previously observed growth inhibition by adenovirus-mediated MKK6 overexpression/p38α phosphorylation (20). These differences probably result from sustained (stable) versus transient p38α phosphorylation. These results further establish a distinct role of p38γ versus p38α in regulating Ras transformation.

p38 MAPK proteins by nature are stress kinases, and we wished next to determine whether stably expressed p38γ and p38α fusion proteins may have distinct roles in regulating stress response. Of interest, stably expressed MKK6-p38γ or MKK6-p38γ/AGF blocks ARS-induced JNK/c-Jun phosphorylation, whereas transfected MKK6-p38α fusion proteins are without effect (Fig. 4, A and B). The inhibitory effects of p38γ fusion proteins on ARS-induced JNK/c-Jun activation are different from their intrinsic activities on c-Jun phosphorylation (Fig. 2C), as the latter depends on p38γ phosphorylation and occurs without the concomitant JNK regulation. Importantly, stably expressed p38γ but not p38α fusion proteins suppress ARS-induced cell death, and both MKK6-p38γ and MKK6-p38γ/AGF showed a similar protective activity (Fig. 4, C and D). The cell death inhibitory activity of the p38γ fusion proteins was further confirmed by an increased ARS-induced toxicity after small interfering RNA-mediated p38γ protein depletion in IEC-6/K-Ras cells (data not shown); the viability decreased from 46.0 ± 3% in the ARS-treated control to 19.8 ± 5.0% in p38γ-depleted ARS group (p < 0.05) with the sub-G1 population correspondingly increased from 27.3 to 46.8%. These results together reveal a stress inhibitory activity of p38γ independent of phosphorylation.

A cell death inhibitory activity of p38γ promotes us to further explore if there is an increased sensitivity to stress-induced cell death in p38γ knock-out (p38γ−/−) MEFs (28). Treatment of p38γ−/− and wild-type (p38γ+/+) MEFs with ARS induces similar cell death, which couples with an increased JNK but not p38α phosphorylation in both lines (Fig. 4, E and F). Because ARS induces p38α phosphorylation in IEC-6/K-Ras but not in MEF cells (Fig. 4E and 5A), these results indicate that p38γ may only be anti-apoptotic when p38α is phosphorylated. Consistent with the JNK inhibition by p38γ fusion proteins in IEC-6/K-Ras cells, however, there was an increased JNK expression/activation in p38γ−/− cells (Fig. 4E). These results together indicate that in addition to the positive role in Ras transformation, p38γ also suppresses the JNK stress pathway, albeit this activity only leads to a cell death inhibitory response in IEC-6/K-Ras cells.

**Stress Preferably Phosphorylates p38α over p38γ, and Phosphorylated p38α Primes p38γ for a Down-regulation**—p38γ has been shown to be activated by several types of stresses (17, 18, 42, 43). Most of these studies, however, were performed by overexpressing p38γ proteins and/or through isolating activated p38γ kinases through immunoprecipitation. As a result, a physiological role of endogenous p38γ in regulating stress response remains un-established. We sought to address this question in IEC-6/K-Ras cells that express both p38α and p38γ proteins (20) (Fig. 5A, top left). In response to either ARS or sorbitol, a single band around 39 kDa was induced that was reacted with a specific p-p38 antibody that recognizes all phosphorylated p38 isoform proteins (Fig. 5A, top left). Because p38α is 38 kDa in size, whereas p38γ is about 45 kDa, these results suggest that it is p38α and not p38γ that is phosphorylated by stress signaling in these cells. This speculation was further confirmed by Western analyses of p-p38 (Fig. 5A, top right) or p38 γ precipitates (Fig. 5A, middle left). Direct Western analyses of 293T cells in which all four p38 family proteins are expressed further showed that only p-p38α is induced by ARS or sorbitol (data not shown), and increased p-p38γ protein is only detectable through examining p38γ precipitates (Fig. 5A, middle right). These results together indicate that p38α is preferably phosphorylated over p38γ in cells expressing both proteins in stress response.

The phosphorylation-independent stress-inhibitory property of p38γ promotes us to further explore mechanisms for its resistance to stress-induced phosphorylation. Because p38α and p38γ oppose each other in regulating c-Jun phosphorylation, Ras transformation, and cell death and p38γ is preferably phosphorylated, p38α phosphorylation may directly suppress p38γ activation. To test this possibility, MKK6 was overexpressed through adenovirus infection to examine if increasing endogenous p38α phosphorylation directly antagonizes p38γ activity. Although MKK6 phosphorylates co-transfected p38α and p38γ (31), there was a decrease in endogenous p38γ protein expression in response to ad-MKK6-induced p38α phosphorylation in IEC-6/K-Ras cells with and without p38γ fusion pro-
protein expression (Fig. 5B). Consistent with the previous observation (20), MKK6 infection results in a more than 50% inhibition of the soft-agar growth as compared with the control infection in all three sublines (data not shown). Because p-p38α suppresses and p38γ promotes Ras transformation, these inhibitions probably represent a combination effect of transient p38α phosphorylation and the resultant p38γ depletion.

A down-regulation of p38γ protein expression by p38α activation suggests a novel cross-talk between these two p38 family proteins. We sought next to examine if inhibiting p38α phosphorylation reverses the p38γ depletion. Indeed, treatment of IEC-6/K-Ras cells with SB203580 (SB) that inhibits p38α but not p38γ activity (10) elevates cellular p38γ proteins dose-dependently (Fig. 5A, bottom left), which also couples with an increased soft-agar growth (data not shown). Although ectopically expressed p38γ, but not p38β, inhibits endogenous p38α phosphorylation, there are no alterations in p38α protein expression (Fig. 5A, bottom right), indicating a specific antagonism between p38γ protein expression and p38α phosphorylation. Moreover, experiments in human breast cancer 231 cells showed that the p38γ protein depletion event occurs specifically to p38α (and less JNK) phosphorylation by MKK6 but not to JNK (less p38α) phosphorylation by taxol-independent of Tet-inducible estrogen receptor protein expression (Fig. 5C) (21). Different than the growth inhibition in IEC-6/K-Ras cells, p38α phosphorylation and p38γ depletion by MKK6 lead to an increased cell death in 231 cells in the absence of estrogen receptor expression (viability, 84 ± 9.0% in control versus 38 ± 15% in MKK6 group, p < 0.05) as previously described (35). These results together suggest that p38α, upon phosphorylation, negatively regulates p38γ protein expression, which may be required for an orchestrated response in regulating Ras transformation and/or cell-death by a cell-type dependent mechanism.

**p38α Phosphorylation Decreases p38γ Protein Expression by Ubiquitin-proteasome Pathways—Protein ubiquitination is an important posttranscriptional regulation that typically marks modified proteins for proteasome-mediated degradation (44, 45). To explore whether p38α phosphorylation decreases p38γ protein expression by ubiquitin-proteasome pathways, 293T cells were transiently transfected with a FLAG-tagged p38α and/or p38γ-expressing plasmid together with a HA-tagged Ub expressing cDNA (36, 37). To stimulate p38α phosphorylation, cells were either co-transfected with MKK6 or pulse-treated with ARS 24 h before their collection. Also, a group of transfected cells were subjected to a short period treatment with a proteasome inhibitor MG132 to determine...
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A

Input control ARS SOR Cont p-p38 p38γ p38α IEC-6/K-Ras ARS SOR Cont p-p38 p38γ p38α

IP: p38γ

293T cells ARS SOR Cont p-p38 p38γ p38α

Stable transfected IEC-6/K-Ras cells

Direct Western

Adenovirus

MKK6-p38α-galactosidase MKK6-p38γ-galactosidase MKK6-p38α MKK6-p38γ p-ERKs p38α p38γ

IEC-6/K-Ras cells

Tet on 231 breast cancer cells

MKK6 Taxol

C

+ - Tet in MIPI

ER

c-Jun c-Jun c-Jun c-Jun MKK6 p38 p-JNK p-JNK p38 p38α p38α

FIGURE 5. p38α is preferably phosphorylated over p38γ by stress signaling, and phosphorylated p38α triggers a down-regulation of p38γ protein expression. A, stress signaling preferably phosphorylates p38α over p38γ, and there is a specific antagonism between p38α phosphorylation and p38γ protein expression. IEC-6/K-Ras cells were treated with ARS or sorbitol (SOR) for 30 min and analyzed for p38α versus p38γ phosphorylation by direct Western (top left) and immunoprecipitations (IP) followed by Western blotting (top right and middle left). In 293T cells stress-induced p38γ phosphorylation was examined by Western analyses of p38γ immunoprecipitates (middle right). To inhibit p38α phosphorylation, IEC-6/K-Ras cells were incubated with and without SB for 24 h and analyzed for p38α phosphorylation/p38γ protein expression by Western (bottom left). To study the effects of p38γ overexpression, NIH3T3 cells were transiently expressed with FLAG-tagged p38γ or p38β proteins, and their effects on endogenous p38α phosphorylation/expression were analyzed by Western (bottom right; the asterisk indicates endogenous p-p38α). B, p38α phosphorylation leads to a depletion of endogenous p38γ proteins in IEC-6/K-Ras cells with and without p38α fusion protein expression. Shown are IEC-6/K-Ras cells stably transfected with MKK6-p38γ, MKK6-p38γ-AGF, or a vector were infected with adenovirus (MKK6 with β-galactosidase (β-Gal) as a control) and analyzed by Western 48 h later. C, p38α but not JNK phosphorylation depletes p38γ protein expression in estrogen receptor (ER)-positive (+Tet) and negative (−Tet) human breast cancer cells. Tet-on estrogen receptor 231 cells (21) were infected with adenovirus (MKK6, β-galactosidase) or treated with taxol (50 μM, 24 h) in the presence or absence of Tet and analyzed for protein phosphorylation and expression.

whether p38α phosphorylation-induced p38γ protein depletion is dependent on proteasome activity.

Direct Western analyses show that levels of expressed p38γ proteins are decreased in response to either MKK6 or ARS, whereas those of transfected p38α proteins remain relatively constant under the same conditions (Fig. 6C, Input Control). Importantly, MG132 treatment blocks the down-regulation, resulting in an increased p38γ protein expression in both MKK6 and ARS group without significant impacts on the p38α protein contents. Similar results were also obtained in a separate experiment in which transfected cells were incubated with another proteasome inhibitor, lactacystin (Fig. 6D). A less substantial reversal effect by lactacystin in this experiment in the ARS group may be due to a slight, instead of significant (as observed in Fig. 6C), decrease in p38γ protein expression. A general consistent down-regulation of p38γ proteins by two types of stress stimuli as well as its reversal by two distinct proteasome inhibitors, on the other hand, strongly indicates that one mechanism by which p-p38α decreases p38γ protein expression may occur by proteasome-dependent pathways. Western analyses of HA precipitates further showed that both transfected p38α and p38γ are constitutively ubiquitinated in the absence of stress, and a unique mono-ubiquitinated band was only detected for the p38γ (about 57 K Da) but not for p38α protein (about 46 kDa) (Fig. 6A). An increase in Ub-p38γ proteins in HA precipitates after MG132 in p38γ plus MKK6 or plus ARS groups but not in p38γ expression alone (Fig. 6A, top, seventh and eighth lanes versus third and fourth lanes from right for the combination and sixth versus second lane for p38γ alone) indicates that only stress-induced, but not constitutively, ubiquitinated p38γ protein is degraded by the proteasome-dependent pathway. Analyses of FLAG precipitates further reveal that a decreased p38γ protein expression by either ARS or MKK6 is reversed by MG132 treatment (Fig. 6B, middle, third and fourth lanes versus the second lane from the right and the seventh and eight lanes versus the sixth lane). Consistent with the results from the HA precipitation, levels of p38γ proteins from FLAG precipitates in p38γ expression alone were not increased by the MG treatment (Fig. 6B, middle, sixth lane versus second lane from the right), further indicating that the constitutively ubiquitinated p38γ may not be sensitive to the proteasome inhibition. This conclusion is further supported by an ineffectiveness of two proteasome inhibitors in increasing total p38γ protein concentrations in the absence of stress as observed from direct Western (Fig. 6C and 6D, sixth versus second lane from the right). These results together indicate that stress-induced but not constitutively ubiquitinated p38γ proteins are degraded by proteasome-dependent pathways.

Both p38α and p38γ Are Ubiquitinated Independent of Phosphorylation but p38α Requires Phosphorylation to Activate c-Jun in Depleting p38γ Protein—One possibility for proteasome-dependent p38γ depletion is that p38γ phosphorylation itself, although undetectable by direct Western under normal conditions, may be able to trigger its own ubiquitination/degradation. To investigate this possibility, fusion proteins were expressed with HA-Ub, and their in vivo ubiquitination was analyzed by Western blotting. Results in Fig. 7A showed that both constitutively active and AGF forms of p38α and p38γ fusion proteins are ubiquitinated to a similar extent, indicating a phosphorylation-independent modification. Consistent with results from the c-Jun co-expression (Fig. 2C), only constitutively active p38α stimulates endogenous c-Jun phosphorylation (Fig. 7A), leading to its increased ubiquitination, as recently reported in response to osmotic stress signaling (46). This c-Jun phosphorylation and consequent ubiquitination effect of
MKK6-p38α again is similar to that observed with MKK6-p38γ/AGF expression (Fig. 7A). These results indicate that p38γ phosphorylation itself does not trigger its own ubiquitination, but p38α may require phosphorylation to activate a c-Jun-associated ubiquitination regulatory cascade.

Stress frequently phosphorylates both p38α and c-Jun (13, 35, 47). c-Jun activity, on the other hand, can integrate various phosphorylation and ubiquitination regulatory events of MAPK signaling (48–51). It is, therefore, important to determine whether c-Jun is required for p-p38α-induced p38γ down-regulation. In this regard, 293T cells were transiently transfected with wild-type or dominant negative c-Jun constructs (35) together with a HA-Ub expressing cDNA, and their effects on ARS-induced p38α phosphorylation/p38γ depletion were analyzed. Results in Fig. 7B, top, showed that expression of the dominant negative c-Jun reversed ARS-triggered p38γ downregulation without affecting p38α phosphorylation, indicating an involvement of c-Jun phosphorylation in p38γ depletion. Similar results were also obtained when experiments were performed without HA-Ub co-transfection (Fig. 7B, middle). The observation that ARS-induced p38α phosphorylation couples to a decreased p38γ protein expression only in c-Jun+/− cells but not in c-Jun−/− cells (Fig. 7B, bottom) further supports the requirement of c-Jun in p-p38α-induced p38γ depletion. Together with the sufficient role of MKK6-p38α in increasing c-Jun phosphorylation, these results indicate that p-p38α requires c-Jun activity to deplete p38γ protein expression.

**DISCUSSION**

The p38 MAPK pathway plays an important role in regulating stress response (13, 52–54) and Ras activity (7, 13, 14, 16, 20). Although stress phosphorylates all p38 family proteins (18, 31), and Ras, on the other hand, stimulates p38α phosphorylation and increases p38γ expression (20), mechanisms by which activated p38 family proteins coordinate for an integrated biological response remain unknown. Here we show that p38α and p38γ have antagonistic activities in Ras transformation and stress response, and p38α phosphorylation primes p38γ protein for depletion by c-Jun-dependent ubiquitin-proteasome pathways (Fig. 7C). These results reveal a novel feed-forward mechanism

**FIGURE 6.** p38α phosphorylation decreases p38γ protein expression by proteasome-dependent mechanisms. Human 293T cells were transiently transfected with p38γ and/or p38α together with a HA-Ub. To stimulate p38 phosphorylations and/or inhibit proteasome activity, cells were either co-transfected with MKK6 or treated with ARS 24 h before lysate collection in which 10 μM MG132 or Me2SO (DMSO) was added for the last 6 h. Protein expression and ubiquitination were analyzed by direct Western (as an input control, C) as well as immunoprecipitations (IP) followed by Western blots (WB) as indicated (A and B). The asterisk in A indicates mono-ubiquitinated p38γ in HA precipitates. In panel D 293T cells were similarly transfected but treated instead with 15 μM of lactacystin (or Me2SO) for the last 6 h before being analyzed for protein expression by direct Western. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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FIGURE 7. p38α requires c-Jun to deplete p38γ proteins through ubiquitin-proteasome pathways. A, p38α and p38γ are both ubiquitinated independent of phosphorylation, but only the active p38α and the inactive p38γ phosphorylate endogenous c-Jun proteins. Human 293T cells were transiently transfected with fusion constructs together with a HA-UB-expressing cDNAs. Protein expression and phosphorylation were examined by a direct Western. B, p38α phosphorylation requires c-Jun activation to down-regulate p38γ protein expression. 293T cells were transiently transfected with a wild-type (WT) or dominant negative (DT) c-Jun constructs in the presence (top panel) and the absence (middle panel) of HA-UB and their effects on ARS-induced p38γ depletion and/or p38γ phosphorylation were analyzed by Western blot. In the bottom panel, wild type (c-Jun+/−) and knock-out (c-Jun−/−) c-Jun cells were treated with ARS and analyzed by Western. ARS-induced c-Jun phosphorylation and protein ubiquitinations were also coupled to p38γ depletion/p38α phosphorylation in IEC-6/K-Ras cells (data not shown). C, an experimental model showing a feed-forward mechanism by which p38α phosphorylation primes p38γ protein for depletion by c-Jun-dependent ubiquitin-proteasome pathways in regulating Ras transformation and stress response. p38α is most frequently phosphorylated in stress response, whereas p38γ expression is typically induced by Ras oncogene, and p38α phosphorylation requires c-Jun activation to deplete p38γ proteins by ubiquitin-proteasome pathways. Because p38γ opposes p38α in regulating Ras transformation and stress response, our results suggest a feed-forward mechanism by which p-p38α cooperates with resultant p38γ protein depletion to inhibit Ras transformation and/or to induce cell death.

by which p38α phosphorylation promotes Ras inhibition and/or pro-apoptotic activity.

This signaling integration mechanism was first suggested by opposite localizations and antagonistic regulations of c-Jun phosphorylation by constitutively active p38γ versus p38α. p38γ to down-regulate p38γ protein expression may explain why p38α is preferably phosphorylated over p38γ in response to stress in cells expressing both proteins (Fig. 5A). Because Ras both stimulates p38α phosphorylation and increases p38γ protein expression (20), its transforming activity in a given system will be determined by an integrated signaling between anti-Ras

fusion proteins. The observation that p38γ both increases Ras transformation and inhibits cell death whereas p-p38α is either Ras inhibitory or pro-apoptotic further supports their opposite functions. A greater enhancing effect of MKK6-p38γ/AGF over its constitutively active counterpart on Ras soft-agar growth (Fig. 3B) but not on cell-death protection (Fig. 4C) may indicate distinct mechanisms involved in p38γ increasing Ras transformation and inhibiting stress response. The coupling of an increased p38α phosphorylation with a decreased p38γ protein expression in every case, however, provides the direct evidence for their integrated activities. An increased Ras transformation by a p38α inhibitor SB that concurrently elevates cellular p38γ proteins further consolidates the co-requiment of inhibiting p38γ proteins further amplifies the p38γ protein expression for increased Ras activity. Additional analyses reveal that this interfamily cross-talk is triggered by p38α phosphorylation leading to a c-Jun-dependent p38γ protein depletion through ubiquitin-proteasome pathways. p38α, upon phosphorylation, may, therefore, act as a gatekeeper of the p38 family through depleting the antagonistic p38γ protein via proteasome degradation pathways to amplify its Ras inhibitory and/or pro-apoptotic signal.

The demonstration of p38α phosphorylation triggering a depletion of p38γ protein expression has important implications. Because p38α is the most abundant family protein expressed in all types of cells/tissues and is easily phosphorylated by various environmental stresses, these results may explain the general phenomenon of a lower level of p38γ protein expression as compared with p38α (10). Moreover, the ability of phosphorylated
p38α phosphorylation and pro-Ras p38γ protein expression (Fig. 7C). In response to stress, on the other hand, increased cell death may only be envisioned when a pro-apoptotic p38α phosphorylation couple with a depletion of anti-apoptotic p38γ protein expression.

One intriguing aspect of p38α and p38γ signaling integration is that this cross-talk occurs between p38α phosphorylation and p38γ protein expression. Although high levels of p38γ protein expression inhibit endogenous p38α phosphorylation (possibly as a result of their competition for a common activator(s)), levels of p38α protein expression remain unaltered. Moreover, this inhibitory effect is not specific for p38α, as p38γ also blocks JNK phosphorylation. A transient regulating of p38α phosphorylation, on the other hand, consistently leads to an opposite alteration in cellular p38γ protein concentrations. Because signals regulating p38α phosphorylation are more abundant and p38α is preferably phosphorylated, these two-way cross-talks will conceivably favor the feed-forward mechanism by which increased p38α phosphorylation augments with the resultant p38γ protein depletion to inhibit Ras transformation and/or increase cell death. It should be pointed out that p38α phosphorylation does not inhibit p38γ transcription, as demonstrated by analyses of a human p38γ promoter,4 but instead triggers proteasome-dependent p38γ degradation. Because the active p38α induces c-Jun phosphorylation that is required for p38γ protein phosphorylation, p38α upon phosphorylation may cooperate with its resultant c-Jun activity to downregulate p38γ protein expression. These results together illustrate an interesting scenario in which a phosphorylation event of one p38 family member initiates an ubiquitinated modification of another p38 family protein to counteract its antagonistic activity for a coordinative response.

Experiments with transient transfections show that both p38γ and p38α are constitutively ubiquitinated in the absence of stress, which, however, is not regulated by the proteasome inhibition. Whether these constitutively ubiquitinated p38α/γ proteins, especially the unique mono-ubiquitinated p38γ, dictate their distinct cellular localizations as recently described for PTEN (55) remains for further explorations. In response to stress, however, both MG132 and lactacystin increased levels of the decreased p38γ proteins. These results indicate that stress-induced but not constitutively ubiquitinated p38γ proteins are degraded, at least in part, by proteasome-dependent pathways. Ubiquitination is one major inducible and reversible protein modification that regulates stability and localizations of many key signaling proteins including c-Jun (36, 48). Of interest, we show that p38α phosphorylation alone is sufficient to activate c-Jun, and this activation is required for its p38γ-depleting activity. JNK has been shown to phosphorylate/activate E3 ligase family proteins in regulating protein ubiquitination (49, 51). p38α may act through similar mechanisms to prime p38γ for ubiquitination and proteasome-dependent degradation. Because c-Jun can form a complex with key ligases in these reactions (50), c-Jun may serve as a platform to facilitate this reaction. It would be of interest to explore further why the p38α/c-Jun cascade, instead of the classical JNK/c-Jun pathway, is involved in regulating p38γ ubiquitination and degradation.

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