Mechanism of p38 MAP kinase activation in vivo

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The p38 mitogen-activated protein kinase (MAPK) is activated in vitro by three different protein kinases: MKK3, MKK4, and MKK6. To examine the relative roles of these protein kinases in the mechanism of p38 MAP kinase activation in vivo, we examined the effect of disruption of the murine Mkk3, Mkk4, and Mkk6 genes on the p38 MAPK signaling pathway. We show that MKK3 and MKK6 are essential for tumor necrosis factor-stimulated p38 MAPK activation. In contrast, ultraviolet radiation-stimulated p38 MAPK activation was mediated by MKK3, MKK4, and MKK6. Loss of p38 MAPK activation in the mutant cells was associated with defects in growth arrest and increased tumorigenesis. These data indicate that p38 MAPK is regulated by the coordinated and selective actions of three different protein kinases in response to cytokines and exposure to environmental stress.

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Studies of the ERK1/2 signaling module demonstrate that MEK1 has an essential function in placentation during embryonic development, but the role of MEK1 to activate ERK1/2 appears to be largely redundant with MEK2 [Giroux et al. 1999]. In contrast, studies of the JNK signaling module have demonstrated nonredundant functions of both MKK4 and MKK7 in JNK activation. JNK is phosphorylated preferentially on Tyr by MKK4, whereas MKK7 preferentially phosphorylates JNK on Thr [Lawler et al. 1998; Tournier et al. 2001; Wada et al. 2001]. Because dual phosphorylation on Thr and Tyr is required for full activation of JNK [Derijard et al. 1994], these data suggest that MKK4 and MKK7 may cooperate to activate JNK. Strong support for this conclusion has been obtained from studies of Mkk4 and Mkk7 gene disruption. Ultraviolet (UV) radiation causes activation of both MKK4 and MKK7 [Tournier et al. 1999], and loss-of-function mutations in either Mkk4 or Mkk7 cause reduced UV-stimulated JNK activation [Nishina et al. 1997; Yang et al. 1997; Ganiatsas et al. 1998; Tournier et al. 2001; Wada et al. 2001; Kishimoto et al. 2003]. Significantly, compound mutations of both Mkk4 and Mkk7 eliminated the ability of UV radiation to activate JNK, indicating that these MAPKK isoforms are essential for JNK activation [Tournier et al. 2001].

The observation that MKK4 and MKK7 have nonredundant functions in the activation of JNK suggests that other MAPKK may also have specialized functions in mammalian MAPK signaling modules. The purpose of the study reported here was to examine the role of MAPKK isoforms in the activation of p38 MAPK. Previous studies have identified the isoforms MKK3 and MKK6 as specific activators of p38 MAPK [Derijard et al. 1995; Raingeaud et al. 1996]. However, in vitro studies suggest that MKK4, an activator of JNK, may also contribute to p38 MAPK activation [Derijard et al. 1995; Doza et al. 1995; Lin et al. 1995]. Furthermore, it has also been reported that some cell surface receptors, including tumor necrosis factor (TNF) receptors, may activate p38 MAPK by a MAPKK-independent mechanism [Ge et al. 2002, 2003]. Our approach was to examine the effect of loss-of-function mutations in the murine Mkk3, Mkk4, and Mkk6 genes. We show that all three MAPKK isoforms can contribute to p38 MAPK activation and that the repertoire of MAPKK isoforms that cause p38 MAPK activation in vivo depends on the specific stimulus that is examined. Loss of p38 MAPK regulation in the mutant cells causes defects in growth arrest and increased tumorigenesis.

**Results**

**Targeted disruption of Mkk3 and Mkk6**

We have previously reported phenotypes of mice with targeted disruptions of the Mkk3 and Mkk6 genes [Lu et al. 1999, Wysk et al. 1999; Tanaka et al. 2002]. The Mkk3−/− and Mkk6−/− mice were viable with no obvious developmental abnormalities. Mice with compound mutations in Mkk3 and Mkk6 were created by breeding these mutant mice. Because Mkk3 and Mkk6 are both located on mouse Chromosome 11, we screened mice for the presence of a chromosome containing disruptions of both Mkk3 and Mkk6. Subsequent breeding resulted in the generation of mice with compound mutations in Mkk3 and Mkk6. Mice lacking expression of both Mkk3 and Mkk6 were not viable. The Mkk3−/− Mkk6−/− embryos died during midgestation at embryonic day 11.0–11.5 (E11.0–E11.5). Major defects in the formation of the placenta and deficiencies in the development of the embryonic vasculature were observed [Fig. 1]. The mutant mice appeared to be developmentally delayed and exhibited symptoms of severe anoxia. This phenotype of Mkk3−/− Mkk6−/− mice resembles that previously described for p38 MAPK−/− embryos [Adams et al. 2000; Allen et al. 2000; Tamura et al. 2000]. The similar embryonic phenotype of these mice is consistent with the known role of Mkk3 and Mkk6 to activate p38 MAPK selectively [Raingeaud et al. 1996]. Together, these data indicate that Mkk3 and Mkk6 serve redundant roles that are essential for survival.

To characterize the effects of Mkk3 and Mkk6 deficiency biochemically, we isolated fibroblasts from wild-type and mutant embryos. Immunoblot analysis demonstrated that Mkk3−/− mice did not express Mkk3, that Mkk6−/− cells did not express Mkk6, and that Mkk3−/− Mkk6−/− cells did not express Mkk3 or Mkk6 [Fig. 2A]. In contrast, these cells expressed similar amounts of JNK, p38 MAPK, Mkk4, and Mkk7 [Fig. 2A]. These data indicated that defects in the expression of Mkk3 and Mkk6 did not cause marked changes in the expression of other components of stress-activated MAP kinase pathways. Phase contrast microscopy demonstrated that the wild-type, Mkk3−/−/−, Mkk6−/−, and Mkk3−/− Mkk6−/− cells displayed the typical flattened appearance of embryonic fibroblasts [Fig. 2B]. The rate of proliferation of these cells in medium supplemented with 10% fetal calf serum was similar, although the Mkk3−/− Mkk6−/− fibroblasts were found to reach a slightly higher saturation density compared with wild-type fibroblasts [Fig. 2C].

Mkk3 and Mkk6 are essential for TNFα-stimulated p38 MAPK activation

To examine the role of Mkk3 and Mkk6 in p38 MAPK activation in vivo, we investigated the effect of TNFα on wild-type and mutant fibroblasts by immunoblot analysis using antibodies that bind phosphorylated and activated MAPK and MAPKK. Control studies using wild-type cells demonstrated that TNFα caused a marked increase in MAPK activation [JNK, ERK, and p38; Fig. 3A] and a more moderate activation of both Mkk3 and Mkk6 [Fig. 3A]. Mkk6 gene disruption did not cause obvious changes in MAPK activation in cells treated with TNFα. In contrast, Mkk3 gene disruption caused reduced activation of p38 MAPK, but did not alter JNK or ERK activation. Interestingly, compound mutant cells lacking both Mkk3 and Mkk6 were severely defective in TNFα-stimulated p38 MAPK activation [Fig. 3A]. This defect in TNFα-stimulated p38 MAPK activation in
MKK3 and MKK6 contribute to p38 MAPK activation caused by UV radiation

MKK3 and MKK6 are essential for TNFα-stimulated p38 MAPK activation [Fig. 3]. To investigate whether MKK3 and MKK6 are required for other stimuli that activate p38 MAPK, we examined the effect of an environmental stress, UV radiation. Control studies using immunoblot analysis with phospho-specific antibodies demonstrated that exposure of wild-type cells to UV radiation caused increased activation of MAPK (MKK3, MKK4, and MKK6; Fig. 4A) and caused increased activation of ERK, JNK, and p38 MAPK [Fig. 4A]. Deficiency of MKK3 or MKK6 did not cause marked defects in p38 MAPK activation. Strikingly, UV radiation also caused p38 MAPK kinase activation in Mkk3/− Mkk6/− cells, although the extent of p38 MAPK activation was diminished compared with wild-type cells [Fig. 4A]. This observation was confirmed using in vitro kinase assays [Fig. 4B]. Together, these data indicate that although MKK3 and MKK6 contribute to p38 MAPK activation in cells exposed to UV radiation, these protein kinases are not essential for UV-stimulated p38 MAPK activation [Fig. 4]. This observation markedly contrasts with the essential role of MKK3 and MKK6 in TNFα-stimulated p38 MAPK activation [Fig. 3].

MKK4 contributes to UV-stimulated p38 MAPK activation

The observation that Mkk3/− Mkk6/− cells can respond to UV radiation with increased p38 MAPK activation demonstrated that a mechanism must exist in UV-stimulated cells to activate p38 MAPK in the absence of MKK3 and MKK6. Previous studies have indicated two possible mechanisms. First, p38 MAPK may be activated by a MAPKK-independent mechanism. For example, it has been reported that the TAB1 adapter protein may cause p38 MAPK activation in the absence of MAPKK involvement [Ge et al. 2002, 2003]. This TAB1-dependent [and MAPKK-independent] mechanism has been implicated in p38 MAPK activation caused by TNFα [Ge et al. 2002, 2003]. However, because we now show that TNFα-stimulated p38 MAPK requires MKK3 and MKK6 [Fig. 3], the conclusion that TNFα causes MAPKK-inde-
To test whether MKK4 may have a redundant role in the activation of p38 MAPK, we examined the effect of MKK4 loss of function in wild-type and Mkk3−/−Mkk6−/− fibroblasts. Attempts to construct triple knock-out mice {Mkk3−/−Mkk4−/−Mkk6−/−} by breeding were not successful, in part because all three genes are linked.

To test the role of MKK4 in the UV-stimulated activation of p38 MAPK, we compared the activation of p38 MAPK in wild-type and Mkk4−/− fibroblasts. As expected [Nishina et al. 1997; Yang et al. 1997; Ganiatsas et al. 1998; Tournier et al. 2001], Mkk4 gene disruption caused decreased activation of JNK following exposure to UV or TNFα [Fig. 5A]. In contrast, the loss of MKK4 expression caused no marked decrease in p38 MAPK activation in response to UV or TNFα [Fig. 5B]. These data confirm the conclusion that MKK4 has a nonredundant role in the activation of JNK and demonstrate that MKK4 has either no role, or a redundant role, in the activation of p38 MAPK.

Figure 3. Targeted disruption of Mkk3 and Mkk6 prevents activation of p38 MAPK by tumor necrosis factor. [A] Wild-type [WT], Mkk3−/−, Mkk6−/−, and Mkk3−/−Mkk6−/− fibroblasts were treated without and with 10 ng/mL TNFα [10 min]. Extracts prepared from these cells were examined by immunoblot analysis using antibodies to JNK, ERK, p38 MAPK, Mkk3, Mkk4, Mkk6, Mkk7, and α-tubulin. [B] The proliferation of wild-type [WT], Mkk3−/−, Mkk6−/−, and Mkk3−/−Mkk6−/− fibroblasts cultured in medium supplemented with 10% fetal calf serum was examined. The relative cell number was measured by staining with crystal violet [OD595 nm]. The normalized data presented are the mean of triplicate determinations and are representative of three independent experiments.
on mouse Chromosome 11. We therefore used an alternative approach to test the role of MKK4 in p38 MAPK activation using siRNA [Fig. 5C]. Decreased expression of MKK4 caused by siRNA caused no change in UV-stimulated activation of p38 MAPK in wild-type cells, but strongly suppressed p38 MAPK activation in Mkk3−/− Mkk6−/− fibroblasts [Fig. 5C]. Similarly, inhibition of MKK4 with dominant-negative JNK caused no change in UV-stimulated p38 MAPK activation in wild-type cells, but inhibited p38 MAPK activation in Mkk3−/− Mkk6−/− fibroblasts (data not shown). Together, these data indicate that MKK4 serves a role that is redundant with MKK3 and MKK6 in the activation of p38 MAPK in cells exposed to UV radiation.

Altered p38 MAPK regulation causes defects in growth arrest and increased tumorigenesis

It is been proposed that the p38 MAPK pathway regulates growth arrest (Bulavin et al. 2002a). Because Mkk3−/− Mkk6−/− fibroblasts exhibit severe defects in p38 MAPK regulation, we investigated whether these cells might have altered proliferative responses. Control studies demonstrated that although p38 MAPK activation was markedly reduced in Mkk3−/− Mkk6−/− fibroblasts [Fig. 2A], the rate of proliferation of wild-type and Mkk3−/− Mkk6−/− fibroblasts was similar [Fig. 2B]. However, differences in proliferation between these cells were observed following serum starvation [Fig. 6B]. Wild-type cells cultured in serum-free medium were not observed to proliferate. In contrast, the Mkk3−/− Mkk6−/− fibroblasts were found to have increased proliferation potential in serum-free medium [Fig. 6B]. To biochemically characterize the difference between the wild-type and Mkk3−/− Mkk6−/− fibroblasts, we examined Rb phosphorylation [a hallmark of G1/S progression] in serum-starved wild-type and mutant fibroblasts. Serum starvation caused Rb dephosphorylation in wild-type fibroblasts, but not in Mkk3−/− Mkk6−/− fibroblasts [Fig. 6D]. Cyclin-dependent protein kinases are thought to represent the major group of Rb kinases in vivo. We therefore examined cyclin expression in the wild-type and Mkk3−/− Mkk6−/− fibroblasts. Ribonuclease protection assays demonstrated that serum starvation caused decreased expression of D-type cyclins in wild-type cells [Fig. 6C]. In contrast, the expression of D-type cyclins was maintained in serum-starved Mkk3−/− Mkk6−/− fibroblasts. Immunoblot analysis confirmed that D-type cyclins were selectively expressed in serum-starved Mkk3−/− Mkk6−/− fibroblasts, but not in the serum-starved wild-type fibroblasts [Fig. 6D]. This deregulated expression of D-type cyclins in Mkk3−/− Mkk6−/− fibroblasts is consistent with the established role of p38 MAP kinase as an inhibitor of cyclin D gene expression (Lavoie et al. 1996).

The p38 MAP kinase pathway has also been implicated in the regulation of c-Jun gene expression (Han et al. 1997; Hazzalin et al. 1997). Defects in the p38 MAPK pathway in Mkk3−/− Mkk6−/− fibroblasts could therefore cause disrupted c-Jun expression and consequently altered cellular proliferation. We therefore examined c-Jun mRNA expression in wild-type and Mkk3−/− Mkk6−/− fibroblasts [Fig. 6E]. Contrary to expectations, c-Jun expression was increased in Mkk3−/− Mkk6−/− fibroblasts. These data indicate that although the MKK3/6 pathway may contribute to the regulation of c-Jun expression, this role of MKK3/6 can be compensated by the function of other signal transduction pathways in fibroblasts. Indeed, previous studies have demonstrated that c-Jun ex-
pression is regulated by multiple functionally redundant signaling pathways (Chiariello et al. 2000).

Together, these data indicate that Mkk3\(^{-/-}\) Mkk6\(^{-/-}\) fibroblasts exhibit a deregulated cell cycle associated with a failure to growth arrest in serum-free medium and altered expression of D-type cyclins and c-Jun (Fig. 6). Consistent with this conclusion, a dramatic increase in tumor burden was observed when SV40-large-T-antigen-immortalized Mkk3\(^{-/-}\) Mkk6\(^{-/-}\) fibroblasts (compared with wild-type cells) were injected subcutaneously in athymic nude mice (Fig. 7).

**Discussion**

The protein kinases Mkk3 and Mkk6 have been reported to specifically activate p38 MAP kinase (Derijard et al. 1995; Raingeaud et al. 1996). Consequently, these MAPKKs have been considered to be critical for p38 MAPK activation in vivo (Schaeffer and Weber 1999; Kyriakis and Avruch 2001). However, biochemical studies indicate that Mkk4, an activator of JNK, can also activate p38 MAPK in vitro (Derijard et al. 1995; Doza et al. 1995; Lin et al. 1995). The physiological significance of this observation has been questioned. Indeed, the role of Mkk4 in p38 MAPK activation in vivo is controversial because studies of Mkk4\(^{-/-}\) mice demonstrate major defects in JNK activation without obvious changes in p38 MAPK activation (Nishina et al. 1997; Yang et al. 1997). Furthermore, the proposed dual function of Mkk4 to activate two separate groups of MAPK [JNK and p38] is unprecedented.

In this study, we have examined the role of Mkk3, Mkk4, and Mkk6 using targeted gene disruption in mice and siRNA approaches. We show that all three MAPKK isoforms contribute to the activation of p38 MAP kinase in cells exposed to UV radiation. These data confirm the importance of Mkk3 and Mkk6 in p38 MAP kinase activation. In addition, we demonstrate that Mkk4 also contributes to p38 MAP kinase activation in cells exposed to UV radiation. This function of Mkk4 was not detected in previous studies because the role of Mkk4 in fibroblasts exposed to UV radiation is redundant with Mkk3 and Mkk6. Nevertheless, Mkk4 is established by these data to be an activator of two different groups of MAPK: p38 and JNK.

**Mkk3 and Mkk6 are essential for TNFα-stimulated p38 MAPK activation**

TNFα causes p38 MAP kinase activation in wild-type fibroblasts, but not in Mkk3\(^{-/-}\) Mkk6\(^{-/-}\) fibroblasts [Fig. 3]. This observation demonstrates that TNFα causes p38 MAPK activation in a MAPK-dependent manner. However, a significant question relates to the specificity of this response involving Mkk3 and Mkk6. Why is there no discernable contribution of Mkk4 to TNFα-stimulated p38 MAPK activation? One contributing factor may be the observation that TNFα activates Mkk3, Mkk6, and Mkk7, but does not activate Mkk4 [Fig. 3; Tournier et al. 1999, 2001]. However, disruption of the Mkk4 gene does cause reduced TNFα-stimulated JNK activity, indicating that basally active Mkk4 is required for maximal TNFα-stimulated JNK activation [Tournier et al. 2001]. The effectiveness of Mkk4 to activate JNK under these conditions may be accounted for by the observation that Mkk7 primarily phosphorylates JNK on Thr 180, whereas Mkk4 primarily phosphorylates JNK on Tyr 182. Interestingly, phosphoThr 180-JNK is the...
preferred substrate for MMK4 compared with nonphosphorylated JNK [Lawler et al. 1998]. The low Km of phosphoThr180-JNK as a substrate for Tyr phosphorylation by MMK4 most likely accounts for the ability of basally active MMK4 to participate in TNFα-stimulated JNK activation.

The mechanism of MMK4 activation of p38 MAPK is markedly different from that for the activation of JNK. MMK4 preferentially phosphorylates JNK on Tyr [Lawler et al. 1998], but phosphorylates p38 MAPK equally on Thr and Tyr [Doza et al. 1995; Tourner et al. 2001]. Similarly, p38 MAPK is phosphorylated on both Thr and Tyr by MMK3 and MMK6 [Enslen et al. 2000]. The absence of preferential Thr or Tyr phosphorylation of p38 MAPK may contribute to the lack of a role for MMK4 in TNFα-stimulated p38 MAPK activation.

Activation of p38 MAP kinase by MAPKK-independent mechanisms

Our studies of fibroblasts have not revealed a role for a MAPKK-independent mechanism of p38 MAPK activation. However, it is possible that such mechanisms of p38 MAPK activation may exist in other cell types. Similarly, MAPKK-independent mechanisms of p38 MAPK activation may be present in fibroblasts exposed to specific stimuli. Recent studies have established that the adapter protein TAB1 represents an example of a mechanism of MAPKK-independent activation of p38 MAPK [Ge et al. 2002]. TAB1 binds and activates TAK1, a MAP3K that can activate both the JNK and p38 MAPK pathways. However, TAB1 also binds p38 MAPK and causes MAPKK-independent activation by causing p38 MAPK autophosphorylation and activation. Evidence that this function of TAB1 is independent of TAK1 has been obtained from the identification of the splice variant TAB1β that does not bind TAK1, but does bind and activate p38 MAPK [Ge et al. 2003]. The MAPKK-independent activation of p38 MAPK caused by TAB1 has been proposed to regulate the basal activity of p38 MAPK and to contribute to the activation of p38 MAPK by cell surface receptors, including TNF receptors [Ge et al. 2002, 2003]. The observation that TNFα does not activate p38 MAPK in Mkk3−/− Mkk6−/− fibroblasts suggests that TAB1 does not contribute to TNFα-stimulated p38 MAPK activation in this cell type. Further studies are required to determine the physiological context of TAB1-mediated p38 MAPK activation. Importantly, the recent description of Tab1−/− mice, which die during early embryogenesis with cardiovascular and lung dysmorphogenesis, will facilitate this analysis [Komatsu et al. 2002].

The Mkk3/6 pathway regulates the cell cycle

The p38 MAPK pathway has been reported to inhibit cell cycle progression by at least three different mechanisms [Bulavin et al. 2002a]. First, p38 MAP kinase inhibits the expression of D-type cyclins [Lavoie et al. 1996]. Second, p38 MAPK can phosphorylate and inhibit Cdc25B and Cdc25C, two protein phosphatases that activate cyclin-dependent protein kinase activity [Bulavin et al. 2001]. Third, p38 MAP kinase phosphorylates the p53 tumor suppressor on two activating sites in the N-terminal re-
gion (Ser 33 and Ser 46) and causes p53-dependent growth arrest (Bulavin et al. 1999; Sanchez-Prieto et al. 2000). Together, these targets of the p38 MAP kinase pathway (cyclin D, Cdc25, and p53) may cooperate to arrest the cell cycle. This finding suggests that defects in p38 MAPK function may contribute to cell cycle defects and increased tumorigenesis. Indeed, the Ppm1D gene (which encodes a phosphatase that inhibits p38 MAPK) is amplified in many human tumors (Bulavin et al. 2002b). Inactivation of p38 MAPK by gene targeting in mice or by overexpression of PPM1D dramatically increases tumorigenesis (Bulavin et al. 2002b). Similarly, MKK4 may be an important activator of p38 MAPK in cells with low levels of MKK3 and MKK6.

The observation that p38 MAPK is activated by three different protein kinases suggests that this pathway represents a site of signal integration during the response of cells to cytokines, growth factors, and environmental stimuli. This is likely to be biologically significant because of the role of p38 MAPK in the regulation of cell cycle progression (Bulavin et al. 2002a). Indeed, defects in the p38 MAPK pathway are associated with tumorigenesis (Bulavin et al. 2002b).

Materials and methods

Mice

Mkk3−/− mice (Lu et al. 1999; Wysk et al. 1999), Mkk4−/− mice (Yan et al. 1997), and Mkk6−/− mice (Tanaka et al. 2002) have been described. Mkk3−/− Mkk6−/− mice were obtained by interbreeding Mkk3−/− and Mkk6−/− mice. Tumor assays were performed using 12-week-old male athymic nude mice (Charles River) by subcutaneous injection of 1 × 10^6 fibroblasts. All animals were housed in a facility accredited by the American Association for Laboratory Animal Care (AAALAC), and the animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts.

Cell culture

Murine embryo fibroblasts were isolated and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen). The fibroblasts were immortalized using the SV40 large T-antigen expression vector p321-T and the Fugene reagent. In vitro fibroblast proliferation assays were performed by staining with crystal violet (Tournier et al. 2001).

siRNA studies

Fibroblasts were transfected with double-stranded RNA (Dharmacon Research) targeting the sequence AATGGGAGTGT GATTGATTGCCCAT using Lipofectamine 2000 (Invitrogen), following the manufacturer’s recommendations (Elbashir et al.

Figure 7. Compound disruption of Mkk3 and Mkk6 causes increased tumorigenesis. Wild-type (WT) and Mkk3−/− Mkk6−/− fibroblasts immortalized with SV40 large T antigen were injected subcutaneously in athymic nude mice. Representative mice with tumors are illustrated. The mice were euthanized, and the tumors were fixed and processed for histological analysis. Sections of the tumors stained with H&E are shown. The tumor volume was measured and is presented graphically (mean ± S.D.; n = 5).

Conclusions

The results of this study indicate that p38 MAPK is regulated by MKK3, MKK4, and MKK6. The MKK3 and MKK6 protein kinases are specific activators of p38 MAPK. In contrast, MKK4 represents a site of integration of stress-activated MAPK pathways because it can activate both JNK and p38 MAPK. The repertoire of protein kinases that contribute to p38 MAPK activation depends on the specific stimulus that is examined. Thus, exposure of cells to TNF causes p38 MAPK activation by a mechanism that requires MKK3 and MKK6. In contrast, MKK3, MKK4, and MKK6 contribute to p38 MAPK activation caused by UV radiation. This role of MKK4 in p38 MAPK activation in fibroblasts is largely redundant with MKK3 and MKK6.

The contribution of MKK3, MKK4, and MKK6 to p38 MAPK activation may depend on the cell type that is examined. For example, differences in the expression of MKK3 and MKK6 account for the observation that T-cell-receptor-mediated p38 MAPK activation is selectively defective in Mkk6−/− thymocytes and Mkk3−/− CD4+ peripheral T-cells, respectively (Lu et al. 1999; Tanaka et al. 2002). Similarly, MKK4 may be an important activator of p38 MAPK in cells with low levels of MKK3 and MKK6.

The observation that p38 MAPK is activated by three different protein kinases suggests that this pathway represents a site of signal integration during the response of cells to cytokines, growth factors, and environmental stimuli. This is likely to be biologically significant because of the role of p38 MAPK in the regulation of cell cycle progression (Bulavin et al. 2002a). Indeed, defects in the p38 MAPK pathway are associated with tumorigenesis (Bulavin et al. 2002b).
2001). The double-stranded siRNA was designed to selectively suppress expression of mouse MKK4. Control experiments were performed using double-stranded RNA targeting the sequence AAACATGCAGAAAATGCTGTT, which suppresses the expression of luciferase (Elbashir et al. 2001). The cells were examined 48 h posttransfection.

Biochemical assays

Cells were lysed in Triton lysis buffer containing 20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/mL of aprotinin and leupeptin. Extracts (50 µg of protein) were examined by protein immunoblot analysis by probing with antibodies to JNK [PharMingen], phospho-JNK (Cell Signaling), p38 MAPK (Santa Cruz), phospho-p38 MAPK (Cell Signaling), ERK1/2 (Santa Cruz), phospho-ERK (Cell Signaling), MKK3 [PharMingen], phospho-MKK3/6 (Cell Signaling), MKK4 (Santa Cruz), MKK6 [Stressgen], MKK7 [PharMingen], Rh [PharMingen], hypophospho-Rb [PharMingen], and α-tubulin [Sigma]. Immunocomplexes were detected by enhanced chemiluminescence [NEL]. MAP kinase activity was measured by in vitro kinase assays [Raingeaud et al. 1995]. Ribonuclease protection assays were performed using reagents obtained from PharMingen.

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