Murine p38-δ Mitogen-activated Protein Kinase, a Developmentally Regulated Protein Kinase That Is Activated by Stress and Proinflammatory Cytokines*

(Received for publication, September 23, 1998, and in revised form, November 16, 1998)

Mickey C.-T. Hu‡§, You-ping Wang‡, Adel Mikhail¶, Wan Rong Qiu‡, and Tse-Hua Tan†

From the ‡Department of Cell Biology, Amgen, Inc., Thousand Oaks, California 93120, §Phylogenix, Inc., Columbus, Ohio 43212, and the ¶Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

The p38 mitogen-activated protein kinases (MAPK) play a crucial role in stress and inflammatory responses and are also involved in activation of the human immunodeficiency virus gene expression. We have isolated the murine cDNA clones encoding p38-δ MAPK, and we have localized the p38-δ gene to mouse chromosome 17A3-B and human chromosome 6p21.3. By using Northern and in situ hybridization, we have examined the expression of p38-δ in the mouse adult tissues and embryos. p38-δ was expressed primarily in the lung, testis, kidney, and gut epithelium in the adult tissues. Although p38-δ was expressed predominantly in the developing gut and the septum transversum in the mouse embryo at 9.5 days, its expression began to be expanded to many specific tissues in the 12.5-day embryo. At 15.5 days, p38-δ was expressed virtually in most developing epithelia in embryos, suggesting that p38-δ is a developmentally regulated MAPK. Interestingly, p38-δ and p38-α were similar serine/threonine kinases but differed in substrate specificity. Overall, p38-δ resembles p38-γ, whereas p38-β resembles p38-α. Moreover, p38-δ is activated by environmental stress, extracellular stimuli, and MAPK kinase-3, -4, -6, and -7, suggesting that p38-δ is a unique stress-responsive protein kinase.

The mitogen-activated protein kinase (MAPK) cascade is a major signaling pathway by which cells transduce extracellular stimuli into intracellular signals to control the expression of genes essential for cellular processes such as cell proliferation, differentiation, and stress responses (1, 2). In mammalian cells, these kinases include extracellular signal-regulated protein kinases (ERKs) (3), the c-Jun amino-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs) (4–6), and p38 MAPKs (7–10). Whereas ERKs are activated rapidly in response to the binding of growth factors to growth factor receptors (11, 12), JNKs/SAPKs and p38 MAPKs are stimulated by environmental stress (i.e. osmotic shock, ultraviolet irradiation, cytotoxic chemicals, etc.) and proinflammatory cytokines (i.e. interleukin-1 (IL-1) and tumor necrosis factor (TNF)) (4, 5, 7, 8, 13–17). Although the physiological function of the ERKs and JNKs/SAPKs in signal transduction pathways has been extensively studied (1, 3, 18–21), the functional role of the p38 MAPK signaling pathway is relatively less understood (7–9, 17, 22). Nevertheless, p38 MAPKs play an important role in stress and inflammatory responses and are also involved in activation of the human immunodeficiency virus type 1 promoter (23).

These MAP kinases have the unique feature of being activated by phosphorylation on threonine (Thr) and tyrosine (Tyr) residues by upstream dual-specificity kinases, i.e. MAP kinase kinases (MKKs or MEKs) (18, 20). This dual phosphorylation Thr-X-Tyr motif is located within the kinase subdomain VIII where ERK is Thr-Glu-Tyr; JNK/SAPK is Thr-Pro-Tyr; and p38 MAPK is Thr-Gly-Tyr. MKK-1 and MKK-2 phosphorylate and activate ERK-1 and ERK-2 (24, 25), whereas MKK-3 and MKK-6 activate p38 MAPK specifically (26–32). Although MKK-4 (SEK1) stimulates JNK/SAPK and p38 MAPK (26, 27, 33), MKK-5 phosphorylates and activates ERK-5 (34, 35). Recently, MKK-7 has been shown to activate JNKs/SAPKs specifically but not p38 MAPKs and ERKs (36). Each MAP kinase group has a unique substrate specificity and is regulated by a distinct signal transduction pathway (18, 20, 21). For instance, ERK-1 and ERK-2 phosphorylate and activate the transcription factor Elk-1 (37, 38). JNKs/SAPKs phosphorylate and activate the transcription factors c-Jun (4, 5), ATF-2 (39), and Elk-1 (40). The p38 MAPKs phosphorylate and activate the transcription factors ATF-2 (17, 26) and Elk-1 (29).

The first p38 MAPK (hereafter designated as p38-α) was identified initially in lipopolysaccharide-stimulated macrophages and was found later to share significant homology with the yeast HOG1 kinase (7, 41). Subsequently, the human p38-α homologues (CSBPs) were isolated by using radiolabeled and radiophotoaffinity labeled pyridinyl imidazole compounds, which block inflammatory cytokine biosynthesis by monocytes stimulated with lipopolysaccharide (8). Another member (p38-β) of the p38 MAPK family was identified and cloned, which is very homologous (with 75% amino acid identity) to p38-α (42). The third member (p38-γ) of the p38 MAPK family was recently isolated as ERK-6 (43) and SAPK3 (44), which share significant homology (63% amino acid identity) with p38-α. All these p38 MAPK members contain a characteristic Thr-Gly-Tyr motif within the kinase subdomain VIII.

Here, we present a murine p38 MAPK family member, p38-δ,
whose sequence is significantly homologous to p38-α (63% amino acid identity). We showed that expression of p38-δ mRNA was regulated in different developmental stages, suggesting that p38-δ is a developmentally regulated MAPK. We characterized p38-δ by determining its chromosomal location, stimulation by extracellular stimuli, and activation by upstream kinases (MKks). Moreover, we compared substrate specificity and inhibitor sensitivity between p38-δ and p38-α, and we showed that they are discrete. Our results indicate that p38-δ is a unique stress-responsive protein kinase.

**EXPERIMENTAL PROCEDURES**

**Isolation of Murine and Human P38-δ cDNAs**—A rat 1.3-kilobase pair expressed sequence tag (EST) cDNA clone with ~62% homology to mouse p38-β (GenBank™ accession number D83073) cDNA was used as a probe to screen a rat lung cDNA library in CLONTECH Laboratories. For hybridization, replicate filters were prehybridized for 1 h at 68 °C in Express hybridization buffer (CLONTECH Laboratories) and hybridized 12 h at 68 °C in the same solution with the [32P]dCTP-labeled probe. After hybridization, the filters were washed several times at high stringency, at 65 °C in 0.1% SDS, 0.2 SSC (1× SSC, 150 mM NaCl and 15 mM sodium citrate), and subjected to autoradiography. Several positive clones were picked and purified after hybridization of 105 phages. The cDNA inserts of these positive phage clones were subsequently subcloned into pcCR3.1 plasmid vector (Invitrogen). After analysis of the inserts, the longest cDNA clone was sequenced on both strands, using a PCR procedure employing fluorescent dye terminucleotides and a model 373A automated sequencer (Applied Biosystems). Similarly, for human p38-δ cDNA cloning, the same EST cDNA probe was used to screen a human lung cDNA library in λTripEx phage vector (CLONTECH Laboratories). Several positive clones were obtained, and the cDNA inserts of these phage clones were converted into vitro into pcTripEx plasmid vector, according to the manufacturer’s instructions. A candidate full-length cDNA clone was sequenced on both strands as described above. Sequence comparisons were aligned with the Bestfit program of the GCG sequence analysis software package (Wisconsin Package version 9.0).

**DNA, Protein, and Chemical Reagents**—The Flag-tagged p38-δ expression plasmid was constructed from the murine p38-δ cDNA by the PCR technique using oligonucleotides 5'-AAGCTTGGATCCGACGCGCCAT-3' and 5'-CTCGAGCTCGAGCAAGCTAGATATATGCTC-3' (as primers to incorporate a Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) at the amino terminus of p38-δ. The PCR-generated product was cloned into the expression vector pcCR3.1 plasmid vector (Invitrogen) and designated pFlag-p38-δ. The Flag-tagged p38-δ (Flag-p38-δ) plasmid expression was generated from the pFlag-p38-δ plasmid by the PCR technique using oligonucleotides 5'-GATCCGAGGATCCGGACGCGCCAT-3' and 5'-CTCGAGCTCGAGCAAGCTAGATATATGCTC-3' (as primers to replace the Thr56-Gly-Tyr102 motif with Ala56-Gly-Phe102, using a QuietChange™ site-directed mutation kit (Stratagene). The sequences of these cDNA constructs were confirmed by DNA sequencing on both strands as described. The pVA1 containing the adenovirus VA1 RNA gene plasmid was obtained as described previously (45). The Flag-tagged p38-α, MKK-3, MKK-4, MKK-6, MKK-7, and MEK kinase-7 (ASK-1) expression plasmids were kindly provided by Dr. R. Geroski (Amgen Inc.). ATF-2-(1–96) and GST-MAPKAP kinase-2 were purchased from CLONTECH Laboratories. Each sample (2 µg) was denatured and electrophoresed on a 1.2% agarose gel containing formaldehyde and then transferred to a Hybond-N membrane (Amersham Pharmacia Biotech) in 20× SSC as described (46). Murine p38-δ or human β-actin cDNA was labeled with [32P]dCTP to a specific activity of approximately 10⁶ dpm/µg. Membranes were hybridized with either the p38-δ or β-actin cDNA probe, then washed at high stringency, at 65 °C in 0.2× SSC, 0.1% SDS, and subjected to autoradiography. Probes were removed in 0.5% SDS at 95–100 °C.

**In Situ Hybridization (ISH)**—ISH was performed as described (47).

Briefly, fetuses and tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline overnight, dehydrated, and infiltrated with paraffin. Serial sections at thickness of 5–7 µm were mounted on gelatin-coated slides, deparaffinized in xylene, dehydrated, and post-fixed. The tissue sections were digested with proteinase K, post-fixed, and washed several times with triethanolamine buffer, perchloric acid, and dehydrated. The cDNA transcripts were synthesized from linearized cDNA templates to generate antisense and sense probes, according to manufacturer’s conditions (Ambion) and labeled with [35S]-UTP (>1000 Ci/mmol; Amersham Pharmacia Biotech). cRNA transcripts larger than 200 nucleotides were subjected to alkali hydrolysis to give a mean size of 200 nucleotides. The tissue slides were hybridized at 65 °C in 50% deionized formamide, 0.3 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM NaPO4, 5 mM EDTA, 10 mM dextran sulfate, 1× Denhardt’s, 50 µg/ml total yeast RNA, and 5–7.5 × 10⁶ cpm/ml 35S-labeled cRNA probe. The tissue slides were subjected to stringent washing at 65 °C in 50% formamide, 2× SSC, 10 mM dithiothreitol, and washed in phosphate-buffered saline before treatment with 20 µg/ml RNase A at 37 °C for 30 min. Following washes in 2× SSC and 0.1× SSC at 37 °C for 10 min, the slides were dehydrated and dipped in Kodak NTB-2 nuclear track emulsion and exposed for 2–3 weeks in light-tight boxes with desiccant at 4 °C. Photographic development was carried out in Kodak D-19. The tissue slides were counterstained lightly with toluidine blue and analyzed using both light and dark field optics of a microscope. Sense control cRNA probes indicate the background levels of the hybridization signals.

**Cell Culture and Transfections**—293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Cells to be transfected were plated the day before transfection at a density of 2 × 10⁶ cells per 100-mm dish. 293T cells were co-transfected with expression plasmids (10 µg each plasmid per dish) as indicated with pVA1 (10 µg per dish) to enhance transient protein expression, using the calcium phosphate precipitation protocol (Specialty Media, Inc.). The transfected 293T cells were harvested 48 h after transfection. For cell stimulation, 293T cells were treated with human TNF-α (20 ng/ml) for 10 min before harvest.

**Immunoprecipitation and Western Blot Analysis**—Cells were lysed in WCE lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 0.5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM Pefabloc (Boehringer Mannheim) or phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Soluble lysates were prepared by centrifugation at 10,000 × g for 30 min at 4 °C. The lysates were precleared using Pansorbin cells (Calbiochem) and then incubated with specific antibodies. After 16 h of incubation, the immunocomplexes were recovered with the aid of Gamma-Bind Sepharose beads (Amersham Pharmacia Biotech) and then washed four times with lysis buffer. Subsequently, immunoprecipitates were analyzed by Western blotting after SDS-PAGE (10%), electroblotted onto polyvinylidene difluoride membranes (Novex, Inc.), and probed with the corresponding rabbit antisera or mouse monoclonal antibody. Immunocomplexes were visualized by enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech) using goat anti-rabbit or anti-mouse antisera conjugated to horseradish peroxidase as a secondary antibody (Pierce).

**Immunocomplex Kinase Assays**—Immunocomplex kinase assays were carried out as described previously (48). Specifically, cellular p38-δ or p38-α proteins were immunoprecipitated by incubation with anti-Flag M2b and protein A-agarose beads (Bio-Rad) in WCE lysis buffer. After 3 h of incubation at 4 °C, the immunoprecipitates were collected and washed twice with WCE lysis buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-Cl, pH 7.6, and 0.1% Triton X-100), and twice with kinase buffer (20 mM Mops, pH 7.6, 2 mM EGTA, 10 mM MgCl2, 1 mM dithiothreitol, 0.1% Triton X-100, and 1 mM Na3VO4). Pellets were then mixed with 5 µg of substrate, 20 µCi of [γ-32P]ATP, and 15 µM unlabeled ATP in 30 µl of kinase buffer. The substrates included MBP, GST-c-Jun, GST-MAPKAP kinase-2, PHAS-1, and ATF-2-(1–96) in the absence or presence of p38-α inhibitor (SB203580). The kinase reaction was performed for 30 min at 30 °C and terminated by boiling in an equal volume of Laemmli sample buffer, and the products were resolved by SDS-PAGE (10%). The gel was dried and subjected to autoradiography.

**Phosphoamino Acid Analysis**—The phosphorylated proteins obtained from immunocomplex kinase assays were analyzed using electrophoretically to polyvinylidene difluoride membranes. The spots containing phosphoproteins on the membranes were excised according to the bands on autoradiograms and then hydrolyzed in 50 µl of 6 N HCl for 1 h at 110 °C. The supernatant was lyophilized and dissolved in 6 µl of pH 1.9 buffer (2.2% formic acid and 7.8% acetic acid) containing cold phosphoamino acids as markers. The phosphoamino acids were resolved
electrophoretically in two dimensions using a thin layer cellulose (TLC) plate with two pH systems as described (49). The markers were visualized by staining with 0.2% ninhydrin in acetone, and the 32P-labeled residues were detected by autoradiography.

Lymphocyte Culture and Microscope Slides Preparation—Lymphocytes isolated from human blood were cultured in -minimal essential medium supplemented with 10% fetal bovine serum and phytohemagglutinin at 37 °C for 68–72 h. The lymphocyte cultures were treated with bromodeoxyuridine (0.18 mg/ml, Sigma) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block and recultured at 37 °C for 6 h in -minimal essential medium with thymidine (2.5 mg/ml, Sigma). The cells were harvested, and the cell slides were prepared by using standard procedures including hypotonic treatment, fixation, and air-drying.

Chromosome Mapping by Fluorescence in Situ Hybridization (FISH)—The procedure for FISH detection was performed as described previously (50, 51). Briefly, the cell slides were baked at 55 °C for 1 h. After RNase treatment, the slides were denatured in 70% formamide in 23 SSC for 2 min at 70 °C followed by dehydration with ethanol. DNA probes were labeled with biotinylated dATP at 15 °C for 1 h, using the Life Technologies, Inc., BioNick labeling kit (Life Technologies, Inc.). Probes were denatured at 75 °C for 5 min in a hybridization buffer containing 50% formamide and 10% dextran sulfate and loaded onto the denatured chromosomal slides. After 16–20 h hybridization, the slides were washed and incubated with fluorescein isothiocyanate-conjugated avidin (Vector Laboratories), and the signal was amplified as described (51). FISH signals and the 4',6-diamidino-2-phenylindole (DAPI) banding patterns were recorded separately by taking photographs, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with the DAPI-banded chromosomes (52).

RESULTS

Molecular Cloning and Structure of Murine and Human P38-δ cDNAs—A 1328-bp partial cDNA sequence with high homology (62% amino acid identity) to the kinase domain of mouse p38-δ was identified from the Amgen EST database of a rat colon cDNA library. Initially, we termed this cDNA an IKK-like kinase. By using this rat cDNA as a probe, we have isolated a putative full-length cDNA clone from a rat lung cDNA library. The nucleotide sequence of 1577 bp contains a single open reading frame of 1098 bp encoding a protein that shares 62% amino acid identity with mouse p38-δ.

**Fig. 1.** Nucleotide and amino acid sequences of murine p38-δ cDNA and sequence alignment. A, the nucleotide and predicted amino acid sequences of murine p38-δ are shown. The predicted amino acid sequence is indicated below the first nucleotide of each codon, and the termination codon is marked with an asterisk. The polyadenylation signal is underlined. GenBank accession numbers for murine and human p38-δ are AF092534 and AF092535, respectively. B, alignment of the deduced amino acid sequences of murine and human p38-δ (mp38-δ and hp38-δ) with those of human p38-γ (hp38-γ, GenBank accession number X79483), murine and human p38-β (mp38-β and hp38-β, GenBank accession numbers D83073 and U66243, respectively), and human p38-α (hp38-α, GenBank accession number L35264). The sequences (single letter codes) were aligned with the Bestfit program of the GCG sequence analysis software package. Gaps were introduced to obtain optimal alignment and are denoted by dashes. Identical amino acids among at least five proteins are highlighted with solid boxes. Roman numerals on the top line denote the 12 conserved kinase subdomains identified by Hanks and Quinn (55). The bottom consensus sequence indicates amino acids that are invariant (uppercase) or almost invariant (lowercase) in a comparison of the catalytic domains of 100 Ser/Thr protein kinases (56). The asterisks highlight the fully conserved TGY motif within the kinase subdomain VIII.
polypeptide of 366 amino acids, and followed by a 471-bp 3' untranslated region that contains the polyadenylation signal at position 1512 (Fig. 1A). The calculated molecular mass of the deduced amino acid sequence is about 41 kDa. A homology search of the GenBank® data base revealed that the coding sequence of this clone is very similar with those of p38-α (8), p38-β (42), and p38-γ (43, 44), and designated as murine p38-δ.

By using the murine p38-δ cDNA as a probe, we have also isolated a putative full-length cDNA clone from a human lung cDNA library. The nucleotide sequence of 1794 bp contains a single open reading frame of 1095 bp encoding a polypeptide of 365 amino acids and followed by a 678-bp 3′-untranslated region that contains the polyadenylation signal at position 1749 (data not shown). Sequence alignments showed that the deduced amino acid sequences of human and murine p38-δ exhibit 92% identity, and p38-δ is approximately 63, 61, and 67% identical to p38-α, p38-β, and p38-γ, respectively (Fig. 1B).

The putative dual phosphorylation TGY motif within the kinase subdomain VIII is fully conserved among the known mammalian p38 family members.

Expression of P38-δ Is Regulated in Different Developmental Stages—The expression of p38-δ was examined in a variety of mouse adult tissues by Northern blot analysis. A tissue Northern blot was probed with the murine p38-δ cDNA, and a major p38-δ transcript (~3 kilobase pair) was identified in the lung, testis, kidney, and at lower levels in the liver and skeletal muscle (Fig. 2). Furthermore, we examined the expression of p38-δ mRNA in various days of mouse embryos and adult tissues by in situ hybridization using a 35S-labeled antisense p38-δ RNA probe, followed by autoradiography. Whereas p38-δ was expressed predominantly in the developing gut and the septum transversum in the mouse embryo at 9.5 days (Fig. 2A), its expression began to be localized to the gut, heart ventricle, neuroepithelium of the fourth ventricle of the brain, cochlea, and semicircular canal of the inner ear and oropharynx in the 12.5-day embryo (Fig. 2C). At 15.5 days, the expression of p38-δ was further expanded to the adrenal gland, duodenum, intestine, epidermis, kidney, and lung thalamus (Fig. 2, D and E). P38-δ was expressed virtually in most developing epithelia in embryos, suggesting that p38-δ is a developmentally regulated MAPK that may play a role in embryonic development.

FIG. 2. Expression pattern of murine p38-δ mRNA. Poly(A)+ RNAs from the indicated mouse adult tissues were prepared for Northern blot analysis and probed with the murine p38-δ cDNA (upper panel). As a control, the same blot was re-probed with a β-actin cDNA to check the integrity of the RNA (bottom panel).

FIG. 3. In situ hybridization (ISH) analysis of murine p38-δ mRNA expression in mouse embryos and adult tissues. A, ISH of a sagittal section of a 9.5-day embryo (5 × magnification) shows strong expression of p38-δ in the primitive foregut (a), septum transversum, which is the future site of liver development (b), and the ventricle of the primitive heart (c). B, ISH using a sense control probe shows the level of background in the same sagittal section as described in A (5 × magnification). C, ISH of a frontal section of a 12.5-day embryo (2.5 × magnification). Intense labeling is observed in the gut (d), and significant signals are found in the heart ventricle (e), the neuroepithelium of the fourth ventricle of the brain (f and g), the cochlea of the inner ear (h), the semicircular canal of the inner ear (i), and the oropharynx (j). D, ISH of a sagittal section of a 15.5-day embryo abdomen (2.5 × magnification). Labeling is observed in the adrenal gland (k), the duodenum (l), the kidney (m), the small intestine (n), the large intestine (o), and the epidermis (p). E, ISH of a sagittal section of a 13.5-day embryo abdomen and thorax (2.5 × magnification). Strong signals are found in the epidermis (q), the intestine (r), the lung (s), and the kidney (t). F, ISH using a sense control probe shows the level of background in the same sagittal section as described in E (2.5 × magnification).
other positive locus detectable under the condition used; therefore, the gene of p38-d was mapped to mouse chromosome 17, region A3-B (Fig. 4C).

Similarly, the biotinylated human p38-d cDNA probe was used to map the human chromosome. A specific region of one chromosome showed the FISH positive with the p38-d probe (Fig. 5A), and the hybridization efficiency was approximately 70% for this probe. The assignment between signal from the probe and the short arm of chromosome 6 was established (Fig. 5B), and the detailed position was further determined to region p21.3. Since there were no other positive loci detected under the condition used, the gene of p38-d was localized to human chromosome 6, region p21.3 (Fig. 5C).

**P38-d and P38-a Differ in Substrate Specificity**—To investigate whether p38-d and p38-a share substrate specificity, we transfected either the Flag-tagged human p38-a or the Flag-tagged murine p38-d cDNA into 293T cells and prepared lysates from transfected cells in the presence of H2O2 stimulation. After immunoprecipitation with anti-Flag M2 mAb, the p38-a or p38-d kinase activity was determined by an immunocomplex kinase assay, using myelin basic protein (MBP), GST-c-Jun, GST-MAPKAP kinase-2, PHAS-1, and ATF-2-(1–96) as substrates with or without p38-a inhibitor. The immunocomplex kinase assay detected marked phosphorylation of PHAS-1 and ATF-2 by p38-a but little phosphorylation of PHAS-1 and ATF-2 in the presence of p38-a inhibitor (Fig. 6A). In contrast, p38-d phosphorylated PHAS-1 and ATF-2 strongly in the absence or presence of p38-a inhibitor (Fig. 6B). Although p38-a phosphorylated MAPKAP kinase-2 significantly but not GST-
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Fig. 6. p38-δ and p38-α are serine/threonine kinases but differ in substrate specificity. 293T cells were transfected with either the Flag-tagged human p38-α or the Flag-tagged murine p38-δ cDNA (10 μg each). pVA1 plasmid (10 μg) containing adenovirus VA1 RNA gene was also included in each transfection to enhance transient protein expression. The cells were harvested 48 h after transfection without stimulation. After immunoprecipitation with anti-Flag M2 mAb, p38-α (A) or p38-δ (B) kinase activity was measured by immunocomplex kinase assays in the presence of the indicated substrates. The in vitro phosphorylated ATF-2-(1–96) by p38-α (C) or p38-δ (D) were gel-isolated, and phosphoamino acids were analyzed electrophoretically in two dimensions using a TLC with two pH systems. The relative positions of unlabeled phosphoamino acids are indicated below the autoradiographs. S, serine; T, threonine; Y, tyrosine.

DISCUSSION

We have identified and isolated a novel murine and human p38 MAPK family member, p38-δ, whose sequence is most similar to p38-γ (67% amino acid identity) among the p38 family members, whereas p38-α is most homologous to p38-β (75% amino acid identity). It is intriguing that expression of p38-δ was primarily in the developing gut and the septum transversum in the early mouse embryo (at 9.5 days) initially, then its expression began to be expanded to many specific tissues of the later embryo (at 12.5 days). At 15.5 days, p38-δ was expressed virtually in most developing epithelia in embryos, suggesting that p38-δ is a developmentally regulated MAPK that may play a role in embryonic development. Since all four p38 MAPK family members are closely related in structure and function, it is possible that expression of p38-α, β, and γ may also be regulated in different developmental stages. They may play important roles in stress and inflammatory responses in different tissues during embryonic development.

We elected to show different sectional perspectives for embryos at days 9.5, 12.5, and 15.5, rather than presenting a common sectional perspective because different sectioning orientations are advantageous to view certain developing tissues that grow at different rates and change orientation as development proceeds. Additionally, different sectioning orientations provide depth of the labeling for a more comprehensive study. Interestingly, expression of p38-δ correlated with areas of epithelial development in the gut, kidney, adrenal, lung, and skin. The p38-δ mRNA expression was also detected in some neurons that are derived from the ectoderm. Overall, the pattern of expression suggests that p38-δ is expressed in the
proliferating and nonproliferating layers of epithelia.

Since the kinase domains of all four p38 family members are very conserved, it is of interest to test whether they share the same substrate specificity. It has been shown that p38-α and p38-β phosphorylate similar substrates including ATF-2, PHAS-1, and MAPKAP kinases (42). Although p38-γ can also phosphorylate ATF-2, it cannot phosphorylate MAPKAP kinases effectively (44). Here, we showed that p38-δ phosphorylated ATF-2 and PHAS-1 strongly but not MAPKAP kinase-2 which is a physiological substrate for p38-α. This result suggests that p38-δ shares substrate specificity with p38-α. Thus, in terms of sequence similarity and substrate specificity, p38-δ most resembles p38-γ, whereas p38-β most resembles p38-α. In addition, p38-δ differs from p38-α in phosphorylation specificity against serine residues. We showed that p38-α phosphorylated serine and threonine, whereas p38-δ phosphorylated threonine predominantly, suggesting that p38-δ is primarily a threonine kinase and is dissimilar with p38-α in phosphorylation specificity against serine residues.

It has been shown that p38 MAPKs are activated by dual phosphorylation at the Thr180-X-Tyr182 motif within the kinase subdomain VIII (17). P38-δ contains the dual phosphorylation TGY motif that is fully conserved among all four p38 MAPK family members. Mutation of the Thr180 and Tyr182 residues in this TGY motif abrogated the p38-δ kinase activity and its activation by extracellular stimuli or upstream kinases (MKKs). Therefore, the dual phosphorylation TGY motif is indispensable for the kinase activity and activation of p38-δ. Similar to other p38 MAPKs, p38-δ is activated by environmental stress and proinflammatory cytokines. This activation is presumably regulated by dual phosphorylation on Thr180 and Tyr182. P38-δ is also activated by its upstream kinases (MKKs) which may phosphorylate Thr180 and/or Tyr182 in the TGY motif. However, it is unclear whether all these upstream kinases (MKK-3, -4, -6, and -7) phosphorylate the TGY motif of p38-δ in the same manner.

It has been shown that p38-α is preferably activated by MKK-3 in PC-12 cells, whereas p38-α is predominantly activated by MKK-6 in monocytes and KB cells, suggesting that p38-α is activated by different MKKs in a cell type-dependent manner (44). Since MKK-1, -2, and -5 are specific activators for ERKs (24, 25, 34, 35), we examined the other MKKs on p38-δ activation. Unlike p38-α, we found that p38-δ was activated by MKK-3, -4, -6, and -7 approximately equally well in 293T cells, suggesting that the regulation of p38-δ may be distinct from p38-α. However, it is still unknown whether or not the regulation of p38-δ depends on cell type. Additionally, p38-δ was activated in response to a variety of stimuli including environmental stress, TNF-α, IL-1α, and EGF. Although most of these factors stimulated p38-δ to a relatively similar degree in 293T cells, it is possible that the kinase reaction was not in a linear range and ATF-2 might not be the physiological substrate for p38-δ. Therefore, the degrees of stimulation of these factors may not reflect their physiological effects on p38-δ in vivo. Although activation of p38-δ by EGF may be somewhat surprising, it has been shown recently that p38 MAPK (p38-α) can be stimulated by EGF in certain cell types (53, 54). Thus, further investigation of these factors is required to understand fully the physiological activators of p38-δ.

p38-α (CSBP) has been implicated in the regulation of inflammatory cytokine biosynthesis through the use of specific p38-α inhibitors (8). We examined one of the p38-α inhibitors in our phosphorylation studies and showed that it was ineffective in blocking p38-δ activity. Further investigation of other compounds that may inhibit p38-δ function is necessary to understand whether p38-δ is involved in the regulation of inflammatory cytokine production in cells.

We showed that the gene of p38-δ was localized to mouse chromosome 17 region A3-B and human chromosome 6p21.3. Interestingly, the gene of p38-α (CSBP) has also been mapped to human chromosome 6p21.3/21.2 (52). To our knowledge, this is the first description of chromosomal localization of p38-δ. At present, it is unknown whether mutation or deletion of p38-δ gene is involved in any diseases. Mutation analysis in mice or human with monogenic disorders that map to mouse chromosome 17A3-B or human chromosome 6p21.3 will evaluate the
involvement of this gene in diseases. Furthermore, targeted disruption (knock-out) of this gene in mice may provide evidence for the relationship between its function and diseases.

In summary, we have isolated and characterized p38-δ and determined its global tissue distribution, chromosomal localization, and its biological activity. Further investigation of the regulation of p38-δ may contribute to a better understanding of the roles that p38-δ have in normal development and pathological processes.

Acknowledgments—We thank B. Sutton for DNA sequencing; Dr. H. Heng for assisting in FISH; and Drs. L. Souza, R. Bosselman, and W. Boyle for their support.

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