The p38 family of kinases is a subgroup of the mitogen-activated protein kinase family. It is composed of four isoforms and is involved in critical biological processes as well as in inflammatory diseases. The exact unique role of each p38 isoform in these processes is not understood well. To approach this question we have been developing intrinsically active variants of these processes. Recently we described a series of mutants of the human p38α, which were spontaneously active as recombinant proteins purified from Escherichia coli cells. We show here that some of these mutants are spontaneously active in several mammalian cells in culture. The spontaneous activity of some mutants is higher than the activity of the fully activated wild type counterpart. We further produced mutants of the other p38 isoforms and found that p38βD176A, p38γD179A, p38αD176A, and p38δF324S are spontaneously active in vivo. The active mutants are also spontaneously phosphorylated. To test whether the mutants actually fulfill downstream duties of p38 proteins, we tested their effect on activating protein 1 (AP-1)-mediated transcription. Active mutants of p38α induced AP-1-driven reporter genes, as well as the c-jun and c-fos promoters. An active variant of p38γ suppressed AP-1-mediated transcription. When active variants of p38α and p38γ were co-expressed, AP-1 activity was not induced, showing that p38γ is dominant over p38α with respect to AP-1 activation. Thus, intrinsically active variants that are spontaneously active in vivo have been obtained for all p38 isoforms. These variants have disclosed different effects of each isoform on AP-1 activity.

Mitogen-activated protein kinases (MAPKs)² compose a large family of enzymes that are expressed in all eukaryotic cells. Many members of the family are essential for proper development of the organism and for the life and functionality of tissues and cells (1–7). Mammalian MAPKs are commonly subdivided to subfamilies that include the extracellular signal-regulated protein kinases (ERKs), the c-jun N-terminal kinases (JNKs), the p38s, and the big MAPKs, but more subfamilies may exist (1). Each subfamily is composed of several genes, some of which undergo alternative splicing and give rise to several different products (8–10). Consequently, each tissue expresses a distinct repertoire of MAPKs. The exact physiological role of each sub-group, isoform, or splicing variant is not entirely understood. It is clear, however, that each isoform plays a specific physiological function (10–15). The p38 MAPKs are strongly activated under stress conditions and therefore, together with the JNKs, are also known as stress-activated protein kinases (SAPKs). In many cell types p38s induce cell cycle arrest and apoptosis (4, 6, 16–18), but in other systems they are associated with differentiation, inflammation, or cell proliferation (19–24). Studies with knock-out mice and cells showed that p38α is essential for embryonal development (25, 26), but mice lacking either p38β, p38γ, or p38δ are viable (27, 28). Some more studies have described specific roles for different members of the p38 family (11–14), but so far the exact specific role of each given enzyme is not well understood. Abnormal activity of p38s is believed to be part of the cause of various inflammatory diseases, including rheumatoid arthritis and Crohn’s disease, as well as of congestive heart failure (29–31), neuronal degenerative diseases such as Alzheimer disease (32), and cancer (33–36). The linkage between SAPK activity and the etiology of these diseases is not clear. It is still not known for example whether the active MAPK is a direct cause of the disease or an indirect mere consequence. The current understanding of the roles of p38s in health and disease was established mostly through correlative observations or through knock-out and small interfering RNA experiments (25, 26, 37, 38). Only a few studies have tried to study MAPKs via more specific means by activating specifically and controllably a given MAPK pathway (39–44). Because those studies used active variants of upstream components, they were only partially specific. Active variants of MAPKs themselves were hitherto not used because they were not available. In the absence of these tools, it is difficult to understand the role of a given MAPK in a given cell, to reveal its role in disease, or to determine its direct substrates and target genes. In this report we describe the first series of engineered mammalian MAPKs (all isoforms of p38) that are spontaneously active in vivo and may serve as the missing tool for addressing the above questions.

All of the MAPKs are activated via a unique dual phosphorylation mechanism, on a Thr-Xaa-Tyr motif, located in the phosphorylation loop. Dual specificity kinases termed MAP

---

¹ To whom correspondence should be addressed. Tel.: 972-2-658-4718; Fax: 972-2-658-4910; E-mail: engelber@cc.huji.ac.il.

² The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; SAPK, stress-activated protein kinase; MAPKK (and MKK), MAP kinase; MEK, MAPK/ERK kinase; HA, hemagglutinin; GST, glutathione S-transferase; AP-1, activating protein 1; CREB, cAMP-responsive element-binding protein.
kinase kinases (MAPKKs, MEks, or MKKs) catalyze this dual phosphorylation. MAPKKs are not highly specific and may phosphorylate all members of a subfamily or even of two families (1). The basal activity of the unphosphorylated MAP kinase proteins is very low (45–47), making overexpression efforts inefficient. The main obstacle in engineering intrinsically active variants of MAPKs lies in the fact that dual phosphorylation cannot be mimicked. Also, although vast information is available with respect to the structural requirements for MAPK activation (45, 46), it is not known how to impose these structural changes via mutagenesis (48). We therefore took a genetic approach and were able to generate mutants of the human MAPK p38α, which were spontaneously active as recombinant proteins expressed in and purified from Escherichia coli cells (49). These mutants, p38αD176A, p38αF327L, and p38αF327S manifested spontaneous (i.e. in the absence of MAPKK-mediated phosphorylation) activity that reached ~10% of the maximal activity manifested by dually phosphorylated p38α. Combining two activating mutations in the same p38α gene resulted in even more active proteins. Thus, p38αD176A+F327L and p38αD176A+F327S manifested levels of activity that were 25% of that of a fully active (MKK6-treated) p38αWT (49). The active p38α mutants were found to share similar characteristics with the activated p38αWT (e.g. substrate specificity, inhibition by specific p38α inhibitors, and activation by MKK6) (49). Recently we generated similar mutations in p38β, p38γ, and p38δ and found that the mutations rendered these isoforms spontaneously active in vitro (49).

The fact that the active mutants faithfully maintain many properties of the parental wild type enzyme, as tested in vitro, raises the possibility that they might also be active in vivo and perhaps become the missing tool for accurate and specific research of the biology and pathology of p38 isoforms. The goal of this study was to test this possibility, namely to check whether the p38 mutants may be active in cell cultures and disclose the specific activities of each isoform. We show that some of the mutants of all four p38 isoforms are spontaneously active in mammalian cells in culture. In fact, the spontaneous activity (i.e. in the absence of any stimulation) measured in vitro is much stronger than that measured in vivo, reaching 200–700% (depending on the cell type used) of the maximal activity manifested by the respective activated p38αWT isoform. We also show that the active mutants of the different p38 isoforms have different, in some cases opposing, effects on AP-1-mediated transcrption. Thus, the mutants are not only spontaneously active but also readily fulfill downstream activities that reveal cross-talk between p38 isoforms.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**—HEK293, NIH3T3, and DHER14 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Biological Industries, Beit Ha’emek, Israel) and were incubated at 37 °C in 5% CO₂. Wild type mouse embryonic fibroblasts and mouse embryonic fibroblasts lacking MKK3 and MKK6 (MKK3/6−/− cells), a gift of Prof. Roger Davis, were grown in the same medium as above, supplemented with nonessential amino acids, sodium pyruvate, and β-mercaptoethanol (Invitrogen). UV irradiation was employed with a germicidal 254-nm UV lamp at a rate of 2 J/m²/s. Prior to UV irradiation, the entire medium was removed. Fresh medium was added immediately after treatment. The cells were then allowed to grow for 1 h and then harvested. The cells were transfected using either the Exgen500 transfection reagent (Fermentas) according to the manufacturer’s instructions or using the calcium-phosphate method. All of the transfected recombinant p38 cDNAs carried an HA tag and were cloned into pcDNA3 vectors (Invitrogen). All of the plasmids expressing p38 (wild type or mutants) were identical except for the point mutations they possessed. A double mutant, active MKK6 (MKK6-EE), was cloned into the pBabe plasmid and included an HA tag. Unless otherwise stated, 48 h post-transfection the cells were harvested in two ways: (i) For Western blotting, the cells were washed with phosphate-buffered saline, and 60–250 μl of Laemmli’s buffer were added. The cells were scraped using rubber policeman. (ii) For native lysis, all of the steps were performed on ice. The cells were washed twice with cold phosphate-buffered saline, followed by the addition of 0.5 ml of lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 10 μg/ml pepstatin A, 313 μg/ml benzamidine, 1 mM Na₃VO₄, 1 mM p-nitrophenyl phosphate, and 10 mM β-glycerophosphate) and 30 min of incubation under shaking. The cells were scraped, frozen in liquid nitrogen, and thawed on ice. After a 10-min centrifugation at 20,000 × g, supernatant was collected.

**Antibodies and Other Reagents**—The antibodies were obtained as follows: anti-p38 from Santa Cruz Biotechnology; anti-phospho-p38 from Cell Signaling; anti-hemagglutinin (anti-HA) from 12CA5 hybridomas and anti-HA (3F10 high affinity antibodies) from Roche. GST-fused ATF2 was purified as described (49). Unless otherwise stated, all other chemicals were purchased from Sigma.

**Western Blotting**—Thirty micrograms of protein lysates were separated by SDS-PAGE and subsequently transferred to a nitrocellulose membrane. After incubation of the membrane with the appropriate antibodies, specific proteins were visualized using an enhanced chemiluminescence detection reagent.

**Immunoprecipitations and Kinase Assay**—Immunoprecipitated p38—300 μg of lysate were incubated (in lysis buffer, see above) with 20 μl of protein G-Sepharose beads (GE Healthcare) bound to 1 μg of 3F10 anti-HA antibody for 2 h at 4 °C in a rotating wheel. The samples were then washed twice with 1 ml of lysis buffer and twice with 1 ml of kinase buffer (25 mM HEPES, pH 7.5, 20 mM MgCl₂, 1 mM dithiothreitol, 20 mM β-glycerol phosphate, 5 mM p-nitrophenyl phosphate, and 0.1 mM Na₃VO₄). The supernatants were removed and 30 μl of kinase buffer containing 20 μg of GST-ATF2, 20 μM ATP, and 10 μCi of [γ-³²P]ATP were added. Kinase reactions were performed in a 30 °C shaker for 30 min. The reactions were terminated by placing the tubes on ice and the addition of 10 μl of 4X Laemmli’s buffer. The samples were boiled for 3 min, and 30 μl of each sample were separated on 10% SDS-PAGE followed by transfer to nitrocellulose membrane. The membrane was exposed to a phosphorimaging plate (Fuji), and the relative radioactivity was measured. To measure the levels of p38 mol-
ecules that were immunoprecipitated in each reaction, the same membrane was incubated in a blocking solution (Tris-buffered saline, 1% Tween 20, 5% low fat milk), and a Western blot was performed using anti-p38 and/or anti-HA antibodies.

Luciferase Assays—HEK293 cells were plated on 12-well plates (1 x 10^5 cells/well). The cells were transfected with 0.1 µg of either 6X AP-1-luc, c-fos-luc, or c-jun-luc constructs, along with either pcDNA3 empty vector, or pcDNA3 containing the specified types of p38 isoforms (wild type or mutant). In some experiments pBabe-MKK6-EE (0.2 µg) was included. Plasmid (0.1 µg) encoding Renilla luciferase (pRL-TK) was also added to each transfection mixture as a control for transfection efficiency. The total amount of DNA was adjusted to 1 µg 48 h post-transfection, the cells were harvested, and the luciferase activity was measured using the dual luciferase reporter assay system (Promega) according to the manufacturer’s instructions.

Reverse Transcription-PCR—Total RNA was extracted as described (50). DNA was digested by Turbo DNase (Ambion) and RNA (2 µg) was subjected to reverse transcription using the murine leukemia virus reverse transcriptase (Roche Applied Science), with random hexamers as primers. cDNAs were amplified by PCR using the following primers: glyceraldehyde-3-phosphate dehydrogenase, 5’-TGGGTGTGAACCATGAGAAG-3’ and 5’-ACGCCCTGTTCC-3’, with 5’-CTGGGCAGCTGTCTGCGCTGTGTGTAACAGTGTGCTGCAT-3’. DNA was digested by Turbo DNase (Ambion) and total RNA (2 µg) was subjected to reverse transcription using the murine leukemia virus reverse transcriptase (Roche Applied Science), with random hexamers as primers. cDNAs were amplified by PCR using the following primers: glyceraldehyde-3-phosphate dehydrogenase, 5’-TGGGTGTGAACCATGAGAAG-3’ and 5’-ACGCCCTGTTCC-3’, with 5’-CTGGGCAGCTGTCTGCGCTGTGTGTAACAGTGTGCTGCAT-3’.

RESULTS

The Human p38α Mutants Are Spontaneously Active in Vivo—To test whether the p38α mutants, which were shown to be intrinsically active in vitro (49), may also be hyperactive in vivo, we transiently expressed p38αWT and the various p38α mutants in HEK293 cells. 48 h post-transfection, the cells were treated or not with UV radiation. The cells were lysed 1 h later, and the various mutants were immunoprecipitated and tested in a kinase assay using GST-ATF2 as a substrate (Fig. 1A). As expected, the basal activity of p38αWT is very low, almost undetectable. Upon exposure of transfected cells to UV radiation, a strong inducer of the p38α cascade (51), the p38αWT activity increased significantly (Fig. 1A, lane 4). Three of the mutants tested, p38αY106H, p38αA320T, and p38αW337R behaved very similarly to p38αWT (Fig. 1A). In contrast, three other mutants, p38αD176A, p38αA320T, and p38αF327S showed catalytic activity even when immunoprecipitated from nontreated cells. Most importantly, activity manifested by p38αD176A in nonstimulated cells (Fig. 1A, lane 7) was at the levels manifested by p38αWT immunoprecipitated from UV-stimulated cells. Following UV irradiation, the activity of p38αD176A, p38αF327L, and p38αF327S further increased and reached levels higher than those of activated p38αWT (Fig. 1A). The three mutants found to manifest activity in nonstimulated cells are the same mutants that showed spontaneous activity when tested in vitro as recombinant proteins purified from E. coli (49). The p38α mutants that were the most active in vitro carried a combination of two mutations, D176A + F327L and D176A + F327S (49). The spontaneous activity of these mutants in vitro was ~25% of the activity manifested by MKK6-activated dually phosphorylated p38αWT (49). When expressed in HEK293, these double mutants showed spontaneous activity that was ~700% of that of activated p38αWT (Fig. 1A, lanes 17–20). This activity was just slightly enhanced by exposing the cells to UV radiation (Fig. 1A), suggesting that p38αD176A + F327L and p38αD176A + F327S are constitutively active proteins. These p38α mutants provide the first examples of constitutively active molecules of any mammalian MAPK. To test whether the p38α variants tested in HEK293 are active in other cell lines as well, we monitored their activity, following transient transfection, in COS (data not shown), NIH3T3 (Fig. 1B), and DHER14 (Fig. 1C) cell lines. In all cases, results were similar in principle to those obtained in HEK293 cells (Fig. 1, compare A with B and C). However, some differences were observed. For example, p38αD176A showed low
All p38 Isoform Mutants Are Spontaneously Active in Vivo

Mutations of p38β, p38γ, and p38δ Are Also Spontaneously Active in Mammalian Cells—Having identified critical sites in p38α that, when mutated, render the kinase intrinsically active, we sought for equivalent sites in other p38 isoforms. Sequence alignment of all four isoforms shows that several of the sites are conserved in other p38s. Asp176 of p38α was found to be conserved in all p38 isoforms (Fig. 2). The other hand, is conserved only in p38γ (Phe330), but not conserved in p38δ, which contains Val in the equivalent position, nor in p38β that harbors a Leu (Leu328). Based on this alignment we produced mutations p38δD176A, p38δV327S, p38δD176A+V327S, p38δY223S, p38δD179A, p38δE330S, p38δD176A, p38δE324S, p38δL328S, and p38δW338R. We also mutated p38α at Thr324, because this position is equivalent to Phe318 in the yeast Hog1 (Fig. 1). Mutating Thr318 in Hog1 to either Leu or Ser provided some of the most active Hog1 mutants (52). All of the mutants produced were assayed as recombinant proteins expressed in and purified from E. coli, and some were found to be intrinsically active. To test whether they are also active in vitro, all of the mutants and the equivalent wild type isoforms were transiently expressed in HEK293 cells (Fig. 3A) or in DHER14 cells (Fig. 3B), immunoprecipitated, and assayed for their catalytic activity. Several mutants manifested very high spontaneous activity. p38βD179A was spontaneously active at levels higher than those of p38αWT immunoprecipitated from cells exposed to UV light (Fig. 3, A, compare lanes 11 and 12; and B, compare lanes 8 and 9). Similarly, p38δD176A and p38δE324S were spontaneously active to levels higher than that of activated p38αWT (Fig. 3, A, compare lanes 4–6; and B, compare lanes 3–5). The results demonstrate that we have produced intrinsically active variants of all p38 isoforms that are spontaneously active in vivo, in several cell lines.

**The Active Mutants Are Spontaneously Phosphorylated—**

What is the mechanism that makes the mutants spontaneously active? Previously isolated active mutants of Hog1 were shown to be spontaneously phosphorylated (52, 53). This phosphorylation was not observed in pbs2Δ cells, suggesting that active variants are capable of recruiting the MAPKK (52, 53). In addition, the Hog1 mutants gained an autophosphorylation capability (52, 53) that also contributes to the fact that they are spontaneously phosphorylated (52, 53). Our in vitro studies showed that the active p38α variants are also autophosphorylating (52). To test whether the active p38 variants are also spontaneously phosphorylated, we measured their phosphorylation activity in NIH3T3, almost similar to the basal activity of p38αWT (Fig. 1B). This was also the case for p38αF327L (Fig. 1B). However, p38αF327S, p38αD176A+F327S, and p38αD176A+F327S, however, manifested very high spontaneous activity equivalent to or higher than the maximal activity of p38αWT (immunoprecipitated from UV-treated cells). Very high spontaneous activity of the p38αD176A+F327S double mutant was also observed in the DHER14 transformed cell line (Fig. 1C). In this cell line p38αD176A and p38αF327S showed a very high spontaneous activity that was further elevated when cells were exposed to UV radiation. In general, it seems that the single mutants show some variability in the level of their spontaneous activity in the cell types tested, but the double mutants are very active (stronger than activated p38αWT) in all cell types.

---

**FIGURE 2.** Sequence alignment of the four p38 isoforms and the yeast p38 orthologue HOG1. The phosphorylation motif is highlighted in gray. Residues that were mutated are in bold.

| p38 | Variant | α | β | γ | δ |
|-----|---------|----|----|----|----|
| y-HOG1 | VDFDPKKRTIAADALYPSAPYHDPDSPVDAKADFWNFADDPVDPPWVRMSYSEILDF |     |    |    |    |
| h-p38α | VLDSDQRVSSAALANAYFSQYDPEPSPAEP-YDESEGCAREKTELLEKLYTQESL |     |    |    |    |
| h-p38β | VLDSQRTAQAALAYFQAYQHDPEPSPAEP-YDESEGGAREKTELLEKLYTQESL |     |    |    |    |
| h-p38γ | VLDSEQRTAQAALAYFQAYQHDPEPSPAEP-YDESEGGAREKTELLEKLYTQESL |     |    |    |    |
| h-p38δ | ELVDCRKLTAQLTHPFEPPRDPEETEAQQFDSDLKHEKTVWLKOIQYIKEIVNF |     |    |    |    |

**FIGURE 3.** Mutated variants of p38β, p38γ, and p38δ are active in cell cultures. HEK293 (A) or DHER14 (B) cells were transfected with the indicated constructs and immune complex kinase assays were performed as described in Fig. 1. Note the differences in migration of the different p38 isoforms (in the lower panel). WT, wild type; WB, Western blot.

---

3 M. Avitzour, R. Diskin, B. Raboy, N. Askari, D. Engelberg, and O. Livnah, submitted for publication.
All p38 Isoform Mutants Are Spontaneously Active in Vivo

A

FIGURE 4. Spontaneous active p38 molecules are also spontaneously phosphorylated. A, the genes encoding the specified mutants were introduced into HEK293 cells. 48 h post-transfection the cells were treated or not with UV (120 J/m²) and harvested 1 h later. The lysates were subjected to Western blotting, using antibodies that specifically recognize the dually phosphorylated form of p38 (upper panel). The blots were stripped and reincubated with antibodies against p38 (lower panel). The upper bands represent the transfected, HA-tagged p38. The lower bands represent the endogenous wild type p38. B, transfected or not treated with UV (120 J/m²) and harvested 1 h later. The lysates were subjected to Western blot analysis, using antibodies that specifically recognize the dually phosphorylated form of p38 (upper panel). The blots were stripped and reincubated with antibodies against HA (lower panel). The upper bands represent the endogenous wild type p38. The lower bands represent the transfected, HA-tagged MKK6. WT, wild type; WB, Western blot.

B

FIGURE 5. Spontaneous phosphorylation of p38αD176A,F327S is partially dependent on the MKK3 and MKK6 MAPKKs. Mouse embryonic fibroblasts of either wild type background (+/+) or a MKK3/6 double knock-out background (−/−) were transfected with an empty vector, p38αWT, or p38αD176A,F327S. Forty eight hours post-transfection the cells were UV-irradiated (120 J/m²) or left untreated, as indicated. 1 h later, the cells were harvested, and the HA-tagged p38 variants were immunoprecipitated. Immunoprecipitates were subjected to Western blotting, using antibodies that specifically recognize the dually phosphorylated form of p38 (upper panel). The blots were stripped and reincubated with antibodies against HA (lower panel). The upper bands represent the transfected, HA-tagged p38. The lower bands represent the endogenous wild type p38. B, transfected or not treated with UV (120 J/m²) and harvested 1 h later. The lysates were subjected to Western blot analysis, using antibodies that specifically recognize the dually phosphorylated form of p38 (upper panel). The blots were stripped and reincubated with antibodies against HA (lower panel). The upper bands represent the transfected, HA-tagged MKK6. WT, wild type; WB, Western blot.

ation state in transiently transfected 293 cells (Fig. 4). Western blot analysis, using antibodies that specifically recognize the dually phosphorylated form of p38α, showed, as expected, that p38αWT is dually phosphorylated only in cells exposed to UV radiation (Fig. 4A). Similarly, p38αY69H and p38αA320T, which were not spontaneously active (Fig. 1A), were phosphorylated only in UV-treated cells (Fig. 4A). By contrast, mutants that manifested spontaneous activity were also spontaneously phosphorylated. The level of spontaneous phosphorylation was correlated with the level of spontaneous activity, i.e. p38αD176A, p38αA320T, and p38αF327S were weakly phosphorylated, whereas the double mutants were phosphorylated to higher levels (Fig. 4A). We also tested the phosphorylation state of the active variants of p38β, p38γ, and p38δ. All active mutants were spontaneously phosphorylated to some degree, but none of them have reached the maximal phosphorylation (obtained by co-expression of the respective p38WT with the active form of MKK6, MKK6-EE; Fig. 4B). For some of these mutants there is no clear correlation between the levels of activity and the degree of phosphorylation. For example, p38δD324S is far more active than p38δD176A in 293 cells (Fig. 3A), and yet p38δD176A is more strongly phosphorylated (Fig. 4B). Also, some of the mutants manifest activity that is equal or higher than that of maximally active wild type (Fig. 3 and data not shown) although not maximally phosphorylated (Fig. 4B). The basis for the spontaneous phosphorylation of the mutants could be similar to that of the Hog1 mutants, which are also spontaneously phosphorylated in vivo by a combination of recruiting a MAPKK and autophosphorylation. To test whether the phosphorylation of the p38 mutants is dependent on the known upstream activators MKK3 and MKK6, MKK3/6 null mouse embryonic fibroblasts (MKK3/6−/−) were transfected with the p38αD176A,F327S double mutant, and the phosphorylation level of the mutant was monitored (Fig. 5). We found that even in these cells the active mutant is spontaneously phosphorylated to the degree of phosphorylation of the UV-irradiated wild type p38 (Fig. 5, compare lanes 6 and 9). However, the degree of phosphorylation of p38αD176A,F327S was lower in the knock-out cells (Fig. 5, compare lanes 9 and 11), indicating that the mutants’ phosphorylation is dependent, in part, on the upstream MAPKKs. We conclude that the spontaneous phosphorylation is a consequence of both MKK3/6-dependent and MKK3/6-independent (probably autophosphorylated) activity.

Active Variants of p38α Induce, whereas Active Variants of p38γ Inhibit, Transcription of AP-1-driven Reporters—The results above show that some of the p38 mutants are catalytically active in vivo independent of any stimulation. The question remains, however, are these mutants not only catalytically active, but actually mimic natural activation of the p38 cascades and induce p38 downstream activities? We addressed this question first with the p38α mutants because much information has been accumulated about its substrates and target genes. We tested first the ability of the p38αD176A,F327S active variant to induce transcription of a reporter gene driven by an AP-1-responsive cis element. AP-1-responsive cis elements serve as binding sites for transcription activators of the AP-1 family (54, 55) and p38α was shown to stimulate AP-1 activity via one or more of its components (e.g. ATF2 and CREB). Fig. 6A shows that in cells co-transfected with the p38αD176A,F327S and the AP-1-luc constructs, the luciferase activity is significantly higher (50-fold) than in cells transfected with either the empty vector or in cells transfected with vector expressing the p38αWT (10-fold). The luciferase activity in cells expressing the mutants...
All p38 Isoform Mutants Are Spontaneously Active in Vivo

equals that measured in cells co-transfected with both p38α<sup>WT</sup> and MKK6-EE, suggesting that the p38α<sup>D176A+F327S</sup> mutant reaches the maximal activity manifested by phosphorylated wild type. To verify that reporter induction is directly related to the p38α activity, cells transfected with p38α variants and the reporter were treated with SB203580, a specific inhibitor of p38α and p38β. In the presence of SB203580, AP-1 luciferase activity was just marginally induced by the active variants, in a dose-dependent manner (Fig. 6A), strongly suggesting that the induced reporter activity is mediated via p38α. This result also confirms that the active p38α mutants faithfully maintained biochemical and pharmacological properties of native p38α, including sensitivity to SB203580 (49). We further tested whether the p38α<sup>D176A+F327S</sup> mutant is able to spontaneously induce native promoters. Reporter genes driven by the c-fos promoter or c-jun promoter were induced 13- and 2-fold, respectively (Fig. 6B). Finally, we verified that not only reporter genes are affected but also native endogenous genes. For this purpose we used NIH3T3 cells stably transfected with empty vector, p38α<sup>WT</sup>, or p38α<sup>D176A+F327S</sup>. Reverse transcription-PCR performed on RNAs isolated from those clones showed an increase in the mRNA levels of both c-jun and c-fos (Fig. 6C). These results show that the active p38α variants integrate into the endogenous cascade and spontaneously induce downstream activities of the pathway. They also show that activation of p38α is sufficient to provoke AP-1 transcription activity.

We also wished to test whether active variants of p38β, p38γ, and p38δ spontaneously induce downstream activities. These isoforms were not thoroughly studied so far, and their specific unique effects on cell biochemistry and biology are not clear. Particularly, their effect on AP-1, if any, is not clear. A recent study suggested that in human breast cancer cells the main inducer of AP-1 activity is p38β, whereas p38γ and p38δ are inhibitors of AP-1 (14). When we expressed p38γ<sup>WT</sup> with the AP-1 reporter in HEK293, we observed no increase in reporter activity (Fig. 7). Furthermore, when p38γ<sup>WT</sup> was co-expressed with p38α<sup>D176A+F327S</sup>, the p38α active variant was not able to efficiently induce reporter activity (8-fold induction as opposed to 40-fold obtained with no p38γ; Fig. 7). Finally, the active variant of p38γ<sup>Y179A</sup>, suppressed completely the ability of p38α<sup>D176A+F327S</sup> to induce the promoter (Fig. 7). Similarly, when p38γ was expressed together with MKK6-EE and the reporter, it blocked MKK6-mediated induction of the reporter (Fig. 7). These results suggest that p38α activates the AP-1 system, whereas p38γ inhibits it. The results further suggest that the active variant of p38γ is dominant over active p38α. Namely, when p38γ and p38α are co-activated in the cell together, the AP-1 machinery is not induced. These results suggest a cross-talk between the p38 isoforms and a novel mode of p38 control over AP-1 activity. The active variants of either p38β or p38δ showed no activity toward the AP-1-luciferase construct, whether expressed alone or together with MKK6EE (data not shown), suggesting that these MAPKs do not act on the AP-1 system.

**FIGURE 6.** Active variants of p38α spontaneously activate AP-1-mediated reporters and the endogenous c-jun and c-fos genes. A, HEK293 cells were transfected with the specified constructs, the AP-1-luciferase reporter gene and a Renilla luciferase gene (for normalizing; see “Experimental Procedures”). The cells were serum-starved 24 h after transfection, and either SB203580 (10 and 20 μM) or vehicle (dimethyl sulfoxide (DMSO)) was added. 24 h later (i.e. 48 h post-transfection) the cells were harvested, and dual luciferase activity was measured. The results are normalized to the activity of vector-transfected cell lysate, treated with Me<sub>2</sub>SO (leftmost bar), B, cells were treated as in A, but the reporters used were either c-fos-luciferase (c-fos-luc, left panel) or c-jun-luciferase (c-jun-luc, right panel) as indicated (no SB203580 was applied). The results are normalized to the activity of vector-transfected cells. The graphs are the means ± S.E. of three separate experiments, each done in triplicates. C, total RNA was obtained from NIH3T3 cells stably transfected with the specified p38α variant. cDNAs were produced, and PCRs were performed, using primers for c-jun or c-fos and glyceraldehyde-3-phosphate dehydrogenase (see “Experimental Procedures”). Shown are representative results of two experiments. WT, wild type.
All p38 Isoform Mutants Are Spontaneously Active In Vivo

FIGURE 7. Active p38γ suppresses AP-1-mediated reporters. HEK293 cells were transfected with either p38α, p38γ, or a combination of the two, along with the AP-1-luciferase reporter gene, and either an empty vector or MKK6-EE. 48 h post-transfection cells were harvested and dual luciferase activity was measured.

DISCUSSION

In this study we present mutated MAPK molecules that are spontaneously active in mammalian cells. The molecules we present include variants of p38α that were recently shown in vitro to acquire intrinsic activity (49) as well as new mutants of all other p38 isoforms. Their independent spontaneous activity makes the mutants accurate tools for studying the particular specific functions of each p38 isoform. We have already used the active molecules to study the effect of each isoform on AP-1-mediated transcription. We found that active variants of p38α spontaneously induced strong AP-1 activity, whereas active variants of p38γ repressed this activity. Active variants of p38β and p38δ had no effect on AP-1-mediated transcription. Previous studies showed already that p38δ contribute to AP-1 induction, mainly through activation of ATF2 and CREB (22, 56–58), but it was not clear which p38 isoform is directly responsible for this induction. Our results suggest that in HEK293 cells p38α is the only p38 isoform capable of inducing an artificial promoter containing AP-1 elements as its sole activating cis-elements, as well as the natural promoters of c-jun and c-fos. The finding that active p38α could induce the c-jun- and c-fos promoters supports previous studies suggesting that p38 induces the transcription of these two genes (56, 58–60). The use of intrinsically active p38α variant unambiguously shows that p38α activity is in fact sufficient for the induction of the c-fos and c-jun promoters. The target of the other p38 isoforms is not known. It was recently suggested that in human breast cancer cell lines p38γ acts as a down-regulator of AP-1 activity (14). We found that in HEK293 cells as well, p38γ inhibited p38α-mediated induction of AP-1 activity. The intrinsically active variant, p38γD179A, totally blocked this induction. Namely, when both p38α and p38γ are active, p38γ dominates over p38α and prevents AP-1-mediated transcription. This finding suggests the existence of cross-talk between the two p38 isoforms. This cross-talk is another level of the complex regulation of AP-1 activation (54, 61, 62). AP-1 activation must be most accurately regulated because it is critical for processes such as proliferation and apoptosis (55, 61, 63). AP-1 activity is also associated with cancer and other diseases. Interestingly, the case of cross-talk we show here between p38α and p38γ is reminiscent of the cross-talk between members of the AP-1 family. JunB, JDP1, and JDP2 are in fact inhibitors of AP-1-mediated transcription (61, 64–67). JunB is also dominant over c-jun so that a c-jun-JunB heterodimer is transcriptionally inactive (67). Cross-talk between AP-1 proteins occurs via direct dimerization and not only determines levels of activity but also defines the affinity of AP-1 components to a given cis-element (62, 68–70). It could be that MAPK molecules also dimerize (46). Particularly, p38 molecules that acquire autophosphorylation activity may dimerize to allow trans-phosphorylation (73). It could also be that when p38α and p38γ are co-activated, the p38 cascades do not stimulate AP-1 but rather other targets.

Some of the mutants we describe (e.g. p38αD179A/F327S, p38γD179A, and p38γF324S) manifested catalytic activities that were higher than that of activated wild types. Because these mutants are spontaneously active also in vitro as purified recombinant proteins (49), we assume that their spontaneous activity in vivo could be explained by their intrinsic activity. However, when purified from bacteria, the mutants showed activity that was lower than the maximal activity of MKK6-treated p38WT (49). The higher activity observed in vivo can be explained by the fact that in the in vitro assay, the purified protein manifests solely its intrinsic activity, whereas in vivo there is a positive feedback loop that increases the activity. For example, the intrinsic activity may induce a positive feedback loop that culminates in activating MAPKKs that leads to increased phosphorylation of the active p38. The active variants are indeed spontaneously phosphorylated (Fig. 4), in part via the MKK3/6 system (Fig. 5). In addition, it is also possible that their autophosphorylation activity is increased in vivo under the optimal natural milieu. Finally, the mutants seem to be resistant to down-regulation by phosphatases and other factors, further explaining the observation that their activity is higher than that of activated wild types. Although we believe that spontaneous phosphorylation is an important factor in the mechanism underlying the mutant activity, the spontaneous phosphorylation of the p38β, p38γ, and p38δ active mutants was lower than the maximal possible phosphorylation (obtained by MKK6; Fig. 4B), and yet their activity was higher than the maximal activity of the respective wild types (Fig. 3). It seems that phosphorylation is only partially responsible for the activity of these mutants, as we found for the mutants of Hog1 (53, 71).

4 N. Askari and D. Engelberg, unpublished data.
All p38 Isoform Mutants Are Spontaneously Active in Vivo

Our studies with the yeast Hog1 and all isoforms of p38 disclosed several critical locations in these proteins that are preferred targets for mutagenesis for obtaining intrinsically active variants. A highly conserved location is an aspartic acid in the phosphorylation lip (Asp179 in Hog1, Asp178 in p38α, p38β, and p38γ, and Asp179 in p38δ). This Asp is also conserved in the ERK subfamily of MAPKs, offering it as a target in these enzymes as well. It is not conserved in JNKs. Structural studies did not suggest any particular role for this Asp in MAPK activation. We suggest that altering this Asp assists in dimerization and autophosphorylation (73). Another location is a Phe in the L16 domain (Phe118 in Hog1 and Phe272 in p38α). This Phe is not conserved in p38α, p38β, or p38γ, which contain Tyr in the equivalent position (Fig. 2). Interestingly, this Tyr in p38α (Tyr323) is a target for phosphorylation through the bypass pathway (72). Thus, this Tyr may also be a site for mutagenesis. Our recent results suggest that it is indeed a target in p38α. However, mutating this Tyr to Leu did not impose activity on p38β (Fig. 3A). Yet another target is another Phe in L16 (Phe322 in p38α). This Phe is part of the hydrophobic core that serves as a “locker” of p38α activity and should be disrupted for activation (52). On the other hand, neither p38β3275 nor p38γ3280 were active, either in vitro or in vivo (Fig. 3 and data not shown), suggesting mechanistic and structural differences between the isoforms. In any case, it is apparent that modifications of hydrophobic amino acids in L16 along with mutations in Asp176 are key events in unlocking p38α activity. Inserting these modifications to members of the p38 family provided active variants of all isoforms that are spontaneously active in vitro (49) and in vivo (Figs. 1 and 3) that could be applied in various experimental systems.

Acknowledgments—We thank Melanie R. Grably for a critical review of the manuscript. We thank Prof. Roger Davis for the MKK3/6+/− cell line.

REFERENCES

1. Kyriakis, J. M., and Avruch, J. (2001) Trends Biochem. Sci. 26, 257–260
2. Engler, A. G., O’Rourke, K., Fesler, L. A., Zandstra, P. W., Weiss, D. L., et al. (2004) Proc. Natl. Acad. Sci. USA 101, 8410–8415
3. Anderson, J. R., and Noll, S. E. (2004) Curr. Opin. Cell Biol. 16, 73–83
4. Davis, R. J. (2000) Curr. Opin. Cell Biol. 12, 1–13
5. Sabatini, M. D., and Mostoslavsky, R. (2003) Curr. Opin. Cell Biol. 15, 624–629
6. Thorens, B. (2004) Curr. Opin. Cell Biol. 16, 596–600
7. Means, A. R. (2004) Curr. Opin. Cell Biol. 16, 601–606
8. Kurihara, H., Takeuchi, Y., and Yanagawa, Y. (2004) Curr. Opin. Cell Biol. 16, 607–612
9. Yamamoto, M., and Kurihara, H. (2004) Curr. Opin. Cell Biol. 16, 613–618
10. Fruttiger, M., and Yau, K. C. (2004) Curr. Opin. Cell Biol. 16, 619–624
11. Pae, S. R., and Kurihara, H. (2004) Curr. Opin. Cell Biol. 16, 625–631
12. Means, A. R., and Fukami, K. (2004) Curr. Opin. Cell Biol. 16, 632–638
13. Calep, P., and Kurihara, H. (2004) Curr. Opin. Cell Biol. 16, 639–644
14. Takeuchi, Y., and Yamamoto, M. (2004) Curr. Opin. Cell Biol. 16, 645–651
15. Fukami, K., and Kurihara, H. (2004) Curr. Opin. Cell Biol. 16, 652–657
16. Yamamoto, M., and Takeuchi, Y. (2004) Curr. Opin. Cell Biol. 16, 658–664
17. Kurihara, H., and Fukami, K. (2004) Curr. Opin. Cell Biol. 16, 665–670
All p38 Isoform Mutants Are Spontaneously Active in Vivo

6552–6563
52. Bell, M., Capone, R., Pashtan, I., Levitzki, A., and Engelberg, D. (2001) J. Biol. Chem. 276, 25351–25358
53. Yaakov, G., Bell, M., Hohmann, S., and Engelberg, D. (2003) Mol. Cell. Biol. 23, 4826–4840
54. Karin, M. (1995) J. Biol. Chem. 270, 16483–16486
55. Shaulian, E., and Karin, M. (2001) Oncogene 20, 2390–2400
56. Whitmarsh, A. J., and Davis, R. J. (1996) J. Mol. Med. 74, 589–607
57. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) Mol. Cell. Biol. 16, 1247–1255
58. Iordanov, M., Bender, K., Ade, T., Schmid, W., Sachsenmaier, C., Engel, K., Gaestel, M., Rahmsdorf, H. J., and Herrlich, P. (1997) EMBO J. 16, 1009–1022
59. Hazzalin, C. A., Cuenda, A., Cano, E., Cohen, P., and Mahadevan, L. C. (1997) Oncogene 15, 2321–2331
60. Hazzalin, C. A., and Mahadevan, L. C. (2002) Nat. Rev. Mol. Cell. Biol. 3, 30–40
61. Angel, P., and Karin, M. (1991) Biochim. Biophys. Acta 1072, 129–157
62. Karin, M., Liu, Z., and Zandi, E. (1997) Curr. Opin. Cell Biol. 9, 240–246
63. Shaulian, E., and Karin, M. (2002) Nat. Cell Biol. 4, E131–E136
64. Aronheim, A., Zandi, E., Hennemann, H., Elledge, S. J., and Karin, M. (1997) Mol. Cell. Biol. 17, 3094–3102
65. De Cesare, D., Vallone, D., Caracciolo, A., Sassone-Corsi, P., Nerlov, C., and Verde, P. (1995) Oncogene 11, 365–376
66. Schutte, J., Viallet, J., Nau, M., Segal, S., Fedorko, J., and Minna, J. (1989) Cell 59, 987–997
67. Deng, T., and Karin, M. (1993) Genes Dev. 7, 479–490
68. Ryseck, R. P., and Bravo, R. (1991) Oncogene 6, 533–542
69. Hai, T., and Curran, T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3720–3724
70. Smeal, T., Angel, P., Meek, J., and Karin, M. (1989) Genes Dev. 3, 2091–2100
71. Bell, M., and Engelberg, D. (2003) J. Biol. Chem. 278, 14603–14606
72. Salvador, J. M., Mittelstadt, P. R., Guszczynski, T., Copeland, T. D., Yamaguchi, H., Appella, E., Fornace, A. J., Jr., and Ashwell, J. D. (2005) Nat. Immunol. 6, 390–395
73. Diskin, R., et al. (2006) J. Mol. Biol., in press