Molecular Cloning and Characterization of a Novel p38 Mitogen-activated Protein Kinase

(Received for publication, June 4, 1997, and in revised form, July 17, 1997)

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The p38 mitogen-activated protein kinases (MAPK) are activated by cellular stresses and play an important role in regulating gene expression. We have isolated a cDNA encoding a novel protein kinase that has significant homology (57% amino acid identity) to human p38α/CSBP. The novel kinase, p38d, has a nucleotide sequence encoding a protein of 365 amino acids with a putative TGY dual phosphorylation motif. Dot-blot analysis of p38d mRNA in 50 human tissues revealed a distribution profile of p38d that differs from p38α. p38d is highly expressed in salivary gland, pituitary gland, and adrenal gland, whereas p38α is highly expressed in placenta, cerebellum, bone marrow, thyroid gland, peripheral leukocytes, liver, and spleen. Like p38α, p38d is activated by cellular stress and proinflammatory cytokines. p38d phosphorylates ATF-2 and PHAS-I, but not MAPK-activated protein kinase-2 and -3, known in vivo and in vitro substrates of p38α. We also observed that p38d was strongly activated by MKK3 and MKK6, while p38α was preferentially activated by MKK6. Other experiments showed that a potent p38α kinase inhibitor AMG 2372 minimally inhibited the kinase activity of p38d. Taken together, these data indicate that p38d is a new member of the p38 MAPK family and that p38d likely has functions distinct from that of p38α.

Mitogen-activated protein kinases (MAPK) transduce signals from cell membrane to nucleus in response to a wide variety of stimuli (1–3). Four groups of MAPKs have been identified in mammalian cells: the extracellular signal-regulated kinases (ERK) (also referred to as p42/44 MAPK) (1, 4, 5), the c-Jun N-terminal kinases (JNK) or stress-activated protein kinases (SAPK) (6–12), p38/CSBP/RK/MPK2/MXI2 (13–16), and ERK5 kinase (17). The mammalian ERKs are activated by growth factors and mitogenic stimuli (1, 4), whereas p38 and JNK are regulated by stress-inducing signals (i.e. UV irradiation, osmotic shock) and by proinflammatory cytokines (i.e. interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) (6–14, 16)).

MAPKs are activated through phosphorylation on both threonine and tyrosine residues at the Thr-Xaa-Tyr dual phosphorylation motif (18–22). This motif is located in kinase subdomain VIII where Xaa is a Glu, Pro, and Gly for the ERK (19, 20, 22), JNK (6, 14), and p38 (13, 18) group of kinases, respectively. Activation of MAPK is mediated by dual specificity MAPK kinases, MKK or MEK (23–28). MEK1 and -2 catalyze the phosphorylation of ERK1/2 (23, 24), whereas MKK4/SEK1 mediates the activation of JNK and p38 (25–27). MKK3 and MKK6 specifically activate p38 (26–27). Once activated, MAPK phosphorylates several transcription factors at serine and threonine residues, thereby regulating gene expression. Each group of MAPK appears to have different substrate specificity. JNK phosphorylates transcription factors c-Jun (6), ATF-2 (33), and Elk-1 (34), whereas p38 phosphorylates ATF-2 (18), MEF2C (35), and CHOP-1 (36). In addition, p38 phosphorylates and activates MAPK-activated protein (MAPKAP) kinase-2 and -3 (15, 37). Upon activation by p38, MAPKAP kinase-2 phosphorylates the small heat shock proteins HSP25/27 (15).

p38 was originally identified in lipopolysaccharide (LPS)-stimulated mouse macrophages and was found to have substantial homology to the Saccharomyces cerevisiae HOG1 kinase (13, 38). The human homologues of p38 were cloned after p38 was identified with a radiophotoaffinity-labeled pyridinyl imidazole compound (14). Inhibition of p38 by this class of compound prevents the production of IL-1 and TNFα by human monocytes stimulated with LPS (14). In addition to the original isoform of p38 (now referred to as p38α), a second p38 kinase member (p38δ) was identified which shows 74% amino acid identity to p38α (39). p38δ also has a TGY motif in kinase subdomain VIII (39). More recently, a third p38 kinase family member with a TGY motif was cloned and is termed p38γ/ERK6/SAPK3 (40–42). The amino acid sequence of p38γ/ERK6/SAPK3 is 60% identical to p38α (40).

Here we report the isolation of a novel p38 MAPK (p38d) with a TGY motif in its activation domain. p38d was characterized with regard to tissue distribution, stimulus activation, MKK activation, substrate specificity, and inhibitor sensitivity. These studies reveal interesting similarities as well as differences in the properties of p38d as compared with p38α.

**EXPERIMENTAL PROCEDURES**

Reagents—Recombinant GST-c-Jun protein was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Recombinant PHAS-I protein was purchased from Stratagene (La Jolla, CA). ATF-2 was amplified by PCR from human skeletal muscle cDNA using two primers...
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(5'-CATATGCAATACAGGAGCGGTTGGAAT-3' and 5'-CTTCCCTGC- CAGTTAAGAGGGCTTTTTTTTGTACAC-3') and cloned into the pBluescript KS-I vector expression vector pAMG21. Reombinant protein was expressed in Escherichia coli strain F15 and purified by S-Sepharose and Q-Sepharose chromatography.

Reombinant Kinases—MAPKAP kinase-2 was amplified by PCR from human monocyte cDNA using two primers (5'-AACACAGATCC AGGAAAGAAGGCCACATC-3' and 5'-ACAAACCTGGAGCTTGTGACAG -3'). MAPKAP kinase-3 was amplified by PCR from a human lung cDNA library using two primers (5'-CTTCTGCCGAGATGCTTGGGAAGAGG-3' and 5'-CTCCCTGCGAGCTTGTGACAG -3'). The PCR products were cloned separately into a GST fusion vector, pGEX-4T (Pharmacia Biotech Inc.). GST-MAPKAP kinase-2 and -3 were expressed in E. coli strain BL2/D3 (Pharmacia), and fusion protein was purified over a glutathione column (Pharmacia). MKK3 was amplified by PCR from human skeletal muscle cDNA (CLONTECH, Palo Alto, CA) with two primers (5'-ACAAACCTGGAGAAGTACATAGTGCT CATCATCATCATCATCCGCAAGCCCGACCCAC-3' and 5'-TCCCGTCCGAGCTTGTGACAG -3') and then cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). To generate constitutive active MKK3 (ca-MKK3), site-directed mutagenesis (43) was used to replace Ser-189 and Ser-193 with Glu. This DNA was cloned into the baculovirus transfer vector pVL1392 (Invitrogen) and expressed in High Five cells. Recombinant MKK6 was purified by a glutathione column (Pharmacia) chromatography. To generate human FLAG-tagged p38α, two primers from the published nucleotide sequences (14) were used in PCR with human peripheral blood leukocyte cDNA as templates. The PCR product was then cloned into mammalian expression vector pCMVX/V5. HA-tagged MKK6 in pME vector (31) was kindly provided by Dr. Hagi wara Masatoshi and HA-tagged MKK3 and MKK4 (SEK1) in mammalian expression vector pMT (44) were provided by Dr. James Woodgett.

Molecular Cloning of p38α—An expressed sequence tag (EST) (311 base pairs) with homology to p38 was identified in the Amgen EST data base. Gene-specific forward and reverse primers were designed from the EST sequence and used in PCR to clone full-length cDNA with the Marathon-Ready human fetal brain cDNA templates (CLONTECH) following the manufacturer's protocol. These Marathon-Ready cDNAs have adapters ligated at the 5' and 3' ends. The gene-specific forward primer (5'-GAGGCTCAGAGCCAAGCTACGTCG-3') and an adaptor primer (CLONTECH) were used in combination to amplify the 3' portion of p38α. The gene-specific reverse primer (5'-CTGGGCTTGGAGCATCCGGAG-3') and the adaptor primer were used to amplify the 5' portion of p38α. PCR was performed for 30 cycles (95°C for 30 s, 93°C for 30 s, and 72°C for 30 s) followed by an extension at 72°C for 7 min. The resulting PCR product was ligated into the pCR2.1 vector (Invitrogen) and sequenced on both strands. A second murine EST sequence (GenBank™ accession number W53837) that has homology to the Amgen EST sequence was identified in the GenBank data base. This EST fragment was used as a probe to screen a human macrophage library, and two clones were isolated. Sequencing of one of the clones revealed an identical open reading frame as the one cloned by PCR. The clone isolated from human fetal brain library was used for subsequent studies described here.

Full-length p38α cDNA was cloned into a mammalian expression vector pCR3.1 (Invitrogen) by PCR using two primers (5'-ACCCAGATCCAGCAATACAGGAGCGGTTGGAAT-3' and 5'-ACCCCTGGCTTTTTTTTGTACAC-3'). The first primer added a FLAG epitope at the 5' end. PCR site-directed mutagenesis (43) was used to create a p38α mutant (AGF) by substituting Thr-180 and Tyr-182 with an Ala and a Phe, respectively. The inserts were completely sequenced to make sure that no PCR errors were introduced.

Northern and Dot-Blot Analysis of p38α mRNA—A Northern blot filter containing poly(A)+ RNA from multiple tissues and a normalized Master blot filter containing mRNA from 50 different tissues (CLONTECH) were probed with a 32P-labeled DNA fragment generated from the 5' portion of the coding region of p38α (5 nucleotides 1 to 550). Hybridization was performed at 68°C in ExpressHyb Buffer (CLONTECH) followed by two washes in 0.1% SSC, 0.1% SDS at 68°C, then the blot was exposed overnight at -70°C. The same Northern blot was then probed with a 32P-labeled DNA fragment generated from the 5' portion of the coding region of p38α using identical hybridization and washing conditions.

Cell Culture and Transfection—293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin. For transfection, 2 × 106 cells were plated onto 100-mm dishes 16–20 h before transfection. DNA (2.0 μg of p38α, 8.0 μg of all other DNAs) was transfected into 293 cells using LipofectAMINE™ (Life Technologies, Inc.). Transfected cells were incubated for 5 h in serum-free DMEM, further incubated in DMEM with 10% fetal calf serum, and harvested 48 h after transfection.

Immunoprecipitation and Western Blot Analysis—Immunoprecipitation was performed as described previously (45). Briefly, cells were dislodged into lysis buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% Igepal, 150 mM NaCl, 20 mM NaF, 0.2 mM Na3VO4, 1 mM EDTA, 1 mM EGTA) and sedimented (15,000 × g for 60 min) to remove insoluble debris. Total protein in cell lysates was quantified by the Bradford method using a protein assay kit (Pierce). Supernatants containing 100 μg of protein were immunoprecipitated with 5 μg of anti-HA mAb 12CA5 (Berkeley Antibody Co., Berkeley, CA) or anti-FLAG M2 mAb (Sigma) and protein A-Sepharose CL-4B beads (Pharmacia). For Western blot analysis, lysates containing equal amounts of total protein were resolved by 4–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophobized onto nitrocellulose membranes. The blots were then probed with mAb M2, followed by biotinylated rabbit antimouse IgG (Amersham Life Science Inc.) and developed using the enhanced chemiluminescence (ECL) detection system (Amersham Life Science Inc.).

To determine the substrate and inhibitor specificity of p38α and p38α, the kinases were first activated in vitro with ca-MKK3. Activation was performed in the presence of Dulbecco's phosphate-buffered saline (2.7 mM CaCl2, 1.4 mM MgCl2, 0.1% NaN3, and 0.5 mM dithiothreitol), and resuspended in 40 μl of kinase buffer. The beads were then incubated with ATP-2 and 1 μl of (γ-32P)ATP (3000 Ci/mmol) at 30°C for 30 min. Reaction mixtures were then resuspended in 2 × sample buffer (125 mM Tris, pH 6.8, 6% SDS, 20% glycerol) and boiled for 3 min. Phosphorylated proteins were resolved by SDS-PAGE, after which the gels were dried and exposed to radiographic film.

p38 Kinase Assay—Cells transfected with FLAG-tagged p38α or p38β were collected, and recombinant protein was immunoprecipitated using mAb M2 and protein A-Sepharose CL-4B beads. Beads were washed three times with lysis buffer, once with kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl2, 0.1% NaN3, and resuspended in 40 μl of kinase buffer. The beads were then incubated with ATF-2 and 1 μl of [γ-32P]ATP (3000 Ci/mmol) at 30°C for 30 min. Reaction mixtures were then resuspended in 2 × sample buffer (125 mM Tris, pH 6.8, 6% SDS, 20% glycerol) and boiled for 3 min. Phosphorylated proteins were resolved by SDS-PAGE, after which the gels were dried and exposed to radiographic film.

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RESULTS

Molecular Cloning of p38α—To identify novel MAPKs, we searched the Amgen EST data base with the p38 nucleotide sequences as the query sequences. One partial human cDNA was isolated from human fetal brain library was used for subsequent studies described here.

The deduced amino acid sequence alignment of p38αβ/CSBP2 (14), p38α/CSBP2 (14), p38β/CSBP2 (14), p38δ/CSBP2 (14), p38γ/ERK6 (40–42), and the most closely related cDNA clones, p38α/CSBP2 displays 67, 55, and 62% amino acid identity to p38αβ/CSBP2, p38β, and p38γ/ERK6, respectively. p38δ has a putative dual phosphorylation motif (TGY) in kinase subdomain VIII similar to that found in other p38 family members. The amino acid sequence alignment of p38α with human p38αβ/CSBP2, p38β, and p38γ/ERK6 is shown in Fig. 1B.
Tissue Distribution of p38 δ mRNA—The expression of p38 δ was examined in a variety of human tissues by Northern blot analysis using a probe derived from the 5′ end of p38 δ. The p38 δ probe hybridized strongly to a transcript of approximately 1.8 kilobases and weakly to a transcript of 6.0 kilobases, while a probe derived from p38 α hybridized to a single transcript of 4.1 kilobases (14) (data not shown). The same p38 δ probe was used to hybridize a human RNA master blot containing poly(A)́ RNAs from 50 different human tissues. The RNAs in this blot have been normalized to the mRNA levels of eight different housekeeping genes; thus the relative levels of mRNA in different tissues could be determined. Among the tissues examined, strong hybridizing signals were observed in exocrine/endocrine tissues including human salivary gland, pituitary gland, adrenal gland, and placenta (Fig. 2A). Moderate signals were observed in pancreas, trachea, thyroid gland, stomach, prostate, colon, small intestine, lymph node, kidney, and lung. Probing the master blot with p38 α DNA revealed a different tissue distribution profile. Strong hybridizing signals were found in placenta, cerebellum, bone marrow, thyroid gland, peripheral leukocyte, liver, and spleen. Moderate signals were found in occipital lobe, fetal liver, pituitary gland, adrenal gland, aorta, uterus, stomach, lymph node, cerebral cortex, hippocampus, and thymus (Fig. 2B). Probing the master blot with p38 δ DNA found that p38 δ is abundantly expressed in brain tissues such as hippocampus, frontal lobe, cerebral cortex, cerebellum, caudate nucleus, medulla oblongata, whole brain, and fetal brain (Fig. 2C). Interestingly, probing the master blot with p38 γ DNA found that it has a very limited tissue distribution profile. p38 γ was highly expressed in skeletal muscle, while the expression in other tissues appears to be low (Fig. 2D).

Substrate Specificity of p38 δ—Full-length p38 δ cDNA and p38 α were cloned into mammalian expression vectors with a FLAG epitope sequence added at the 5′ end and transfected into 293 cells. Transfected cell lysates were subjected to immunoprecipitation with a FLAG mAb. The immunoprecipitated p38 δ and p38 α were activated using recombinant ca-MKK3, washed, and used in immune complex kinase assays with various substrates. As shown in Fig. 3, p38 δ and p38 α phosphorylated full-length ATF-2 (lanes 1 and 6), but not c-Jun (lanes 2 and 7), a known substrate for JNK (6). We also observed that p38 δ showed minimal phosphorylating activity against MAPKAP kinase-2 and -3 (Fig. 3, lanes 3 and 4), while p38 α phosphorylated these substrates efficiently (Fig. 3, lanes 5 and 10). Control lysates did not phosphorylate any of the substrates (data not shown).

Activation of p38 δ by Extracellular Stimuli—The p38 group of kinases can be activated by a variety of stress stimuli and proinflammatory cytokines (13, 14, 18). Because p38 δ is closely related to p38 α, we determined whether similar stimuli could activate p38 δ kinase activity. 293 cells were transiently trans-
fected with either p38δ or p38α cDNA and treated with various stimuli. p38δ and p38α activity was measured by their ability to phosphorylate ATF-2-(1–109) in an immune complex assay. As shown in Fig. 4A, p38δ was strongly activated by H₂O₂, UV, NaCl, and Na₃VO₄ and moderately activated by anisomycin, IL-1β, TNFα, and epidermal growth factor. p38α was strongly activated by UV, NaCl, H₂O₂, and anisomycin and moderately activated by TNFα, IL-1β, and epidermal growth factor (C). A notable difference is that Na₃VO₄ strongly activated p38δ (Fig. 4A, lane 5) but not p38α (Fig. 4C, lane 5). To eliminate the possibility that changes in p38 kinase activity are due to the variations in protein expression, Western blot analysis was performed. Fig. 4, B and D, shows that similar amounts of p38 were expressed under all conditions tested.

Activation of p38δ by Upstream Mitogen-activated Kinase Kinases—MAPKs are activated by upstream M KK kinases. To determine which M KK(s) can activate p38δ, we co-transfected 293 cells with vectors encoding p38δ and M KK3, M KK4, or M KK6 and then assayed p38δ kinase activity in an immune complex assay. In repeated experiments, co-transfection of cells with p38δ and M KK3 or M KK6 resulted in strong activation of p38δ activity (Fig. 5A, lanes 2 and 4), whereas co-transfection of p38δ with M KK4 had little effect (Fig. 5A, lane 3). Similar studies showed that M KK6 and M KK4 markedly activated p38α (Fig. 5C, lanes 3 and 4), while M KK3 weakly activated p38α (Fig. 5C, lane 2). Western blot analysis indicated that p38δ and p38α were expressed at similar levels under all conditions tested (Fig. 5, B and D).

p38δ Is Activated by Phosphorylation at the Dual Phosphorylation TGY Motif—MAPKs are activated by dual phosphorylation at the Thr-Xaa-Tyr motif within kinase subdomain VIII (18). To determine whether this motif is required for p38δ
activation, we generated a mutant p38δ by replacing the Thr-Gly-Tyr motif with Ala-Gly-Phe (AGF mutant) and tested whether this mutant could be activated. Wild type p38δ phosphorylated ATF-2 when activated by UV irradiation (Fig. 6A, lane 8). However, the AGF mutant was unresponsive to UV stimulation (Fig. 6A, lane 7), whereas p38α kinase activity was inhibited in a dose-dependent manner (Fig. 7B). At 1 μM inhibitor concentration, there was 98% inhibition of p38α (Fig. 7B, lane 3), but less than 25% inhibition of p38δ (Fig. 7A, lane 3).

**Discussion**

In this report, we describe the cloning and characterization of a novel member of the p38 group of protein kinases. p38δ has significant homology at the amino acid level to p38α, -β, and -γ and contains the dual phosphorylation TGY motif that is found in this p38 group of kinases (13, 18). Mutation of the Thr and...
Tyr residues in the TGY motif abolished the kinase activity of p38α and blocked UV or MKK6-induced activation. Thus, like other MAPKs, p38α requires phosphorylation at the Thr and/or Tyr in the TGY motif for its activation.

The tissue distribution of p38α was examined in 50 different human tissues. The pattern of expression of p38α mRNA is distinct from that of p38α, β, and γ. Very high levels of expression of p38α mRNA were observed in human gland tissues, while p38α is abundantly expressed in placenta, brain (cerebellum), and lymphoid tissues. p38β is most abundantly expressed in brain tissues, while p38γ appears to have a limited tissue distribution. These differences in mRNA expression suggest that p38α, p38β, p38γ, and p38δ may have tissue-specific functions.

Similarities among p38α, p38β, p38δ, and p38γ prompted us to investigate whether p38δ can utilize the same substrates. p38δ is activated in 293 cells by a diverse set of stimuli, while MKK3 is the dominant activator of p38α. Similarities among p38α, p38β, and p38δ resemble that of p38α, but not MAPKAP kinase-2 and -3 which are the physiological substrates for p38δ. These results are also in contrast to the substrate profile of p38δ which utilize similar substrates as p38α. The p38δ could phosphorylate ATF-2 and was far less effective in phosphorylating MAPKAP kinase-2 and -3 (42). Thus, the substrate specificity of p38δ resembles that of p38β.

Similar to p38α, p38δ is activated in 293 cells by a diverse array of cellular stresses and proinflammatory cytokines. However, the degree of activation by various stimuli is different for p38δ as compared with p38α. Most notable was the strong activation of p38δ, but not of p38α by NαV04. Because NαV04 inhibits protein tyrosine phosphatase activity, our data suggest that such phosphatases differentially regulate the basal activity of p38δ and p38α.

Differences in activation of p38δ versus p38α were also observed at the MKK level. In cell transfection experiments, p38δ is strongly activated by MKK3 and MKK6, whereas p38α is preferentially activated by MKK6. These data suggest that regulators of p38δ overlap. Like p38α, it is likely that the dominant activator of p38δ in a given cell type will reflect the unique cellular environment. For example, it has been observed that MKK6 is the dominant activator of p38α in monocytes and KB cells, while MKK3 is the dominant activator of p38α in PC-12 cells (46).

p38α/CSBP has been directly linked to inflammatory cytokine production through the use of inhibitors that block its function. We tested one compound that blocks p38α activity and found that it was relatively inactive against p38δ. Thus, other compounds will have to be developed to determine if p38δ is involved in cytokine production. The critical substrates phosphorylated by p38δ leading to cytokine production have not yet been elucidated, although several candidates have been discovered. Of this group, we showed that p38δ phosphorylated ATF-2 and PHAS-1, but not MAPKAP kinase-2 and -3. Additional studies are required to identify in vivo p38δ substrates and to determine if these substrates are involved in cytokine production or other p38δ-mediated processes.
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J. Biol. Chem. 1997, 272:23668-23674.
doi: 10.1074/jbc.272.38.23668

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