p38 Isoforms Have Opposite Effects on AP-1-dependent Transcription through Regulation of c-Jun

THE DETERMINANT ROLE OF THE ISOFORMS IN THE p38 MAPK SIGNAL SPECIFICITY*

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p38 MAPK pathway signaling is known to participate in cell proliferation, apoptosis, and differentiation, in a manner dependent on the cellular context. The factors that determine the specific biological response in a given cell type, however, remain largely unknown. We report opposite effects of the p38 isoforms on regulation of AP-1-dependent activities by p38 activators MAPK kinase 6 (MKK6) and/or arsenite in human breast cancer cells. The p38β isoform increases the activation of AP-1 transcriptional activities by MKK6 and/or arsenite, whereas p38γ/p38δ inhibits or has no effect on the stimulation. The p38γ does so by increasing the levels of phosphorylated c-Jun, whereas the p38γ and -δ isoforms may act by regulating the c-jun transcription. AP-1-dependent processes such as vitamin D receptor gene promoter activation and cellular proliferation were similarly activated by the p38γ or inhibited by the p38γ and/or -δ isoforms. Whereas the human breast cancer cells express all four isoforms, mouse NIH 3T3 and EMT-6 cells express only some of the p38 family members, with p38β higher in 3T3 cells but p38δ only detected in the EMT-6 line. Consistent with the positive and negative roles of p38β and p38δ in AP-1 regulation, MKK6 stimulates AP-1-dependent transcription in NIH 3T3 but not EMT-6 cells. In support of a role of c-Jun regulation by p38 isoforms in determining AP-1 activity, the levels of endogenous c-Jun and its phosphorylated form on p38 activation are higher in NIH 3T3 cells. These results demonstrate the contrasting activities of the different p38 isoforms in transmitting the upstream signal to AP-1 and show that the expression profile of p38 isoforms determines whether the p38 signal pathway activates or inhibits AP-1-dependent processes.

Extracellular signals regulate cellular proliferation, differentiation, and death through activation of kinase cascades of the mitogen-activated protein kinases (MAPKs)1 including ERK, JNK, and p38 (1–4). The ERK MAPKs are most frequently activated by mitogenes, whereas the JNK and p38 MAPKs are strongly responsive to stress and inflammatory signals. The then individual MAPK activities often either collaborate or oppose each other in the regulation of biological responses to different stimuli. ERK activity, for example, has been shown to antagonize the p38 and JNK activities in regulation of apoptosis in PC-12 cells (5). At the level of a target gene expression, ERK kinase stimulates cyclin D1 transcription, which is suppressed by p38 MAPK (6). A similar opposing effect between ERKs and p38 MAPK is also observed in regulation of chondrogenesis of mesenchymes (7). Moreover, an antagonizing effect can occur between the JNK and p38 kinases, despite the fact that both pathways frequently respond to the same classes of stimuli (8, 9). Hypertrophic agonists including endothelin-1 and phenylephrine, for example, stimulate p38 and JNK kinases in myocytes, in which p38 promotes but JNK suppresses the development of myocyte hypertrophy (10). Our previous work of analyzing Ras signal transduction pathways demonstrated that oncogenic Ras stimulates ERK, JNK, and p38 in NIH 3T3 cells, but the p38 activation in this process blocks the Ras signal through its inhibition of Ras-induced JNK downstream effects (11). In addition to their opposing activities, under certain circumstances ERK and p38 kinases cooperate in regulation of c-fos expression in response to UV light (12, 13). It appears, therefore, that signaling cross-talks and integrations among the ERK, JNK, and p38 MAPK pathways are important steps to regulate the final signaling output from MAPK pathway activations. Since each of MAPKs has several subfamily members, it is possible that this signaling cross-talk and integration might also occur inside each type of MAPK among its isoforms to meet cellular requirements for various intricate and delicate biological processing of signals from stimuli.

AP-1 (activating proteins 1) are sequence-specific transcription factors composed of homodimers or heterodimers of the Jun family (c-Jun, JunD, and JunB) or heterodimers of the Jun family member with any of the Fos family members (c-Fos, FosB, Fra1, and Fra2) or other transcription factors such as activating transcription factor-2 (ATF2, CAMP-response element-binding protein, and NFAT (14–16). AP-1 transcription factors are key regulatory molecules that play a central role in control of cell proliferation and transformation (14, 16, 17) by converting MAPK signals into expression of specific target genes (3, 18). Members of the AP-1 family are regulated at both

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‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEF, myocyte-enhancing factor; MKK3 and MKK6, MAPK kinase 3 and 6, respectively; ATP2, activating transcription factor-2; JNK, and p38 (1–4). The ERK MAPKs are most frequently activated by mitogenes, whereas the JNK and p38 MAPKs are strongly responsive to stress and inflammatory signals. The then individual MAPK activities often either collaborate or oppose each other in the regulation of biological responses to different stimuli. ERK activity, for example, has been shown to antagonize the p38 and JNK activities in regulation of apoptosis in PC-12 cells (5). At the level of a target gene expression, ERK kinase stimulates cyclin D1 transcription, which is suppressed by p38 MAPK (6). A similar opposing effect between ERKs and p38 MAPK is also observed in regulation of chondrogenesis of mesenchymes (7). Moreover, an antagonizing effect can occur between the JNK and p38 kinases, despite the fact that both pathways frequently respond to the same classes of stimuli (8, 9). Hypertrophic agonists including endothelin-1 and phenylephrine, for example, stimulate p38 and JNK kinases in myocytes, in which p38 promotes but JNK suppresses the development of myocyte hypertrophy (10). Our previous work of analyzing Ras signal transduction pathways demonstrated that oncogenic Ras stimulates ERK, JNK, and p38 in NIH 3T3 cells, but the p38 activation in this process blocks the Ras signal through its inhibition of Ras-induced JNK downstream effects (11). In addition to their opposing activities, under certain circumstances ERK and p38 kinases cooperate in regulation of c-fos expression in response to UV light (12, 13). It appears, therefore, that signaling cross-talks and integrations among the ERK, JNK, and p38 MAPK pathways are important steps to regulate the final signaling output from MAPK pathway activations. Since each of MAPKs has several subfamily members, it is possible that this signaling cross-talk and integration might also occur inside each type of MAPK among its isoforms to meet cellular requirements for various intricate and delicate biological processing of signals from stimuli.

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ARS, sodium arsenite; ANI, anisomycin; HA, hemagglutinin; BrdUrd, 5-bromo-2′-deoxyuridine; GST, glutathione S-transferase; GFP, green fluorescent protein; MEK kinase.

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p38 activation is known to trigger pleiotropic biological effects, including cell death, differentiation, and proliferation, by mechanisms mostly unknown (1–3). The signal relay along the p38 pathway involves a kinase cascade, which generally consists of three p38 family members: α, β, and γ. In mouse cells, p38α is the predominant isoform. The most important functions of the p38 pathway so far include its role as a central integrator of MAPK signaling to specific target gene expressions (14, 16, 30).

p38 isoforms (p38 called p38, was first cloned as a 38-kDa protein (31). The AFs are p38 mutants that cannot be phosphorylated, since the TGY dual phosphorylation site has been changed to AGF, whereas the kinase-dead KM mutants were generated by a mutation of the ATP-binding site (Lys to Met to K to M). Mutations were generated by PCR-based techniques using the QuickChange site-directed mutagenesis kit from Stratagene as described (40, 41). A p38δ double mutant (p38δ/AF) was created by substituting Thr400 with Ala and Tyr402 with Phe (underlined) using a PCR-based procedure (the primer sequence: GCA-GACCGGAGATGGCTGGTGCTGGTGACC GCCGTG). pGEX for GST-APF1 (1–109) was kindly provided by Dr. Roger Davis (35). pSV- GFP was purchased from Invitrogen. A full length of mouse c-Jun was cloned into a mammalian expression vector pHM6-HA (Roche Molecular Biochemicals). An AP-1 luciferase construct (AP-1-Luc) was kindly provided by Craig Hauser, which was generated by cloning three AP-1 repeats into a luciferase reporter gene containing a minimal Fos promoter (VDR-Luc) and inserted into a PL2 vector base pair (Promega) in front of the luciferase gene as previously described (43, 44). A c-fos luciferase reporter (c-fos Luc, containing −356 to +109 of the murine c-fos promoter) (45) and c-jun luciferase promoter (c-jun Luc, containing base pairs −225 to +150 of the promoter) (26) have been previously described.

Other Reagents—Minimum essential medium, l-glutamine, and antibiotics were supplied by Invitrogen. Fetal bovine serum was obtained from BioWhittaker. DNA was prepared using an Endofree Kit from Qiagen. A DNA transfection kit (calcium phosphate) and a dual luciferase kit were purchased from Promega. Fugene 6 reagent for transfection was purchased from Roche Molecular Biochemicals. Glutathione-Sepharose beads were from Sigma. Proteins were separated by SDS-PAGE and visualized by Coomassie blue staining, or transferred to nitrocellulose membranes containing 10% fetal bovine serum and antibiotics at 37 °C with 5% CO2. Mouse fibroblasts NIH 3T3, the Ras-transformed counterpart, and mammary carcinoma cells EMT-6 have been previously described (11, 46). The wild type and c-Jun knockout MEF were kindly provided by Ron Wisdom (47). For promoter analyses, the protocol of calcium phosphate-mediated transfection from Promega was followed. To increase transfection efficiency, Fugene 6 was used for cell proliferation, kinase assay, and MKK6-p38 binding. The luciferase construct (AP-1-Luc or VDR-Luc) or FLAG-tagged p38 (wild type or the AF or KM mutant p38α, p38β, p38γ, and p38δ) was expressed at a 1:1 ratio with vector or the active MKK6 (MKK6/E). For cell proliferation, MCF-7 cells were transfected together with a pSV-GFP and, after 48 h, were pulse-labeled with 5-bromo-2′-deoxyuridine (BrdU) (Roche Molecular Biochemicals) and fixed in 4% paraformaldehyde. The GFP-positive and BrdU-positive cells were counted under fluorescence microscope (Leica). For luciferase assay, cells were collected 48 h later in the lysis buffer, and the luciferase activity of the promoter was assayed with a dual luciferase kit from Promega by using PRL-TK (encoding Renilla luciferase) as a normalization control in a TD-20/20 Luminometer (Turner Designs). To assess ARS-induced AP-1 activity, cells were treated with 2 μM ARS for 30 min 24 h before the luciferase assay. The results from at least three separate experiments were analyzed with Student’s t test for the statistically significant difference.

Western Blotting Analyses—For Western blot analyses, cells in good growth condition were lysed in modified radioimmune precipitation 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 phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin, leupeptin, and pepstatin). Protein concentration was determined by a DC Protein Assay Kit (Bio-Rad). Typically, 50 μg of protein was separated on a SDS-PAGE, which was transferred to a nitrocellulose membrane for detection of the molecule of interest, using ECL (Amersham Biosciences) as previously described (48). For detection of endogenous or FLAG-tagged p38s, the experimental conditions of previous publications (39, 49) were followed using either p38-specific or anti-FLAG antibodies. The different dilutions of the polyclonal rabbit anti-p38 antibodies have been previously titrated and shown to yield similar binding affinities to their respective proteins (38). The antibody dilutions used in this work were as follows: anti-p38α, 1:5,000; anti-p38β
Opposing Effects of p38β with p38γ and p38δ

Expression of Endogenous p38s in Human Breast Cancer Cells and Their Contribution to MKK6-induced AP-1 Activation—p38 kinase is known to stimulate AP-1 via activation of one or more of its components (12, 25, 26). To assess the contribution of each p38 family member to transduction of AP-1 stimulatory signaling, Western blot analyses were carried out to determine the expression of endogenous p38 family members in the two human breast cancer cell lines, MCF-7 and MDA-MB-468 (468). Cell lysates were prepared and examined for p38 isoform expressions using the isoform-specific antibodies as previously described (38). A similar ECL staining intensity was shown for these antibodies toward the same amount of the purified recombinant p38 isoform proteins under the antibody dilutions utilized here (see “Materials and Methods”). The results of Fig. 1 (upper panel) reveal that all four p38 isoforms are present in MCF-7 and 468 cells, which are migrated differently on SDS-PAGE due to their different molecular weights (3, 38). p38α displayed a major band at 38 kDa in these two human breast cancer cell lines, followed by p38δ (39 kDa), p38β (43 kDa), and p38γ (40 kDa). The higher level of p38α than other isoforms in both cell lines is consistent with the previous literature, indicating that p38α is the major predominant form of the p38 family (3). However, a considerable amount of the p38δ and p38γ proteins is also present in these cells, which isoforms are known to be selectively expressed only in certain tissues or cell types (3). This is perhaps the first report showing that proteins of all p38 family members are expressed in the same cell lines.

Our previous work demonstrated that expression of the p38 stimulator MKK6 in these breast cancer cells increases AP-1 trans-activity (50). Expression of all four p38 family members promoted us to determine which of these family members contribute to transduction of the MKK6 signaling to AP-1. The dominant negative mutant form of each p38 member was co-expressed with or without MKK6, together with AP-1-Luc (a minimal c-fos promoter containing an additional three AP-1 enhancer elements regulating the luciferase gene transcription (42). Since the AF mutants do not have the dual phosphorylation residue TGY and the KM mutant is inactivated in the ATP-binding site, both should function as a kinase-dead form of p38 to block the upstream signals, as previously demonstrated (51). Expression of these kinase-dead forms of p38 alone has different effects on AP-1 reporter activity, with a slight stimulation by p38α/AF and p38β/AF but not by p38γ/AF and p38δ/KM (data not shown). All four mutant forms, however, are able to block MKK6-induced AP-1 activity (Fig. 1, bottom panel). The strongest inhibitions were observed for p38γ/AF (p < 0.01 versus MKK6 alone) and the p38δ/KM (p < 0.01), followed by a moderate suppression of MKK6-induced AP-1 activity by p38β/AF and the p38α/AF (p < 0.05 in both cases). Application of p38δ/AF also achieved a significant inhibition of the AP-1 activation by MKK6, as in the case of p38δ/KM (p < 0.02, data not shown). Of interest, the inhibitory activity of these dominant negatives on MKK6-induced AP-1 activity does not correlate with the expression levels of their endogenous proteins. For example, p38β and p38γ are expressed at a similar level, yet transfection of the same amounts of p38γ/AF and p38β/AF shows that the p38γ/AF is much more efficient in inhibition of MKK6 stimulation of AP-1. These results suggest that each of these family isoforms may have different roles in mediating MKK6 signaling to AP-1.

p38β Potentiates but p38γ and p38δ Inhibit or Have No Effects on MKK6- and ARS-induced AP-1 Activation—MKK6 is a specific upstream kinase that activates all four family members of p38 (1, 3, 25, 39). To further evaluate roles of each p38 family member in transduction of signaling to activate AP-1-dependent transcription, each of the wild-type p38 family members were individually expressed with or without the active MKK6, and their effects were determined on the stimulated AP-1 reporter activity (Fig. 2). Transfection of either wild-type p38α or p38β but not p38γ and p38δ alone increases the basal
treated with 2 mM ARS for 30 min, and the luciferase activity was assayed 24 h later. Results are the mean of three independent experiments (bars, S.E.). *, significantly different compared with vector; **, significantly different compared with ARS alone.

The results further support an opposing role of p38β with p38γ and p38δ in MKK6 and/or ARS stimulation of AP-1 activity in human breast cancer 468 (top panel) and MCF-7 (bottom panel) cells. Each of the wild-type p38 family members was expressed alone or together the active MKK6, in the presence of the AP-1-Luc construct in 468 and MCF-7 cells. The luciferase activity was assayed 48 h after transfection. For regulation of ARS-mediated AP-1 activity, cells were transfected and treated with 2 mM ARS for 30 min, and the luciferase activity was assayed 24 h later. Results are the mean of three independent experiments (bars, S.E.). *, significantly different compared with vector; **, compared with MKK6 alone; ***, compared with ARS alone.

AP-1-Luc activity about 5-fold (p < 0.05). To our surprise, whereas co-expression of wild-type p38α had no effect on the MKK6-induced AP-1 activity, compared with either MKK6 or with p38α alone, although its endogenous content is most predominant. Unexpectedly, transfection of the same amount of wild-type p38β synergistically increased the AP-1 activity by more than 30-fold (p < 0.05 versus MKK6). In contrast, both p38γ and p38δ significantly inhibited the stimulation by MKK6 (p < 0.01, both versus MKK6 alone in 468 cells), indicating an opposing effect of p38β with p38γ and p38δ. The enhancing effect of p38β is kinase-dependent, since this effect is not seen with the phosphorylation-dead form p38βΔF (Fig. 1), but the inhibition by p38γ and p38δ occurs regardless whether the mutants (Fig. 1) or the wild-type forms of the p38s (Fig. 2) are applied.

To confirm this result independently of MKK6 overexpression, ARS, a well known chemical p38 activator (11, 52–54), was utilized. Our previous work demonstrated that ARS stimulates AP-1 trans-activity and AP-1 binding in these breast cancer cells (50). Different p38 isoforms were expressed in 468 cells, which were then treated with 2 mM ARS for 30 min 24 h before the assessing of AP-1 luciferase activity. As for MKK6 activation, ARS treatment alone increased AP-1 activity of about 2-fold, but this stimulation was increased to 10-fold when p38β was also transfected into the cells (p < 0.01 versus ARS alone). Expression of other p38γ and p38δ, however, failed to have a significant impact on the ARS-induced AP-1 activation, implying that these isoforms play a different role in transmitting MKK6 and ARS signaling to AP-1 (Fig. 2, upper panel). The results suggest that p38β is the only p38 family member to transmit both MKK6 and ARS signal to AP-1. To determine whether the opposing effect of p38β with p38γ and p38δ is cell type-specific, another human breast cancer cell line, MCF-7, which has a similar expression profile of p38 family members (Fig. 1), was examined for effects of p38 isoform expression on MKK6 activation of AP-1 activity. As shown in the bottom panel of Fig. 2, expression of p38β again increased MKK6 stimulation of AP-1 from 3.2- to 16.5-fold (p < 0.01 versus MKK6 alone). Expression of p38γ or p38δ, on the other hand, failed to inhibit the AP-1 stimulation by MKK6 (p > 0.05, both versus MKK6 alone). Together, these results demonstrate that at least in these two human breast cell lines in which all of the isoforms are expressed, p38β increases MKK6 and/or ARS signaling to stimulate AP-1-dependent transcription, whereas p38γ and p38δ are either inhibitory or have no effects on the AP-1 activation by MKK6 and/or ARS.

Opposing Effects of p38β with p38γ and p38δ on MKK6-mediated VDR trans-Activation—AP-1 is a key transcription factor that converts MAPK signaling into target gene expression (14, 16). Our previous work has established that MKK6 trans-activates VDR in these human breast cancer cells in a manner dependent on c-Jun/AP-1 activity (50). It is therefore critical to determine whether the opposing effects of p38β with p38γ and p38δ also occur in regulation of this p38/AP-1 target gene transcription. A reporter plasmid containing 0.5 kb of the mouse VDR promoter (43, 44) in front of the luciferase gene was co-transfected with each isoform of the p38 family members in the presence or absence of the active MKK6, and the VDR-luciferase activity was assessed. As illustrated in Fig. 3 (upper panel) in 468 cells, the VDR promoter activity conferred by MKK6 expression was significantly increased from 2.2- to 11-fold by p38β (p < 0.05, versus either MKK6 or p38β alone). Expression of each of the other family members (p38α, p38β, and p38γ with MKK6), on the other hand, reduced the MKK6 stimulation of the VDR gene promoter (p < 0.05, the p38 isoform and MKK6 combination versus MKK6 alone). These results further support an opposing role of p38β with p38γ and p38δ (probably also p38α) in regulation of an AP-1-dependent
VDR gene transcription by MKK6. When the same experiment was carried out in MCF-7 cells (Fig. 3, bottom panel), an inhibitory effect was similarly observed for p38γ, p38δ, and p38α (p < 0.05 versus MKK6 alone) but not for p38β on the MKK6 activation of the VDR promoter. The stimulation of AP-1 and VDR–Luc activities by expression of p38α or p38γ alone observed in the estrogen receptor-negative 468 cells but not in estrogen receptor-positive MCF-7 cells (Figs. 2 and 3) implies either a cell line–specific phenomenon or estrogen receptor-related effect. The enhancing effect of p38β on MKK6 activation of AP-1 (Fig. 2) but not VDR promoter (Fig. 3) activity in MCF-7 cells may suggest that p38β can mediate MKK6 signaling to VDR via AP-1–independent mechanisms in this cell line. Nevertheless, a clearly different effect is observed for p38β–mediated transduction on MKK6 stimulation of VDR transcription than from the rest of other three p38 isoforms in both human breast cancer cell lines.

Expression Profiles of Endogenous p38 Family Members Determine a Specific Response of a p38–Activating Signal—Our results of the antagonizing activities of p38β with p38γ/δ in MKK6 activation of AP-1/VDR suggest that effects of p38 activation on AP-1–dependent transcription are determined by the expression pattern of endogenous p38 isoforms. p38 activation would be stimulatory to the AP-1–dependent transcription if p38β is the major form, whereas it would be inhibitory in cells predominantly expressing p38γ and/or p38δ. To directly test this possibility, a series of cell lines were screened for expression of p38 isoforms by Western blot analyses as described above. As shown in Fig. 4A, the p38β protein level is about 2 times higher in mouse NIH 3T3 cells compared with mouse mammary carcinoma EMT-6 cells, and p38δ, on the other hand, is only detected in EMT-6 but not in the other cell lines screened. Exactly as predicted, MKK6 expression in 3T3 cells significantly activates AP-1 (Fig. 4B) and VDR promoter activity (Fig. 4C, p < 0.05 in both cases), but the same MKK6 transfection in EMT-6 cells has no effects on the basal AP-1 activity (Fig. 4B) and inhibits the VDR promoter activity by more than 50% (p < 0.05, Fig. 4C). These results provide direct evidence that the endogenously expressed p38 isoforms may determine the final outcome of the p38 activation signaling on AP-1–dependent gene transcription.

Increase by p38β but Suppression by p38γ of Cell Proliferation by MKK6—In addition to regulation of target gene expression, AP-1 activity can be either growth-inhibitory or growth-stimulatory by itself and/or as a result of integration of the effects on multiple cellular AP-1–dependent target genes (14). To examine whether the AP-1 activity in our analyses corresponds to a proliferative or growth-inhibitory signal, cell proliferation assays were performed in which p38β or p38γ was co-expressed with MKK6 in MCF-7 cells, together with a marker plasmid pSV-GFP. Following transfection, cells were pulse-labeled with BrdUrd and examined for BrdUrd-positive cells in the transfected population (GFP-positive, about 10–20%) under a fluorescence microscope. Expression of MKK6 or p38β or p38γ alone had no significant effect on BrdUrd labeling over vector control (Fig. 5). Co-expression of MKK6 with p38β, however, increased the labeling, and that with p38δ decreased BrdUrd incorporation (p < 0.05 for MKK6 plus p38β or MKK6 plus p38δ versus MKK6 alone). The slight inhibition of BrdUrd incorporation by MKK6 alone (26%, p > 0.05 versus control) may reflect the net effects of transfected MKK6 on the four p38 endogenous isoforms shown to be expressed in these cells. The lack of significant growth-regulatory effects of p38β or p38γ by itself suggests that these activities require activation by upstream signals such as MKK6. The significant increase of growth promoted by co-transfection of MKK6 plus p38β and the growth inhibition by co-transfection of MKK6 plus p38γ indicates that the positive or negative growth regulatory effects of the isoforms can be observed when these proteins are over-produced on a background in which all four isoforms are detected. Furthermore, in order to see an effect, the p38 pathway must be activated by an upstream activator such as MKK6. These growth-regulatory activities correlate with the changes of AP-1 transcriptional activity observed in these human breast...
Fig. 6. Activation of p38 kinases by MKK6 and ARS. A, expression of FLAG-tagged p38s in MCF-7 and 468 cells. FLAG-tagged p38s were expressed and immunoprecipitated with anti-FLAG antibody. The precipitates were examined for expression of p38 by Western analysis using anti-FLAG antibody. B, activation of p38 by MKK6 or ARS. Wild-type p38s were expressed alone, co-expressed with MKK6, or treated with ARS 24 h after the transfection, as described in the legend to Fig. 2. Transfected p38s were isolated by anti-FLAG immunoprecipitation and assessed for their *in vitro* kinase activity against ATF2. The results at the top represent the basal activity of transfected p38 (control) in comparison with p38 stimulated by MKK6 overexpression (middle) and by ARS (bottom). A similar result was obtained from a separate experiment.

The similar levels of these four exogenous p38s in 468 cells and p38 isoforms were also treated with ARS, the FLAG-tagged p38s were isolated by immunoprecipitation, and their *in vitro* kinase activity was determined as above. Results shown in Fig. 6B (bottom panel) demonstrated that ARS activates all p38s similarly in 468 cells, whereas the effect on p38α and p38β was greater than p38γ and p38δ in MCF-7 cells, similar to the activation by MKK6 (middle panel). Once again, none of these kinase activities explain the opposing effects of p38β with p38γ and p38δ in AP-1-dependent transcription. Since the kinase-dead form of p38β does not increase MKK6-induced AP-1 transcription, whereas the dominant negatives of either p38γ or p38δ may inhibit the AP-1 stimulation by MKK6 (Fig. 1), these results suggest that p38β mediates MKK6 and/or ARS signaling to AP-1 dependent on its kinase activity, whereas p38γ and p38δ inhibit the AP-1-dependent transcription in a manner independent of kinase activity.

Enzyme binding to a substrate is required for MKK6 activation of the p38s (56, 57). To examine whether the opposing effects may be due to different associations of the p38 isoforms with MKK6, an *in vivo* binding experiment was performed by co-expression of HA-MKK6 with wild-type and dominant negative FLAG-p38s in MCF-7 cells. Transfected p38s were isolated by immunoprecipitation with anti-FLAG antibody and examined for the presence of co-precipitated HA-MKK6. As in Fig. 7A, HA-MKK6 was detected in every group whether the wild-type or dominant negative p38 isoforms were transfected. If the FLAG-p38 isomorphism was normalized by co-precipitated HA-MKK6 in each of the transfections (p38/MKK6 ratio constant), there appeared to be more p38α and p38β binding to MKK6 with both wild-type and the dominant negative p38 mutant forms (Fig. 7A). These results may explain why the *in vitro* kinase activity of both p38α and p38β is relatively higher in these cells after co-expression with MKK6 (Fig. 6B), but once more this result does not correlate with their effects on AP-1-dependent transcription.

**Contributions of c-Jun Transcription and c-Jun Phosphorylation to the Opposing Activities**—AP-1 activity most frequently consists of a heterodimer of c-Jun and c-Fos, both of which can...
be activated by p38 kinases (3, 14, 16). In order to dissect whether c-Fos or c-Jun is involved in p38 isoform-specific regulations of AP-1 activity by MKK6, the luciferase activity driven by either the c-fos (45) or c-jun (26) promoter was analyzed. Results in Fig. 7B showed that MKK6 alone selectively trans-activates c-Jun (left) but not c-Fos (right). These results are consistent with our published data that c-Jun but not c-Fos or ATF2 is the major component of the AP-1 activity induced by MKK6 in these breast cancer cells (50). Of interest, co-transfection of p38β or p38δ inhibited the c-jun promoter activity induced by MKK6, whereas p38β appears to increase the stimulation. These results indicate that the opposing effects of p38β with p38γ and p38δ correlate with their different effects on MKK6-induced c-jun transcription.

Alterations in c-jun transcription by p38 isoforms in combination with MKK6 could contribute to the opposing effects on AP-1 activity. The p38 isoform-specific effect on c-jun transcription may be a result of their effects on regulation of other AP-1 components or regulators such as MEF2s (26, 29). Alternatively, it could occur as a consequence of AP-1 regulation via the c-Jun/AP-1 enhancer element in c-jun promoter by its positive autoregulatory loop (23). Besides transcriptional regulation, c-Jun phosphorylation is the second major mechanism by which c-Jun/AP-1 is activated by MAPKs, especially by JNK (14, 16). Whereas p38s were reported by several investigators not to phosphorylate c-Jun in vitro (32, 41, 58), recent studies suggest that immunoprecipitated and transfected human p38 (59) or murine p38δ in mammalian cells (60) phosphorylates c-Jun in an in vitro kinase assay.

We have previously demonstrated that adenovirus-mediated MKK6 gene delivery in these breast cancer cells phosphorylates endogenous c-Jun (50). To determine whether p38 isoforms may differently regulate c-Jun phosphorylation by MKK6, HA-c-Jun was transfected with MKK6 in the absence or presence of wild-type or dominant negative p38s. Following immunoprecipitation with anti-HA antibody, c-Jun phosphorylation status was examined by Western blot using specific anti-phospho-c-Jun (Ser-63) antibody, and the same membrane was stripped off and reprobed with anti c-Jun antibody. As shown in Fig. 7C, MKK6 induced higher levels of both c-Jun and phosphorylated c-Jun, the later consistent with the observation with the adenovirus infection (50). Of great interest, wild-type p38β increased phosphorylated c-Jun, but c-Jun levels were equal (p38β plus MKK6 versus MKK6 alone). Both p38γ and p38δ, on the other hand, inhibited the MKK6 induction of c-Jun as well as phosphorylated c-Jun levels. The decrease of c-Jun phosphorylation by either p38γ or p38δ in this case may represent a consequence of inhibition of the total c-Jun protein. These results indicate that p38β may increase the AP-1 activity predominantly by stimulation of the c-Jun phosphorylation, whereas p38γ or p38δ may inhibit primarily by suppression of the c-Jun trans-activation by MKK6. The moderate inhibitory effects on c-Jun phosphorylation and to a lesser extent c-Jun levels by each of the dominant negative p38 isoforms, on the other hand, may explain their moderate inhibitory activities on MKK6-induced AP-1 activation (Fig. 1). The different c-Jun phosphorylation is not due to the potential presence of active JNK or p38 in the anti-HA complex, as detected with phosphor-JNK or phosphor-p38 antibody (data not shown). Since c-Jun but not c-Fos or ATF2 is the major component of MKK6-induced AP-1 activity in these cells as shown by Western blotting and gel retardation (50), these results strongly suggest that the p38 isoform-specific regulation of c-Jun represents one mechanism for the opposing effects of p38β with p38γ and p38δ on AP-1-dependent transcription.

Correlations of Expression of Endogenous p38 Isoforms with Endogenous c-Jun Expression and Phosphorylations—the c-Jun regulation by MKK6 and p38δ may provide an important mechanism for the opposing effects of p38β with p38γ/δ in AP-1-dependent transcription. Results obtained above, however, are based on experiments with co-transfection and over-expression and consequently need to be further confirmed in physiologically relevant conditions. Mouse NIH 3T3 and EMT-6 cells were applied here again to assess the total and phospho-c-Jun levels by p38 activation by virtue of their distinct expression pattern of endogenous p38 isoforms. NIH 3T3 cells contain higher concentrations of p38β, and p38δ is only detected in EMT-6 cells (Fig. 4). If these two p38 isoforms indeed contribute to regulations of the endogenous c-Jun expression and phosphorylations, the total and phosphorylated c-Jun should be higher in NIH 3T3 than in EMT-6 cells. Total and phospho-c-Jun levels were determined in NIH 3T3 and EMT-6 by Western analysis. Exactly as expected, the level of total c-Jun protein is about 3 times higher in NIH 3T3 cells over that in EMT-6 cells (p < 0.01, Fig. 8, A and B). These results thus consolidate our conclusion obtained above with the
Moreover, evidence is presented indicating that the enhancing effect of p38 on regulation of AP-1 activity was also observed with breast cancer cells (Figs. 2, 3, and 5). A similar effect of the p38 activating signal (MKK6 or ARS) stimulates AP-1-dependent transcription through c-Jun regulation via p38β, but p38γ or p38δ inhibits this process. p38-activating signal would be stimulatory to AP-1-dependent activities in cells expressing high levels of p38β, but it is inhibitory in cells predominantly expressing p38γ and/or p38δ. The net response in cells expressing all p38 family members is determined by integrations of the positive (p38β) and the negative (p38γ and -δ) AP-1 regulatory signaling.

These effects were further demonstrated by both transfection and in endogenous systems, at least in part, to be due to the isoform-specific regulations of c-jun transcription/expression and phosphorylations. The results thus show that specific outcomes of p38 MAPK activation in regulation of AP-1-dependent transcription and proliferation in a given cell type depend on the expression profile of p38 family members in those cells.

Different activities of p38 isoforms have been previously suggested, but the opposing effects within the same family members have not been described (3). p38α is the first isolated family member and has been mostly studied and characterized (3). It is not clear why its expression has the least effect on AP-1 stimulation by the upstream stimulators MKK6 and/or ARS. Published studies suggest that p38β may be mitogenic and/or antiapoptotic, whereas p38γ or p38δ may be involved in stress response. In HeLa cells, for example, adenovirus-mediated p38β delivery was demonstrated to protect SB202190-induced apoptosis (62). Furthermore, p38β but not p38α was shown to protect mesangial cells from tumor necrosis factor-α-induced apoptosis (63). Our results, however, showed that expression of either p38β or p38γ alone has no substantial effects on cell proliferation, and their opposing effects only became obvious when their upstream activator, MKK6, is also co-expressed (Fig. 5). The role of p38γ in stress response was, on the other hand, suggested by the observation in which inhibition of p38γ by p38γ/AF expression suppressed γ-radiation-induced G2 arrest in human osteosarcoma U2OS cells, whereas inhibition of other family members by the dominant negatives had no effect (38). There are also, however, exceptions to this distinction: both p38α and p38γ are involved in hypoxia-induced down-regulation of cyclin D1 in PC12 cells (64). Green tea polyphenol, on the other hand, selectively stimulates p38δ phosphorylation, but this effect is not associated with inhibition, rather activation of AP-1 activity in human keratinocytes as a result of simultaneous stimulation of the Ras/MEKK pathways (65). These results are consistent with the concept that each p38

**FIG. 8.** Higher levels of total and phosphorylated c-Jun in NIH 3T3 cells. A and B, higher levels of endogenous c-Jun protein in NIH 3T3 cells. Total cell lysates were prepared, and the total c-Jun protein expression was determined by Western analysis with a representative result shown in A, and means of three separate experiments were plotted in B (bar, S.E.). C, ANI induces c-Jun phosphorylation in NIH 3T3 but not EMT-6 cells. The phosphorylated c-Jun was detected by using anti-phospho-c-Jun (Ser-63) antibody by Western blot, and the same membrane was reprobed with actin antibody as a loading control.

**FIG. 9.** An experimental model describing a determinant role of expression profiles of p38 family members in p38 signal specificity. p38-activating signal (MKK6 or ARS) stimulates AP-1-dependent transcription through c-Jun regulation via p38β, but p38γ or p38δ inhibits this process. p38-activating signal would be stimulatory to AP-1-dependent activities in cells expressing high levels of p38β, but it is inhibitory in cells predominantly expressing p38γ and/or p38δ. The net response in cells expressing all p38 family members is determined by integrations of the positive (p38β) and the negative (p38γ and -δ) AP-1 regulatory signaling.

The p38 MAPKs are universal signaling cascades that transmit extracellular signals into target gene expressions through their regulation of transcription factor activities. Whereas various mechanisms for signaling specificity such as complex formation and selective recognition of specific docking sites and the activation the T-loop of the p38s have been proposed, it remains unclear why the same p38 activation in different cellular contents triggers different biological response (1–4). Here our results demonstrate that upstream stimulatory signals of the p38 pathway may induce different cellular outcomes, depending on the expression profile of p38 isoforms and in a manner independent of the upstream pathway stimulator. This conclusion is supported by our finding that p38β increases, but p38γ and/or p38δ inhibit, AP-1-dependent transcription and cell proliferation induced by MKK6 in human breast cancer cells (Figs. 2, 3, and 5). A similar effect of the p38 isoforms on regulation of AP-1 activity was also observed with activation of the pathway by ARS. The stimulatory activity of p38β and the inhibition effect of p38δ were further confirmed in NIH 3T3 and EMT-6 cells in which endogenous p38β protein level is higher in 3T3 than that in EMT-6 cells, respectively, whereas p38δ is expressed in EMT-6 but not NIH 3T3 cells. Moreover, evidence is presented indicating that the enhancing effect of p38β requires the kinase activity, whereas the inhibition by p38γ and p38δ is independent of p38 phosphorylation.

**DISCUSSION**

The p38 MAPKs are universal signaling cascades that transmit extracellular signals into target gene expressions through their regulation of transcription factor activities. Whereas various mechanisms for signaling specificity such as complex formation and selective recognition of specific docking sites and the activation the T-loop of the p38s have been proposed, it remains unclear why the same p38 activation in different cellular contents triggers different biological response (1–4). Here our results demonstrate that upstream stimulatory signals of the p38 pathway may induce different cellular outcomes, depending on the expression profile of p38 isoforms and in a manner independent of the upstream pathway stimulator. This conclusion is supported by our finding that p38β increases, but p38γ and/or p38δ inhibit, AP-1-dependent transcription and cell proliferation induced by MKK6 in human breast cancer cells (Figs. 2, 3, and 5). A similar effect of the p38 isoforms on regulation of AP-1 activity was also observed with activation of the pathway by ARS. The stimulatory activity of p38β and the inhibition effect of p38δ were further confirmed in NIH 3T3 and EMT-6 cells in which endogenous p38β protein level is higher in 3T3 than that in EMT-6 cells, respectively, whereas p38δ is expressed in EMT-6 but not NIH 3T3 cells. Moreover, evidence is presented indicating that the enhancing effect of p38β requires the kinase activity, whereas the inhibition by p38γ and p38δ is independent of p38 phosphorylation.

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Opposing Effects of p38β with p38γ and p38δ

family member has a distinct function. However, whether these effects are mitogenic or proapoptotic may depend on the individual cellular content.

p38s are serine/threonine protein kinases, and the biological effects of p38 MAPks have been so far exclusively ascribed to the kinase activities (1–3). Our result that the increase in c-Jun phosphorylation and AP-1-dependent transcription is observed with wild-type but not kinase-dead mutant p38δ is consistent with this notion. Paradoxically, the kinase activities may not be required for the inhibitory effects of p38γ and p38δ on MKK6-induced c-Jun phosphorylation and AP-1 activation, although the wild-type p38γ and p38δ appear to be more effective (Figs. 1, 2, and 7C). However, since all dominant negatives of p38δ inhibit c-Jun phosphorylation (Fig. 7C), which more or less correlates with their inhibition on AP-1-dependent transcription (Fig. 1, bottom), the inhibitory effects by these mutants do not appear to be isoformal-specific. These results suggest that p38δ increases the AP-1 by stimulation of c-Jun phosphorylation, whereas p38γ and p38δ inhibit this by mechanisms involving suppression of the c-Jun transcription. Mechanisms operative in this process are unclear at present but may involve p38 isoform-specific protein-protein interactions in which the wild-type y and δ isoforms act as dominant negatives. Efforts in this research direction will facilitate identification of novel functions of p38 MAPks.

The opposing activity of p38δ to p38γ and p38δ in AP-1 trans-activation of transcription has important implications for understanding p38 signaling specificity. p38 activation would correspond to an increase in AP-1 activity if p38δ is the dominant form. However, if p38γ and/or p38δ are the major isoforms, p38 activation would correspond to an inhibition of AP-1-dependent gene expression. AP-1 activity induced by p38 activation in a cell type in which all four p38 family members are expressed, as in the case of 468 and MCF-7 cells, would correspond to an increase in AP-1 activity if p38γ and/or p38δ is the dominant form. However, in the case of 468 and MCF-7 cells, which express all four members of the p38 family, only p38γ and p38δ appear to be effective. This suggests that p38δ may play a key role in regulation of AP-1-dependent gene expression. AP-1 activity induced by p38γ and/or p38δ is consistent with this notion.

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