Mitogen-activated protein (MAP) kinase cascades represent one of the major signal systems used by eukaryotic cells to transduce extracellular signals into cellular responses. Four MAP kinase subgroups have been identified in humans: ERK, J NK (SAPK), ERK5 (BMK), and p38. Here we characterize a new MAP kinase, p38β, that is a 372-amino-acid protein most closely related to p38. It contains a TGY dual phosphorylation site, which is required for its kinase activity. Like p38, p38β is activated by proinflammatory cytokines and environmental stress. A comparison of events associated with the activation of p38 and p38β revealed differences, most notably in the preferred activation of p38β by MAP kinase kinase 6 (MKK6), whereas p38 was activated nearly equally by MKK3, MKK4, and MKK6. Moreover, in vitro and in vivo experiments showed a strong substrate preference by p38β for activating transcription factor 2 (ATF2). Enhancement of ATF2-dependent gene expression by p38β was 20-fold greater than that of p38 and other MAP kinases tested. The data reported here suggest that while closely related, p38β and p38 may be regulated by differing mechanisms and may exert their actions on separate downstream targets.

Activation of the mitogen-activated protein (MAP)1 kinases plays an important role in many cellular processes such as cell proliferation, cell death, and compensation for changes in the extracellular environment (1–5). One member of the MAP kinase family, p38, was first identified from studies involving lipopolysaccharide (LPS, or endotoxin), a complex glycolipid found in the outer membrane of Gram-negative bacteria (6).

Further studies have shown that p38 activation can be observed not only in LPS-treated macrophages, but also in cells treated with cell wall components from Gram-positive bacteria, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), physical stress such as high osmolarity, and chemical stress such as H₂O₂ (7–10). An important role of p38 activation in the LPS-induced inflammatory response was established from its specific inhibition by a class of pyridinyl imidazoles that includes SB 202190 (11). In vivo, administration of this drug to mice blocked LPS-induced TNF-α and IL-1β production. In vitro, SB 202190 inhibited p38 phosphorylation of myelin basic protein (MBP) but had no effect on the activity of extracellular signal-regulated kinase (ERK), c-j un amino-terminal kinase (J NK, or stress-activated protein kinase (SAPK)). Thus, p38 activation may be essential for some cellular responses associated with acute or chronic inflammation.

Several upstream kinases have been implicated in the activation of p38. In co-transfection experiments, p38 was activated by the dual specificity kinases, MAP kinase kinase 3 (MKK3), MKK4, and MKK6 (12–14). Low molecular weight GTP-binding proteins RAC1 and Cdc42 also were shown to regulate p38 activation when co-expressed in several cultured cell lines (15, 16). Potential substrates of p38, identified from in vitro kinase assays, may be MAP kinase-activated protein kinase 2 (MAPKAPK2) and ATF2 (8–10). However, very little is known about the substrate targets of activated p38 under physiological conditions.

Sequence comparison of p38 with other MAP kinases identified a Thr-Gly-Tyr (TGY) dual phosphorylation motif in p38. The other MAP kinases have Thr-Glu-Tyr (TEY) or Thr-Pro-Tyr (TPY) dual phosphorylation motifs (7, 17–23). All three phosphorylation motifs are part of the linker loop 12 region (24) defined by analysis of the three-dimensional crystal structure of ERK2. The length of this loop varies among the different MAP kinases (22). The phosphorylation motif and linker loop 12 length are the structural features used to divide the MAP kinase family members into four subgroups: 1) ERK, 2) J NK (SAPK), 3) ERK5 (BMK), and 4) p38. Isoforms of the ERK and J NK MAP kinase subgroups have been identified (1–4, 21, 32, 33).

Here we describe the molecular cloning of a new MAP kinase, p38β. The protein p38β appears to be an isoform of p38β in that it is 74% identical to p38, has the TGY dual phosphorylation motif, and has the same linker loop 12 length. Despite their structural similarities, p38β displays considerably more activity toward ATF2 than p38 does in vitro. Consistent with this observation, we found that overexpression of p38β but not p38 potentiates the expression of an ATF2-dependent reporter gene. p38β and p38 also respond differently to upstream kinases and some extracellular stimuli. The present findings suggest that these two kinases have distinct functions in cellular responses.

Complete reference list is available upon request.

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help us to understand how p38β and p38 may regulate cellular responses to a variety of extracellular signals.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning of p38β—The oligonucleotide ATCCAGTGGCATGATCTCAGGTGC was 3'-end 32P-labeled with a 3'-end-labeling system (Promega, Madison, WI) and used to screen a λZAPIII human placenta library purchased from Stratagene (La Jolla, CA). 1 × 106 phage were screened as described (14). Clones isolated from the screen were sequenced using a kit from U.S. Biochemical Corp. The original clone (p38β) (27) from the Washington University-Merck Expression Sequences Tag (EST) project was sequenced using a model 373A automated sequencer (Applied Biosystems, Foster City, CA). Northern Blot Analysis—A tissue blot containing 2 μg of poly(A) + RNA isolated from different human tissues, fractionated by denaturing agarose gel, and transferred onto a nylon membrane was purchased from Clontech (San Francisco, CA). The blot was hybridized to a probe prepared by labeling the coding region of p38β cDNA with [γ-32P]ATP by random priming (25). After autoradiography, the blot was reprobed with [γ-32P]ATP-labeled p38 cDNA. Hybridization was performed overnight at 50°C using 50% formamide, 5 × saline/sodium/pHosphate/EDTA; 5 × Denhardt's, 1% SDS, and 200 μg/ml single-stranded fish sperm. The blot was washed 2 times with 1 × SSC, 0.1% SDS, and 1 mM EDTA and 2 times with 0.1 × SSC, 0.1% SDS, 1 mM EDTA at 65°C prior to autoradiography.

cDNA Constructs and Expression Plasmids—A p38β double mutant (p38β(AF)) was created by substituting Thr193 with Ala and Tyr190 with Phe using a PCR-based procedure (26). The flag epitope tag DYGKD-DDDDK was added to the amino-terminal region by PCR recombination (27). For p38β expression in bacteria, the cDNA coding region was subcloned into the PET14b vector (Novagen, Madison, WI). MKK3 and MKK4 (JNK1, JNK2) with a hemagglutinin (HA) tag in pSRa vector were constructed as described (12, 27). HA-tagged MKK6 cDNA is in the pDNA3 vector (14). A PCR-based procedure was used to create a double mutant of MKK3(JNK3E1) by replacing the phospho-tyrosine sites Ser106 and Thr173 with Glu, a double mutant of MKK6 (MKK6(E)) by replacing Ser151 and Thr155 with Glu, and a double mutant of MKK6 (MKK6(E)) by replacing Ser207 and Thr214 with Glu. The cDNAs were subcloned into vector pCDNA3.

Preparation of Recombinant Proteins—The PET14b vectors containing p38β or p38 were transformed into the BL21(DE3) strain of Escherichia coli. The E. coli was grown at 37°C in Luria broth until the A600 = 1.0 at which time isopropyl-β-D-thiogalactopyranoside (1 mM final) was added for 2 h. The cells were collected by centrifugation at 8,000 × g for 10 min. For 100 ml of original bacterial culture, the bacterial pellet was resuspended in 5 ml of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The bacteria were lysed by sonication onsonication by sonication. The supernatant was applied to a Ni2+-NTA-agarose (Novagen) column (0.5 ml). The column was washed with 10 ml of 50 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9. The expressed p38βi and p38 proteins were soluble and were recovered with a yield of 10 μg of bacterial protein. After washing the NTA-agarose columns with the binding buffer, the proteins were eluted with 150 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9.

**RESULTS**

Molecular Cloning of p38β—To identify proteins related to p38, the EST division of GenBank™ data base was searched with the blastn program. An EST-sequence of 223 base pairs from the EST division of GenBank™ data base was searched with the blastn program. An EST-sequence of 223 base pairs

The glutathione S-transferase (GST) fusion proteins of the amino-terminal portion of ATF2 (base pairs 1–109) (14), c-jun (1–93) (21), and full-length MAPKAPK2 (28) were prepared as described (29) by affinity chromatography over GSH-agarose (Sigma). MBP was purchased from Sigma.

Transient Co-expression of Various cDNAs—cOS-7, HeLa, and CHO-K1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. Cells on 35-mm plates were transfected with 1 μg of plasmid DNA using lipofectamine. After 48 h, the cells were treated with stimuli as described in the text. Transfection efficiency was evaluated by co-transfection with plasmid pCMV(β-Gal) (Clontech). Cell lysates were normalized for transfection efficiency by quantifying β-galactosidase activity (30).

In vivo kinase activity of transiently expressed p38β, p38β(AF), or p38 in HeLa cells was measured by an immunokinase assay with M2 as described (21).

We evaluated the in vivo inhibition of p38β by the pyridinyl imidazole FHT-381, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole (FHIPI), which is identical to the published compound SB202190 of SmithKline Beecham (King of Prussia, PA) (11). Inhibition was evaluated by adding different concentrations of this compound to the in vivo kinase reaction.

Reporter Gene Expression—The GAL4-responsive reporter plasmid, pG5E1bLuc, contains five GAL4 sites cloned upstream of a minimal promoter and luciferase gene (31). pG5E1bLuc was co-transfected into CHO cells with a construct encoding the GAL4 binding domain fused to the ATF2(1–505) (31), Elk-1(307–428) (32), or c-jun(1–223) (33). Activation of these transcription factors by p38β or p38 was examined by co-transfection with these plasmids as described above.

**RESULTS**

Molecular Cloning of p38β—To identify proteins related to p38, the EST division of GenBank™ data base was searched with the blastn program. An EST-sequence of 223 base pairs from the EST division of GenBank™ data base was searched with the blastn program. An EST-sequence of 223 base pairs...
in this region of 7 amino acids and 1 amino acid (Fig. 1B).

We obtained and sequenced the EST clone 156272. This clone contains a portion of p38\(\beta\) cDNA (base pairs 53–707). Interestingly, this 156272 clone contains an 86-base pair insertion before base pair 325.2 Common features of introns, such as GT and AG dinucleotides at the ends, were found to suggest that this clone contains an unspliced intron. Otherwise this sequence would disrupt the mRNA by introducing an in-frame stop codon. To determine if this insertion was an intron, we made a mammalian expression plasmid containing flag-tagged p38\(\beta\) cDNA that contained the insertion and transfected it into COS-7 cells. We detected the expression of flag-p38\(\beta\) with the correct molecular mass of \(42\) kDa rather than \(13\) kDa, which would be the case if the mRNA containing the in-frame stop codon were translated (data not shown). However, when we did a PCR using human placenta cDNA to examine p38\(\beta\) cDNA, cDNAs containing this insertion were found several times. The incompletely spliced mRNA may be caused by immature mRNA in our preparation or represent a mechanism by which p38\(\beta\) protein expression is controlled.

Northern hybridizations show that p38\(\beta\) mRNA is expressed in human brain, heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Fig. 2A). The transcript length of p38\(\beta\) was \(2.5\) kilobases. Reprobing of the blot with p38 revealed a second transcript of \(3.5\) kilobases with similar tissue distribution (Fig. 2B). The distribution of p38\(\beta\) and p38 resembles the wide tissue distribution observed for other MAP kinase subgroups (21, 33).

Enzymatic Activity of p38\(\beta\)—The autophosphorylation and substrate specificity of recombinant p38\(\beta\) and p38 from bacteria were compared. Both kinases were observed to autophosphorylate to the same extent (data not shown). In vitro kinase assays demonstrated that both p38\(\beta\) and p38 phosphorylate MBP, ATF2, and MAPKAPK2 but not c-Jun (Fig. 3). One difference observed between p38\(\beta\) and p38 was the extent of ATF2 phosphorylation. ATF2 was phosphorylated 5-fold higher by p38\(\beta\) than p38. One likely explanation is a higher substrate affinity of p38\(\beta\) for ATF2 relative to the ATF2 affinity of p38.

Similar in vitro kinase results were obtained by using flag-tagged p38\(\beta\) and p38 immunoprecipitated from transfected COS-7 cells (data not shown).

The pyridinylimidazole derivative SB202190 previously was shown to inhibit p38 phosphorylation of MBP with no effect on the activity of the ERK or JNK MAP kinase subgroups (11). We observed that FHPI, which is an identical compound of SB202190, worked for p38 (data not shown).
SB 202190, also inhibits the kinase activity of p38β (Fig. 4). The IC_{50} values for inhibition of p38β and p38 phosphorylation of ATF2 by this compound were 0.35 and 0.28 μM, respectively. Similar IC_{50} values were observed for inhibition of p38β and p38 phosphorylation of MBP and their autophosphorylation (data not shown).

To determine if regulation of p38β activity occurs through the dual TGY phosphorylation site previously defined for p38 (8), the kinase activity of the mutant flag-tagged p38β(AF) was evaluated. Neither autophosphorylation nor phosphorylation of the kinase substrates ATF2 and MBP by p38β(AF) immunoprecipitated from COS-7 cells was observed (data not shown). Thus, as with p38, Thr^{188} and Tyr^{190} are most likely the phosphorylation sites involved in regulating the kinase activity of p38β.

Extracellular Stimuli of p38β—Because p38β and p38 share a high degree of amino acid identity, we sought to determine if they are similarly regulated. We and others have shown that p38 is activated when a variety of cell types are stimulated with proinflammatory cytokines, bacteria pathogens, or chemical-physical stress (7, 8). To compare p38β with p38, we transiently expressed flag-tagged p38β and p38 in HeLa cells. We selected HeLa cells because they are easily transfected and previously had been used with p38 (8). Western blot analysis of the cell extracts demonstrated the presence of equal levels of these proteins (data not shown). As shown in Fig. 5, p38β and p38 were similarly activated when the HeLa cells were treated with TNF-α, arsenite, anisomycin, high osmolality, H_{2}O_{2}, or UV, whereas treatment with epidermal growth factor and phorbol-12-myristate-13-acetate (PMA) had little or no effect on their activation. A considerable difference in activation was observed when IL-1β was used as a stimulus. After IL-1β stimulation, p38 was activated ~4-fold higher than p38β. This phenomenon was highly reproducible in all three experiments and cannot be attributed to variability in protein expression, since Western blot analysis of cell extracts consistently detected equal amounts of p38β and p38 (data not shown). The observed difference in IL-1β activation of p38β and p38 suggests p38β and p38 may be differentially regulated.

To further compare the activation of p38β and p38, we evaluated the dose response and kinetics of the TNF-α-induced activation. Fig. 6 shows that while p38β and p38 have similar sensitivities to TNF-α, their activation kinetics are different. Fig. 6A shows that maximum activation of both p38β and p38 is observed between 10 and 100 ng/ml of TNF-α. Fig. 6B shows that maximum activation of p38β took twice as long as that of p38. Activation of p38β peaked at 30 min, whereas that of p38 peaked at 15 min. Distinct activation kinetics between p38β and p38 further support the idea that these kinases are differentially regulated.

Regulation of p38β by MKKs—MAP kinases are activated by MAP kinase kinases (MKK or MEK), and substantial evidence exists that distinct MKK subsets activate specific MAP kinase subgroups (12, 13, 17, 34, 35). MKKs known to phosphorylate and activate p38 in vitro and when co-expressed in culture cells are MKK3, MKK4 (J NKK1 or SEK1), and the two splicing isoforms MKK6 and MKK6b (13, 14, 32). To determine if similar MKKs regulate p38β, we evaluated p38β kinase activ-
ity when p38β was co-expressed with MKK3, MKK4, and MKK6b in COS-7 cells. As shown in Fig. 7, MKK6b appears to be a stronger activator of p38β than MKK3 or MKK4. As we and others reported (13, 14, 32), p38 was activated similarly by MKK3, MKK4, and MKK6. The different preference of p38β for the upstream kinase MKK6 suggests that p38β and p38 are not identically regulated.

p38β Increases ATF2 Reporter Gene Expression—Rangneaud et al. (32) reported that the expression of ATF2- and Elk-1-dependent reporter genes is modulated only when p38 is co-expressed with the activated form of MKK3, MKK3(E). Over-expression of p38, ERK1, or JNK1 alone had little effect (31, 32). As shown in Fig. 8, over-expression of p38β alone was sufficient to increase expression of the ATF2-dependent reporter gene; ATF2-dependent reporter gene expression increased nearly 30-fold in p38β-transfected CHO-K1 cells. The in vivo enhancement of ATF2-dependent reporter gene expression by p38β is consistent with the in vitro enhanced phosphorylation of purified ATF2 by p38β (Fig. 3). Overexpression of the enzyme-dead mutant of p38β, p38β(AF), had no effect on ATF2-dependent reporter gene expression (Fig. 8).

When p38β is co-expressed with MKK3(E), ATF2-dependent reporter gene expression was not further enhanced (Fig. 9). However, ATF2-dependent reporter gene expression did increase when p38β was coexpressed with the activated MKK6b mutant, MKK6b(E). This contrasts with the behavior of p38, p38 increased ATF2-dependent reporter gene expression when cotransfected with either MKK3(E) or MKK6b(E) (Fig. 9). The results shown in Fig. 9 are consistent with the observation that MKK6b is the preferred activator of p38β (Fig. 7). The increase in reporter gene expression by p38β was specific for ATF2.
Co-expression of p38β with MKK3(E) or MKK6b(E) had little effect on the Elk-1- or c-Jun-dependent reporter gene expression (Fig. 9). The magnitude and selectivity of p38β for ATF2 strongly suggest that p38β is involved in regulating the transactivation activity of ATF2 under physiological conditions.

**DISCUSSION**

Herein we describe the cloning and characterization of a novel member of the MAP kinase family, p38β. p38β shares ~74% sequence identity with p38 and contains the TGY dual phosphorylation motif observed in p38 (7). Further analysis of p38β revealed that it is activated by diverse extracellular stimuli in a fashion quite similar to p38. The one exception we observed was activation by IL-1β. While IL-1β activated p38β 8-fold over background, p38β was activated only ~2-fold. Another difference we noted was the rate of activation upon stimulation with TNF-α. Maximal activation of p38 peaked in 15 min, whereas maximal activation of p38β took about twice as long. These results imply that the MAP kinase isoforms p38β and p38 have not only overlapping regulation pathways, but also independently regulated pathways. Differential regulation of kinase isoforms by extracellular stimuli has been reported (36, 37). For example, Oncostatin-M only activated ERK2 in KS cells, although both ERK1 and ERK2 were expressed (37). The differential regulation of MAP kinase subgroups members suggests that the activities of closely related kinase isoforms are not simply redundant but have specific functions.

One controlling factor in the differential regulation of p38β and p38 is the direct upstream kinase. We observed preferential activation of p38β by MKK6b but not by its closely related isoform, MKK3. In contrast, p38β was activated almost equally by the two MKKs. In the ERK pathway, a further upstream kinase, A-Raf, was found to selectively activate MEK1 but not MEK2 in HeLa cells (38), while C-Raf equally activated the two MEKs. These unique regulatory steps of closely related MAP kinase isoforms seem to occur at many levels of the signal transduction pathway.

In addition to differential regulation by MKKs, p38β and p38 also demonstrated different substrate specificity. In vitro and in vivo, p38β phosphorylated ATF2 to a much greater extent than p38. Although we cannot exclude the possibility that other ATF2 activators such as JNK regulate ATF2 under select conditions, the magnitude of ATF2 activation by p38β suggests p38β participates in the in vivo regulation of ATF2. Structural differences may account for the distinct substrate specificity of p38β and p38. Based on the three-dimensional structure of other kinases such as ERK2 (24), the extra eight amino acids created by alignment of p38β and p38 (Fig. 1b) are located in a loop structure (L8) between two helices, α7 and α10. The α8 helix is the putative substrate binding pocket. The loop length could affect the position of αα7, which influences the substrate binding pocket size. When compared with the structure of cAPK-substrate peptide complex (39), L8 does not directly interact with the phosphorylation site of substrate but rather participates in high affinity substrate binding (39). The difference in this region may account for the different substrate specificities of p38β and p38 we observed. The role of L8 in substrate specificity can be tested by exchanging this region in p38β and p38 and then testing the chimeras for substrate specificity. A similar case has been reported for the JNK MAP kinase subgroup. JNK2 has a higher affinity for c-Jun than does JNK1. The structural basis for this difference was found to be the sequence between kinase domains IX and X (33). Such difference of specific regions of closely related kinase isoforms appear to play a role in their substrate selectivity. Since substrate specificity of the kinase plays an important role in conferring biological specificity, p38β and p38 may elicit different biological responses.

The putative substrate of p38β, ATF2, binds to cAMP-responsive element-like elements (T/G)ATCACT in the promoters of many genes and has been implicated in regulating the expression of many proteins such as interferon β, E-selectin, and transforming growth factor-β. ATF2 not only functions alone, but also interacts with other transcription factors such as NF-κB and c-Jun (43–45). NF-κB and c-Jun have been shown to play key roles in the expression of many cytokine and adhesion molecules (46–49). Thus, ATF2 is clearly involved in the gene expression of inflammation-related molecules. Regulation of ATF2 by p38β suggests a role for p38β in inflammatory reactions. Given that both p38β and p38 were inhibited by the anti-inflammatory reagent, pyridinyl imidazone, we are also interested in the putative substrate of p38β for p38β in the inflammatory response. Gene targeting of p38β or p38 in culture cells or mice may shed light on this issue.

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