Characterization of the Structure and Function of the Fourth Member of p38 Group Mitogen-activated Protein Kinases, p38δ

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We have cloned and characterized a new member of the p38 group of mitogen-activated protein kinases here termed p38δ. Sequence comparisons revealed that p38δ is approximately 60% identical to the other three p38 isoforms but only 40–45% to the other mitogen-activated protein kinase family members. It contains the TGY dual phosphorylation site present in all p38 group members and is activated by a group of extracellular stimuli including cytokines and environmental stresses that also activate the other three known p38 isoforms. However, unlike the other p38 isoforms, the kinase activity of p38δ is not blocked by the pyridinyl imidazole, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole (identical to SB202190). p38δ can be activated by MKK3 and MKK6, known activators of the other isoforms. Nonetheless, in-gel kinase assays provide evidence for additional activators. The data presented herein show that p38δ has many properties that are similar to those of other p38 group members. Nonetheless important differences exist among the four members of the p38 group of enzymes, and thus each may have highly specific, individual contributions to biologic events involving activation of the p38 pathways.

Mitogen-activated protein kinases have been shown to play an important role in transducing extracellular signals into cellular responses (1, 2). There are at least four groups of MAP1 kinases that have been identified in mammalian cells; these can be categorized by the sequence of the canonical dual phosphorylation site threonine-Xaa-tyrosine (TXY) or other conserved features within the primary amino acid sequence. In mammalian cells the MAP kinase groups currently include ERK1/2 (Xaa = Glu), JNK or SAPK (Xaa = Pro), Erk5/BMK (Xaa = Glu), and p38 group (Xaa = Gly) (2–11). There are numerous examples supporting the contention that each MAP kinase pathway has unique regulatory features; nonetheless coordinate activation of multiple pathways initiated with the same stimulus often occurs (6, 12, 13). We were the first to describe the structure and some functions of p38 MAP kinase, and we suggested a potential role in regulating host response to phlogistic stimuli (6, 7). This was further supported by the studies of Lee et al. (14) showing that p38 activity is inhibited by a class of pyridinyl imidazoles that includes the prototypic compound SB 202190 (here termed FHP1); such compounds block a variety of biological responses associated with inflammation including production of proinflammatory cytokines (14). Two MAP kinase isoforms, MKK3 and MKK6, have been identified as the dual specificity kinases that activate p38 (15–20). Moreover, a group of substrates for p38 has been identified including MAPKAPK2/3 (8, 9, 21, 22), ATP2 (7, 23), CHOP10 (24), and as we have reported, MEF2C (25).

During the past year two additional members of the p38 group of enzymes have been described by us and others (23, 26–29). Each of these isoforms contains a TGY dual phosphorylation site, and amino acid sequence comparisons revealed 60% identity across the entire sequence. Of the three known isoforms p38α (also known as CSBP, RK) and p38β ubiquitously expressed, whereas p38γ (also known as ERK6 and SAPK3) expression is most prominent in muscle (23, 26–29). Several splicing variants of p38β and p38δ have been reported, but the role of differential splicing of these molecules is at present unknown (14, 30, 31). Comparative studies revealed similarities among this group of enzymes; each is activated by MKK3 and MKK6 and inhibited by FHP1 (14, 23, 27). Interestingly these isoforms appear to have different substrate specificities. For example, p38δ phosphorylates and up-regulates the trans-activation activity of ATF2 more than 20-fold more effectively than does p38 (23, 27).

Here we describe the molecular cloning and characterization of a fourth member of p38 group MAP kinase which we termed p38δ. Like the other p38 isoforms, p38δ has the TGY dual phosphorylation site. A variety of studies show that many of the properties of p38δ are very similar to those of the other three p38 isoforms. In contrast we have noted some important differences between p38δ and other p38 isoforms including a lack of sensitivity to the inhibitory properties of pyridinyl imidazoles and the existence of MKKs with properties distinct from MKK3 or MKK6 which can activate p38δ. The information provided herein establishes the basis for further studies of the function of this important group of enzymes.

MATERIALS AND METHODS

p38δ cDNA Cloning—EST clones (GenBank™ number W53837 and (GenBank™ number W13523) were obtained from Research Genetics (Huntsville, AL). Oligonucleotides were used for 5’-rapid amplification of cDNA ends of p38δ cDNA from a liver cDNA ligated with an adapter.
p38 MAP Kinase

30123

(derived from CLONTECH, Palo Alto, CA) as follows: 30 cycles of PCR were performed for 1 min at 55 °C for primer-template annealing, 1 min at 72 °C for chain elongation, and 30 s at 95 °C for strand separation. A TA cloning kit (Novagen, Madison, WI) was used to clone the PCR products. cDNA clones were sequenced using a model 373A automated sequencer (Applied Biosystems, Foster City, CA). Human p38 was cloned from a brain cDNA using the same method.

**Northern Blots**—A tissue blot containing 2 µg of poly(A)⁺ RNA isolated from different human tissues was purchased from CLONTECH (San Francisco, CA). The blot was hybridized as described (15, 25) to a probe prepared by labeling the coding region of p38 cDNA with [³²P]ATP using random priming (32). Western Blots—Total cell lysates were prepared using a lysis buffer A: 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 10% glycerol, 1 mM Na₃VO₄, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100. Equal protein loading of cell extracts in SDS-PAGE was determined by Bio-Rad protein assay solution (Bio-Rad) and by staining the transferred nitrocellulose membrane with Ponceau S solution (Sigma). Standard Western blot methods were used. Rabbit polyclonal antibodies raised against C-terminal peptide of p38 (VISFPVPPDLQEMES) or p38y (FKPPRQLGARVSKEPTL) and recombinant protein of p38y of p38w were used in the Western blot. Each of these antibodies has been tested to have specificity to one of the p38 isoforms (data not shown).

cDNA Constructs and Expression Plasmids—A p38 double mutant (p38ΔAF) was created by substituting Thr¹⁸⁰ with Ala and Tyr¹⁸² with Phe using a PCR-based procedure (33). The Flag epitope tag DYD- DDK was added to the N-terminal region by PCR as described (23). The resultant cDNA was cloned into the mammalian expression vector pCDNA3 (Invitrogen, San Diego, CA).

**Recombinant Proteins**—The expression of p38y or p38y proteins in COS-7 cells was achieved by transient transfection of vectors containing Flag-tagged p38y, p38y, p38y, or p38 DNA, and the protein were immunoprecipitated as described using M2 beads (anti-Flag monoclonal antibody) (7). Western blot analysis were performed with M2. Glutathione S-transferase (GST) fusion proteins of the N-terminal portion of ATF2(1–109) (15), c-Jun(1–93) (4), ELK1(307–428) (34), c-MyC(1–149) (35) were prepared as described (23). PHA-I was first chased from Stratagen (San Diego, CA). MBP was from Sigma.

**Protein Kinase Assays**—In vitro kinase assays were carried out at 37 °C for 30 min using ~0.2 µg of recombinant kinase, 5 µg of kinase substrate, 250 µM ATP, and 10 µCi of [γ-³²P]ATP in 20 µl of kinase reaction buffer. Reactions were terminated by the addition of Laemmli sample buffer. Reaction products were resolved by 12% SDS-PAGE, and the extent of protein phosphorylation was visualized by autoradiography.

**Enzymatic Activity of p38**—Activated p38 was assayed on native gel using recombinant p38 or p38 by washing the gel with a fixing solution containing 10 mM sodium pyrophosphate and 5% trichloroacetic acid. The gel was dried and subjected to autoradiography.

**RESULTS AND DISCUSSION**

**Cloning of p38y cDNA**—To identify new members of the p38 group of MAP kinases, the EST division of GenBank data base was searched with TBLASTN program. Two sequences of 484 and 603 base pairs, respectively (GenBank number W53837 and W13523), were found encoding a polypeptide with high degree of similarity to p38. We obtained and completely sequenced these two clones. The first clone with ~1-kilobase pair cDNA from a mouse embryo library covers part of the coding region and the 3′-untranslated region (base pairs 372–1363). The second clone from a murine library contains part of coding region and 3′-untranslated region (base pairs 714–1363). Sequence information indicated that the second clone contained an overlapping portion of the first clone. Data base sequence comparisons indicated the clones encoded a novel protein that is most closely related to the p38 group of MAP kinases. We therefore termed this protein p38y since three other isoforms of p38 have already been described (23, 26–28). An adaptor-ligated murine liver cDNA was used to amplify a 5′-portion of p38y. A 450-base pair fragment was produced and subcloned into pT7blue vector. Four clones were randomly picked up and sequenced. The clones have identical sequence that overlapped with p38y. Human p38y cDNA was obtained from a adaptor-ligated human brain cDNA library by using PCR. Because we did not find an in-frame stop codon in the 5′-end portion of the cDNAs, we have tentatively predicted the first ATG as the starting codon. The validity of this prediction is supported by comparing the sequence of human and mouse p38y clones, since the coded amino sequence 5′ upstream of the first ATG is not conserved (27% identity) between human and mouse, whereas the coding sequence after this predicted starting codon is highly conserved (93% identity). Furthermore, the molecular mass of endogenous p38y (38 kDa) determined by Western blotting agrees very well with our predicted size of p38y (see Figs. 1 and 2). The cDNAs of human and murine p38y encode 265 and 366 amino acid polypeptides, respectively (Fig. 1B). Common structural features of p38 group MAP kinases such as the TGY dual phosphorylation site between kinase domain VII and VIII and the short length of linker loop 12 are found in p38y (Fig. 1A). p38y is 61, 59, and 65% identical to p38, p38y, and p38y, respectively, and in contrast the identity between p38y and other group MAP kinases was much lower. For example, p38y is 44, 45, and 41% identical to ERK1, JNK1, and BMK1/ERK5 respectively. Computer analysis using a progressive pair-wise comparison supported our notion that p38y should classified as a member of p38 group of MAP kinases (Fig. 1C). Human and murine p38y are 93% identical at the amino acid level.

**Examining 38y and 38 Activities in Rat Anti-glomerular Basement Membrane Glomerulonephritis Model**—Induction of anti-glomerular basement membrane glomerulonephritis in Wistar-Kyoto rats has been described in previous publications (36). Anti-glomerular basement membrane antibody was kindly provided by Dr. Tadashi Yamamoto (Kyoto, Japan). Inbred female Wistar-Kyoto rats, aged 12–16 weeks, were purchased from Charles River Laboratories (Wilmington, MA). The experimental group of rats received 100 µl of the anti-glomerular basement membrane antiserum intravenously per 100 g body weight, and the control group of rats received the same amount of premunme serum. Experimental and control rats were sacrificed 8 weeks after injection to obtain the kidneys. One kidney was divided into two parts. The first part was fixed by formalin and stained with periodic acid-Schiff. The renal cortex of the second part was separated and homogenized in lysis buffer A; a soluble fraction was isolated by centrifugation (15,000 × g) and used further in analysis of kinase activity using an immunokinase assay. Immunoprecipitation of p38 or p38y was accomplished using rabbit polyclonal antibodies raised against recombinant p38 or p38y. In-gel Kinase Assay—10 µg of lysates of cells treated with different stimuli were separated by SDS-PAGE using a gel polymerized with 0.5 mg/ml kinase inactive p38 or p38y. The separated proteins were denatured and renatured as described (37). The kinase reaction was performed in 5 ml of kinase buffer described above containing 50 µM ATP and 50 µCi of [γ-³²P]ATP for 1 h at 30 °C. The reaction was terminated by washing the gel with a fixing solution containing 10 mM sodium pyrophosphate and 5% trichloroacetic acid. The gel was dried and subjected to autoradiography.
compared, p38 demonstrated about 3-fold more activity than p38 for ATF-2 (Fig. 3).

The pyridnyl imidazole derivative FHPI (SB202190) has been shown to inhibit the enzymatic activity of p38, p38β, and p38γ (14, 23, 27), and in contrast there has been no effect on the activity of ERKs or JNKs. Interestingly, the activity of the fourth member of the p38 group of MAP kinases is not inhibited by this compound. Similar results were obtained by using either activated p38 and p38 transiently expressed in COS-7 cell treated with anisomycin (Fig. 4) or recombinant enzymes expressed in bacteria (data not shown). This suggests that the activity of p38 must be considered when interpreting effects of inhibitors such as FHPI on cellular responses to various stress stimuli considered to activate the p38 group of enzymes.

**Regulation of p38 Activity**—To begin to define the functional characteristics of p38, we next evaluated a panel of stimuli for their ability to regulate this kinase. We and others (6, 7, 23, 26, 27, and 44) have shown that p38, p38β, and p38γ can be activated by diverse extracellular triggers such as cytokines, products of microbial pathogens, and changes in the physical-chemical properties of the extracellular medium. We transiently expressed Flag-tagged p38 and p38 in 293 cells. p38 and p38 were similarly activated when the 293 cells were treated with anisomycin, arsenite, increased extracellular os-
molarity, platelet-activating factor, H2O2, tumor necrosis factor, interleukin-1, interleukin-6, or UV (Fig. 5, upper panel). In contrast serum, insulin, and EGF had minimal effects on p38 activation. We established that equal amounts of p38δ or p38 protein were present in each kinase assay (Fig. 5, bottom panel).

Increased extracellular osmolarity has been shown to activate p38 group members in yeast as well as in mammalian cells (7, 38). Thus we compared the activation of p38δ and p38 when the extracellular osmolarity was altered to include both hypo- and hyperosmolar conditions. Interestingly, p38 is activated more vigorously than p38δ under hypoosmolar conditions; both isoforms were activated when the extracellular osmolarity was increased above 400 mOsm (Fig. 6).

To investigate further the regulation of p38δ in vivo, we examined activities of p38δ and p38 in the renal cortex of normal rats and the rats with crescentic glomerulonephritis. The anti-GMB glomerulonephritis model had been well characterized and documented previously (36, 39, 40). Rats receiving anti-GMB antibody developed reproducible severe glomerulonephritis with proteinuria and decreased renal function which progressed to glomerulosclerosis and interstitial fibrosis (41). As shown in Fig. 7A, 8 weeks after injection of anti-GMB antibody, glomerular sclerosis is advanced with marked extracellular matrix accumulation and decreased resident glomerular cells in sclerotic regions. p38δ and p38 protein were immunoprecipitated from the renal cortex extracts using specific antibodies to each of these two isoforms. Kinase activity in the immunoprecipitates was determined using ATF2 as substrate (Fig. 7B). Equal amounts of p38δ or p38 protein in the immunoprecipitates from control and experimental animals were determined by Western blot analysis (Fig. 7B, bottom line). The activities of p38δ and p38 in the renal cortex of experimental rats were 26- and 13-fold higher than control rat (Fig. 7B, top line). It is not possible at this time to interpret the meaning of the up-regulation of p38δ and p38 activity in the pathogenesis of glomerulonephritis. Nonetheless, these data suggest that the p38 pathway is subject to activation in tissues with ongoing immunologic disease processes.

Activation of p38δ by MKKs—The dual specificity kinases MKK3 and MKK6 appear to be principally responsible for activation of p38δ in cells (19, 42); MKK4 may also activate p38, but this does not appear to be a significant pathway under physiological conditions (43). To determine which MKKs regulate p38δ, we measured the p38δ kinase activity when it was co-expressed with MEK1, MKK3b (47-kDa form), MKK4, MKK5, or MKK6b (39-kDa form) in 293 cells. As shown in Fig. 8, co-expression of MKK3b and MKK6b leads to an increase of p38δ activity (~5–6-fold) that is comparable to the activation of p38. p38δ and p38 are also activated by MKK4 but to a lesser extent.
extent when compared with MKK3b and MKK6b under the same conditions in a co-transfection assay. MKK4 is consistently a better activator for p38 than p38 in our experimental system, and this may be an indication that the two isoforms are differentially regulated. No activation of p38 was observed when MEK1 and MEK5 were co-expressed. Taking together, these data suggest that MKK3 and MKK6 are likely to have important roles for activation of p38 under physiologic conditions.

To study further the activation of p38, we used an in-gel kinase assay to compare the major kinases for p38 and p38 in 293 cells. Stimulation of 293 cells with anisomycin, arsenite, and tumor necrosis factor leads to activation of a 39-kDa protein as the major kinase that phosphorylates p38 (Fig. 9A).

Lesser activity is observed with proteins migrating at ~80 and 47 kDa. In contrast, the major kinases for p38 in the same cell lysates are 47-kDa and 80-kDa proteins (Fig. 9B). These data suggest that regulation of p38 and p38 activation may occur via distinct MKKs insofar as 293 cells are concerned. Further investigations will establish the identities of these kinases.

To confirm if regulation of p38 activity occurs through the dual TGY phosphorylation site, the kinase activity of p38 and p38 isolated by immunoprecipitation was measured with MBP as substrate. The levels of Flag-tagged protein in the cell lysates were determined by Western blotting. Comparable results were obtained in two experiments.

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were compared included distinct substrate specificities and the possibility of differential activation by MKKs as revealed by in-gel kinase assays. The totality of the data provided herein as well as information already published in previous studies of the other p38 isoforms (23, 27, 44) supports the contention that these closely related p38 isoforms are not simply redundant but have specific functions that are determined by both the specificity of the upstream activators and the identities and functions of preferred downstream substrates.

The finding that FHPI does not have an effect on p38 activity may provide a useful lead toward fully understanding how this class of potential drugs exerts its action. It is likely that the binding site for FHPI present in the three other p38 isoforms, which are sensitive to FHPI, is different in p38δ, although this will require experimental proof to verify this contention. A recent report has shown that a compound closely related to FHPI, SB203580, does not inhibit one p38 isoform identical to p38γ (termed SAPK3 from rat) (44). These findings do not agree with our published studies of p38γ and may result from subtle differences in sequence of the isoforms in different mammalian species. Further studies are required to clarify these conflicting sets of findings related to sensitivity of p38 isoforms to pyridinyl imidazoles. A careful analysis of sequence differences among the four isoforms of p38 and the difference between the species might provide some insights into binding sites for the pyridinyl imidazoles. Such information would become useful in the design of mutagenesis strategies to localize the drug binding site and to design drugs with more selections.

The dual specificity kinases that activate the p38 isoforms are key factors in regulating the activity of these enzymes. There is no significant difference observed in the activation of p38δ and p38γ and other p38 isoforms by MKK3 or MKK6 in a co-transfection assay. However, in-gel kinase assays provided evidence that the phosphorylation of p38δ and p38γ might be carried out by distinct enzymes (Fig. 9). Although the 39- and 47-Da protein may correspond to MKK6b and MKK3b, respectively, there appears to be an activable kinase that has a mobility of 80 kDa. At present we are not able to assign the exact identity of these kinases and to determine if the phosphorylation of p38δ or p38γ by these kinases leads to their activation. We are currently investigating how the endogenous p38δ isoforms are activated and what roles MKK3 and MKK6 play in endogenous activation pathways. There are a number of examples that support the contention that the endogenous pathways used by MAP kinases are different than those identified from either in vitro kinase assays or from co-transfection strategies which may often change the balance of protein concentrations due to overexpression (15, 43).

Conservation of the p38 MAP kinases across a very broad evolutionary span from yeast to mammalian cells suggests that this MAP kinase family regulates important cellular functions. The growing number of p38 isoforms in mammalian cells suggests a role in increasing complexities of function during evolution. The yeast p38 counterpart Hog1 or Sck1/ Sty1 have been shown to play a role in osmoregulation and to have responses to a variety of extracellular stress stimuli, cell-cycle events (38, 45, 46), etc. It would be reasonable to predict that p38 isoforms evolved from a single gene might carry the function derived from their ancestor. Indeed, all p38 isoforms identified so far can be activated by high osmolarity as well as by a variety of other stress signals generated in the extracellular environment. Although this suggests functional redundancy may exist, our findings of the existence of multiple isoforms with different tissue distribution support the notion of functional differentiation of this group of MAP kinases in mammals. The differences we have noted regarding the substrate selectivity of different p38 isoforms demonstrated that although all of the isoforms respond to a similar panel of stimuli, the consequences of activation of each p38 isoform may be different. With all of our current tools we now are in a position to define exactly how each isoform participates in cellular responses to different extracellular stimuli.

Acknowledgments—We thank Dr. J.-D. Lee for MEK5 expression plasmid and Betty Chastain for excellent secretarial assistance.

Addendum—While we were revising this manuscript, Goedert et al. (Goedert, M., Cuenda, A., Craxton, M., Jakes, R., and Cohen, P. (1997) EMBO J. 16, 3565-3571) and Kumar et al. (Kumar, S., McDonnell, P. C., Guo, R. J., Hand, A. T., Lee, J. C., and Young, P. R. (1997) Biochem. Biophys. Res. Commun. 235, 533-538) reported the sequence of a human kinase termed SAPK4 that is identical to p38δ. These two articles have shown that SAPK4 (p38δ) has similar in vitro substrate specificity with that of p38δ and can be activated by cytokines and cellular stresses.

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FIG. 9. The major kinase of p38δ and p38. In-gel kinase assays using p38δ (A) or p38 (B) polymerized inside SDS-PAGE. The cell lysates of 293 cells treated with or without stimuli for 20 min were analyzed. The major kinases of p38δ and p38 are different in the whole cell lysates.
p38 MAP Kinase

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