Protein Science (1997), *6*:242–245. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Crystallization and preliminary crystallographic analysis of recombinant human p38 MAP kinase

S. PAV,[1] D.M. WHITE,[1] S. ROGERS,[1] K.M. CRANE,[1] C.L. CYWIN,[1] W. DAVIDSON,[2] J. HOPKINS,[1] M.L. BROWN,[1] C.A. PARGELLIS,[1] AND L. TONG[1]

[1]Department of Inflammatory Diseases, Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road, P.O. Box 368, Ridgefield, Connecticut 06877
[2]Department of Analytical Chemistry, Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road, P.O. Box 368, Ridgefield, Connecticut 06877

(RECEIVED August 28, 1996; ACCEPTED September 30, 1996)

Abstract: The recombinant human p38 MAP kinase has been expressed and purified from both *Escherichia coli* and SF9 cells, and has been crystallized in two forms by the hanging drop vapor diffusion method using PEG as precipitant. Both crystal forms belong to space group $P2_12_12_1$. The cell parameters for crystal form 1 are a = 65.2 Å, b = 74.6 Å and c = 78.1 Å. Those for crystal form 2 are a = 58.3 Å, b = 68.3 Å and c = 87.9 Å. Diffraction data to 2.0 Å resolution have been collected on both forms.

Keywords: crystallization; mitogen-activated protein kinase (MAPK); p38; RK; serine/threonine kinase; X-ray diffraction

The members of the mitogen-activated protein kinase (MAPK) superfamily of enzymes act in parallel cascades to modulate intracellular responses to external signals. Extracellular stimuli such as osmolarity changes, heat shock, UV irradiation, and exposure to phorbol esters, growth factors, and inflammatory cytokines may elicit specific cellular responses including gene transcription, protein synthesis, cell growth and differentiation, motility, or apoptosis. Constituent MAP kinases are activated via sequential phosphorylation events on Thr and Tyr residues by an upstream dual-specificity kinase, and in turn phosphorylate downstream substrates on Ser or Thr adjacent to proline residues, thus activating downstream tiers of the cascades. Parallel pathways involving several hundred such enzymes have been found thus far in eukaryotic cells (Zhang, 1996), and individual components of these signaling networks continue to be elucidated (for reviews, see Cano & Mahadevan, 1995; Cobb & Goldsmith, 1995; Seger & Krebs, 1995). Whereas MAPK cascades in yeast regulate separate phenomena, mammalian cascades can be activated in parallel in response to the same stimulus via distinct cascades (Cano & Mahadevan, 1995). Both the unique and overlapping specificities of each cascade for distinct external signaling events have warranted closer investigations of the individual kinases involved as potential target sites for possible therapeutic intervention along these pathways.

Several laboratories have recently reported a new MAPK family member, named p38/CSBP/RK stress response kinase (Lee et al., 1994; Rouse et al., 1994; Raingeaud et al., 1995). The p38 MAP kinase has been isolated from human THP.1 cells in two highly homologous forms, CSBP1 and CSBP2, which have a sequence difference of fourteen amino acids (Lee et al., 1994). The sequence is 51% identical to yeast HOG1 and 46–49% identical to the mammalian ERKs. This kinase is activated by dual phosphorylation on Tyr and Thr in several cellular systems following treatment with lipopolysaccharides (LPS), physical or chemical stress such as changes in osmolarity or heat shock, and exposure to the pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF). Cytokine-suppressive anti-inflammatory drugs (CSAIDs), which inhibit cytokine biosynthesis, are selective inhibitors of this kinase activity and are currently being used to study signaling cascades involving inflammatory cytokines (Cuenda et al., 1995; Lee & Young, 1996).

Three-dimensional structural data can be invaluable in the elucidation of enzyme mechanism and function. The first reported crystal structure of a protein kinase was of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase (cAMP-dependent PK) (Knighton et al., 1991). Numerous X-ray structures have subsequently been reported (for reviews, see Bossemeyer, 1995; Johnson et al., 1996), including the MAP kinase ERK2 (Zhang et al., 1994). To further our understanding of p38 MAP kinase, we have expressed, purified, and crystallized three recombinant forms of the human enzyme in quality and quantity sufficient to undertake macromolecular crystallographic studies.

Results and discussion: Human p38 MAP kinase has been purified from *Escherichia coli* and baculovirus-infected SF9 insect cell expression systems. The identity of the protein from each system was confirmed by electrospray mass spectrometry (ESMS). ESMS

Reprint requests to: Susan Pav, Department of Inflammatory Diseases, Boehringer Ingelheim Pharmaceuticals, 900 Ridgebury Road, P.O. Box 368, Ridgefield, Connecticut 06877; e-mail: pav@mhv.net.

and Western blotting with anti-phosphotyrosine antibodies suggest that the proteins may be largely in an unphosphorylated form. ESMS of a tryptic digest of SF9-expressed p38 showed acetylation of the N-terminal alanine. Studies on the mechanism of p38 inhibition are currently in progress. p38 from all three expression systems crystallized reproducibly utilizing similar precipitant solutions as described below, in the presence of inhibitors (Lee et al., 1994) and non-hydrolyzable ATP analogs. Two different morphologies were observed for the crystals. Form 1 crystals are long, thin prisms, the largest of which measured $0.15 \times 0.15 \times 0.70$ mm^3 (Fig. 1A, B). Form 2 crystals are also prismatic, but the three dimensions of the prism are roughly the same, with the

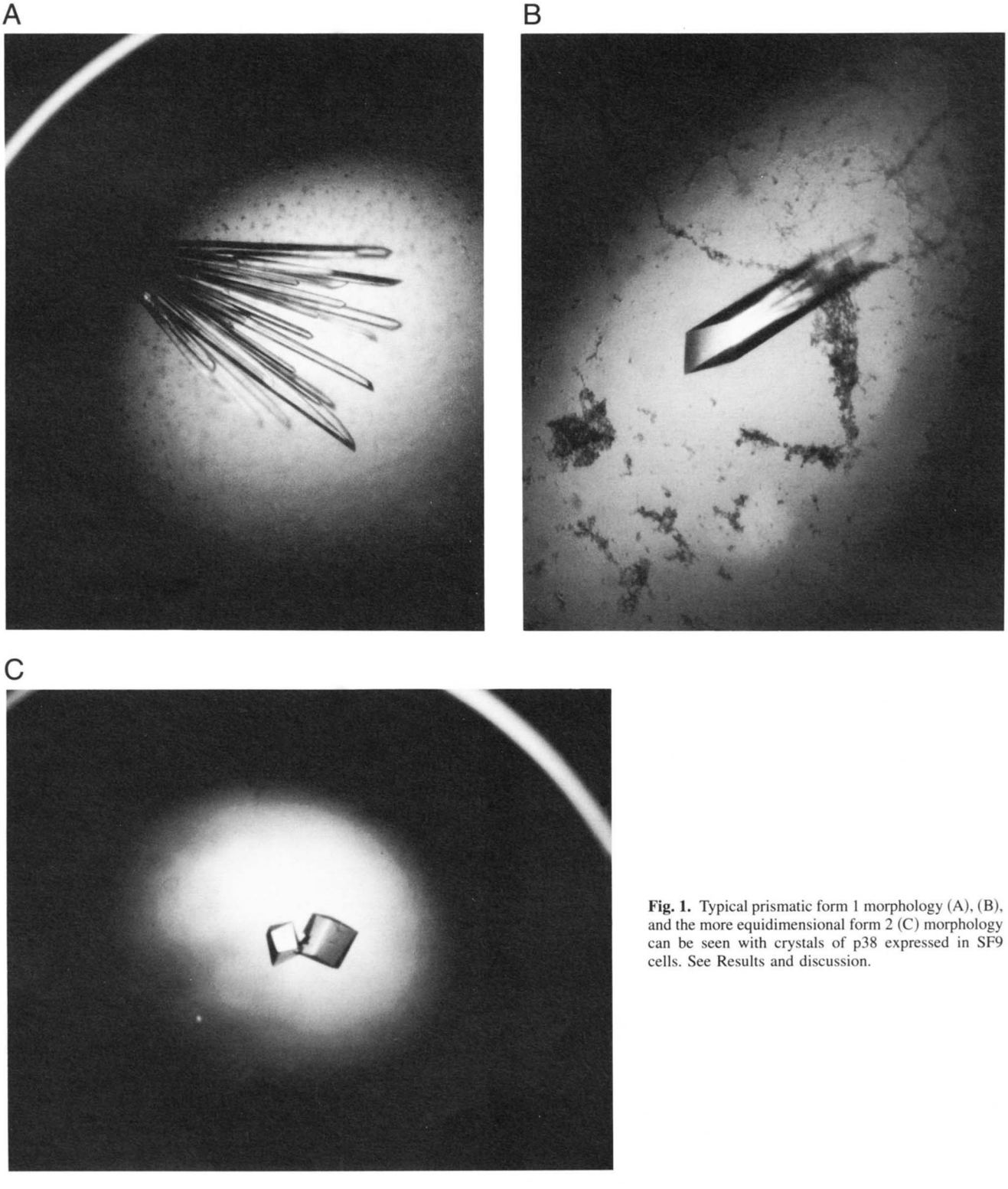

Fig. 1. Typical prismatic form 1 morphology (A), (B), and the more equidimensional form 2 (C) morphology can be seen with crystals of p38 expressed in SF9 cells. See Results and discussion.

largest crystals measuring about 0.1 × 0.1 × 0.1 mm^3 (Fig. 1C). Form 1 crystals have been grown using protein from both bacterial and insect cell expression systems. Form 2 crystals have been obtained predominantly with protein expressed in insect cells. The crystallographic analysis showed that both crystal forms belong to space group $P2_12_12_1$. The cell parameters for crystal form 1 are a = 65.2 Å, b = 74.6 Å, and c = 78.1 Å. Those for crystal form 2 are a = 58.3 Å, b = 68.3 Å, and c = 87.9 Å. Assuming one molecule of p38 per asymmetric unit, the Vm value was calculated to be 2.3 $Å^3$/Dalton for crystal form 1 and 2.1 $Å^3$/Dalton for crystal form 2, suggesting that crystal form 2 is more tightly packed. Diffraction data to 2.7 Å resolution have been collected on both crystal forms on the R-Axis mounted on a Rigaku rotating anode X-ray generator. Recently, data sets to 2 Å resolution were collected for both crystal forms on the X4A and X-25 beamlines at the Brookhaven National Laboratory. The structure determination of p38 MAP kinase by the multiple isomorphous replacement and the molecular replacement methods are currently in progress. In parallel, crystals containing seleno-methionyl residues have also been obtained in order to utilize the seleno-methionyl multiple wavelength anomalous diffraction technique (Hendrickson, 1991).

Materials and methods: *Expression:* p38 cDNA was cloned and expressed in *E. coli* and baculovirus-infected SF9 cells utilizing protocols similar to those reported earlier (Lee et al., 1994) for CSBP2. The open reading frame of p38 cDNA was originally cloned by reverse transcription/polymerase chain reaction (PCR) of U937 cell RNA. The resulting product was subjected to a second PCR reaction with primers that incorporated an NcoI site, an alanine and a histidine tag at the amino terminus, and a stop codon and BamHI site at the carboxy terminus. After sequencing to confirm its identity, the NcoI/BamHI CSBP2 ORF was subcloned into the NcoI and BamHI sites of the pET 15b vector (Novagen), for pRVR-2, and into pQe60 (Qiagen), for p1015. Large scale cultures of p1015 were induced with 2 mM IPTG; cells were harvested 2 h after induction. For expression of p38 with seleno-methionine substitution in *E. coli*, a single colony of B834 (DE3) pLysS containing pRVR-2 was used to inoculate 120 mL of DLM medium, supplemented with Kao and Michayluk vitamin solution and 50 mg/mL seleno-methionine, and cultured overnight at 37 °C. This was used to inoculate six liters of DLM (i.e., 20 mL/L), supplemented as above. Cells were grown at 37 °C to A_{600} = 0.6, induced with 1 mM IPTG and harvested at 5 and 10 h. The NcoI/BamHI CSBP2 ORF was also subcloned into the baculovirus transfer vector pVL1393 (Pharmingen) for infection of SF9 insect cells. Cultures were grown to a cell density of approximately 1 × 10^6/mL in a shaking incubator at 27 °C. The cells were then infected with viral stock at a multiplicity of infection (MOI) of 10 and harvested after three days of infection.

Purification: For all expression systems, soluble p38 was extracted from cell pellets stored at −80 °C. *E. coli* pellets were resuspended in lysis buffer consisting of 20 mM HEPES, 10% (v/v) glycerol, 15 mM imidazole, 1 mM dithiothreitol (DTT) and 325 mM NaCl, pH 8.0 at 4 °C, supplemented with 1 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 1.0 μg/mL okadaic acid, 0.2 mg/mL AEBSF, 0.5 mg/mL leupeptin, 60 μg/mL antipain, 2.0 μg/mL pepstatin, and 2.0 μg/mL aprotinin. For lysis of SF9 cells, NaCl was omitted and added back post-sonication. For *E. coli* samples, lysozyme (chicken egg white, Sigma) was added to 0.25 mg/mL and suspensions were nutated for 15 min at 4 °C. For all samples, $MnCl_2$ and $MgCl_2$ were then added to 5.0 mM, followed by Benzonase (Sigma) to 80 U/mL and nutation for another 15 min. Samples were sonicated on ice for 15 min using 2 second pulses. Lysate was centrifuged for 1 h at 18,000 RPM (38,000 × g) in a Sorvall SS-34 rotor at 4 °C. Supernatant was loaded on a 1.6 × 15 cm column packed with NTA-agarose (Qiagen) equilibrated in lysis buffer. NaCl and 15 mM imidazole were incorporated into the equilibration buffer to minimize non-specific or marginally specific protein binding to the NTA-agarose. A 15–225 mM imidazole gradient was used for elution. Fractions were analyzed by SDS-PAGE and activity assessed by a modification of a tyrosine kinase activity assay (Caverzasio & Bonjour, 1992) using myelin basic protein as substrate. The eluant peak containing p38 was concentrated in a stirred cell (Amicon) using a YM10 membrane, and loaded on either a 1.6 × 60 cm S-100 column (Pharmacia) equilibrated in 25 mM Tris, 200 mM NaCl, 5% (v/v) glycerol, 1 mM DTT, at 4 °C, or on a 1 × 15 cm DEAE-FF column (Pharmacia) equilibrated in 25 mM Tris, 5% (v/v) glycerol, 50 mM NaCl, 1 mM DTT, pH 7.4 at 4 °C. Samples loaded onto DEAE were eluted with a 50–400 mM NaCl gradient, concentrated and loaded on an S-100 column as above. Final S-100 fractions were concentrated as above to about 6.0 mg/mL, as calculated by A_{280} (Mach et al., 1992), prior to crystallization experiments.

Crystallization: The initial search for suitable crystallization conditions was performed by vapor diffusion using p38 from *E. coli* as free enzyme or in combination with several small molecule inhibitors. SB 203580 and analogs were prepared as described (Adams et al., 1993; Cuenda et al., 1995), purified by reverse phase HPLC, and added at 10:1 molar inhibitor excess. Conditions similar to those utilized for crystallization of the MAP kinase ERK2 (Zhang et al., 1994) did not yield crystals. Crystals suitable for X-ray diffraction studies were found using the sparse matrix crystal screening kits (Hampton) (Jancarik & Kim, 1991). Subsequent trials determined that p38 expressed in SF9 cells and in the *E. coli* seleno-methionyl p38 also crystallized under similar conditions. The best crystals were obtained by mixing 6.0 mg/mL enzyme (140 μM) + inhibitor (1.4 mM), pre-incubated at 4 °C for 1–2.5 h, in equal volumes of 1.4 μL with reservoir solution consisting of 28–32% (w/v) PEG 1500 (Fluka), 5mM DTT. In some experiments, 14 mM $MgCl_2$ in combination with 1.4 mM AMP-PNP or AMP-PCP (Fluka) were added to the enzyme prior to inhibitor addition. These conditions yielded crystals at ambient temperatures (21–25 °C) within 12–18 days for *E. coli*-expressed p38 and within 5–26 days for p38 from insect cells. Nucleation and crystal growth were accelerated to 4–6 days for all recombinant forms using macro- or micro-seeding or cross-seeding. This was achieved using a shotgun approach with seed prepared from small crystals or pieces of crystals of desired morphology which were pre-diluted into 20–27% (w/v) PEG 1500, vortexed and added at 0.2 μL per drop before suspending above the wells.

X-ray diffraction studies: Crystals of p38 were characterized on an R-Axis imaging plate system mounted on a Rigaku RU-200 X-ray generator. A 0.6 μL aliquot of ethylene glycol was introduced into the hanging drop and the crystal was flash-frozen about 30 minutes later.

Acknowledgments: The authors would like to thank Dr. Peter Grob, Dr. James Stevenson and Dr. Klaus Lubbe for their encouragement, support, and helpful discussions with this project.

References

Adams JL, Gallagher TF, Lee JC, White JR. 1993. Imidazole derivatives and their use as cytokine inhibitors. Int Patent Applic Number WO 93/14081.

Bossemeyer D. 1995. Protein kinases—structure and function. *FEBS Lett 369*: 57–61.

Cano E, Mahadevan LC. 1995. Parallel signal processing among mammalian MAPKs. *Trends Biochem Sci 20*:117–122.

Caverzasio J, Bonjour J-P. 1992. Tyrosine phosphorylation selectively regulates renal cellular phosphate transport. Evidence that it mediates the stimulatory effect of insulin-like growth factor-1. *Endocrinology 130(1)*:373–380.

Cobb MH, Goldsmith EJ.1995. How MAP kinases are regulated. *J Biol Chem 270(25)*:14843–14846.

Cuenda A, Rouse R, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stress and interleukin-1. *FEBS Lett 364*:229–233.

Hendrickson WA. 1991. Determination of macromolecular structures from anomalous diffraction of synchrotron radiation. *Science 254*:51–58.

Jancarik J, Kim S-H. 1991. Sparse matrix sampling: A screening method for crystallization of proteins. *J Appl Crystallog 24*:409–411.

Johnson LN, Noble MEM, Owen DJ. 1996. Active and inactive protein kinases: Structural basis for regulation. *Cell 85*:149–158.

Knighton DR., Zheng J, TenEyck LF, Xuong N, Taylor SS, Sowadski JM. 1991. Structure of a peptide bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science 253*:407–414.

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter S, Strickler J, McLaughlin MM, Siemens IR, Fisher SM, Livi GP, White JR, Adams JL, Young PR. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature 372*:739–746.

Lee JC, Young PR. 1996. Role of CSBP/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J Leukoc Biol 59*:152–157.

Mach H, Middaugh CR, Lewis, RV. 1992. Statistical determination of the average values of the extinction coefficients of tryptophan and tyrosine in native proteins. *Anal Biochem 200*:74–80.

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem 270(13)*:7420–7426.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Liamazares A, Zamanillo D, Hunt T, Nebreda AR. 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP Kinase-2 and phosphorylation of the small heat shock proteins. *Cell 78*:1027–1037.

Seger R, Krebs EG. The MAPK signaling cascade. 1995. *FASEB J 9*:726–735.

Zhang F, Strand A, Robbins D, Cobb MH, Goldsmith E. 1994. Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution. *Nature 367*:704–711.

Zhang C-C. 1996. Bacterial signalling involving eukaryotic-type protein kinases. *Mol Microbiol 20(1)*:9–15.