Developmental and Tissue-specific Expression of Mouse Pelle-like Protein Kinase*

(Received for publication, April 23, 1996, and in revised form, May 23, 1996)

Marina Trofimova, Amy B. Sprenkles, Melissa Green, Thomas W. Sturgills, Mark G. Goebl, and Maureen A. Harrington†

From the †Departments of Biochemistry and Molecular Biology, and the Walther Oncology Center, Indiana University, Indianapolis, Indiana 46202-5121, and the ‡Howard Hughes Medical Institute, University of Virginia, Charlottesville, Virginia 22908.

The NF-κB/c-Rel proteins are a family of evolutionarily conserved transcription factors activated during development that in the adult, mediate many processes including the immune response. A high degree of sequence similarity is shared between the NF-κB/c-Rel family of transcription factors and the Drosophila dorso- sal protein as well as between its cytoplasmic inhibitor, IκBα, and the Drosophila Cactus protein. Genetic analyses of Dorsal have defined components of a signaling pathway for Dorsal activation, including a serine/threonine kinase, Pelle, placed upstream of Dorsal and Cactus. We demonstrate that this pathway is likely to be conserved in mammals by the isolation of a cDNA that encodes a novel mouse protein highly related to Pelle, mPLK (mouse Pelle-like protein kinase). Expression of mPLK mRNA is developmentally regulated in the mouse and in adult tissue mPLK expression is greatest in the liver, a tissue that expresses a high level of NF-κB. Recombinant mPLK produced in bacteria is a protein kinase capable of autophosphorylating and phosphorylating IκBα.

Acquisition of immune competence in adult mammals depends on a series of developmentally-linked changes in gene expression. How these changes are coordinated during development is unknown. Insight into these processes in mammals can be gained from an understanding of related events in other model developmental systems. The NF-κB/c-Rel family of evolutionarily conserved transcription factors are key mediators of genes expressed during activation of immune and inflammatory responses (1–3). NF-κB/c-Rel family members bind DNA as homo- or heterodimers (4–6), but under basal conditions, NF-κB dimers are sequestered in the cytoplasm in a complex with IκBα (7–9). In response to cellular stimulation, IκBα becomes phosphorylated (1–3,10–13) and in some instances subjected to proteolytic degradation through a ubiquitin-dependent pathway (14–16). Removal of IκBα unmasks a nuclear localization sequence (7–19), thus allowing NF-κB to translocate to the nucleus, bind to its cognate cis-acting element, and transcriptionally activate a variety of genes. IκBα has been shown to be a substrate for several kinases including protein kinase C, eIF-2 kinase, casein kinase II and a newly described monocyte-specific kinase (1–3,10–13). Several of these kinases phosphorylate IκBα on serine and threonine residues present in the COOH terminus. Phosphorylated serine residues have also been detected in the NH2-terminal region of IκBα. Phosphorylation of NH2- and COOH-terminal residues has been linked to changes in IκBα protein stability. Currently, it is unclear whether other kinases are also responsible for IκBα phosphorylation and whether different NF-κB/c-Rel activators operate via distinct kinases.

A high degree of sequence similarity is shared between the NF-κB/c-Rel family of transcription factors and the Drosophila Dorsal protein as well as IκBα and the Drosophila Cactus protein (20). In response to activation of the Toll receptor by Spätzle, a serine/threonine protein kinase encoded by the pelle gene is activated and is believed to phosphorylate Cactus, resulting ultimately in activation of Dorsal. Given the striking parallel between the NF-κB/c-Rel and Dorsal pathways, we set out to determine whether a mammalian homolog of the Drosophila Pelle protein existed.

EXPERIMENTAL PROCEDURES

Isolation of mPLK cDNA—Primers, 20 and 21 nucleotides long, were designed to anneal to DNA sequences that encode two regions of the Pelle protein kinase catalytic domain and included degenerate nucleotides of up to 2048-fold degeneracy to ensure efficient priming of unidentified sequences. The sequence of the primer designed to recognize kinase subdomain I: 5′-GGIG(T/G)CT(T/G)TTG(G/C)TT(T/G)AT(T/G)TTG(T/G)TTG-3′ and the sequence of the primer designed to recognize kinase subdomain VIb: 5′-ATG(T/G)TTG(G/C)TT(T/G)AT(T/G)TTG(T/G)TTG-3′. PCR amplifications were performed with mouse embryonic cDNA (1 ng; Clontech Laboratories, Palo Alto, CA) as a template and 100 μM of each degenerate primer. After 40 cycles of PCR, products with expected size (400 base pairs) were purified from 1.2% agarose gel using GeneClean II kit (Bio 101, Vista, CA) and cloned using TA cloning kit according to manufacturer’s specifications (U. S. Biochemical Corp.). A 400-base pair cDNA fragment was radiolabeled with [α-32P]dCTP by primer extension with a multiprime DNA labeling kit (Amersham Corp.). Probes were purified with QIAquick columns (Boehringer Mannheim) and used to screen approximately 1 × 109 plaques from a λgt11 mouse embryo 5′-stretch cDNA library according to the manufacturer’s specifications (Clontech Laboratories). After a third round of library screening, 20 positive clones containing inserts ranging from 1500 to 2700 base pairs were isolated. All clones were partially sequenced over the putative catalytic domain, and amino acid sequence comparisons to all available data bases were made with the program BLAST (21). Both strands of the longest clone were sequenced using the Fmol DNA sequencing system (Promega, Madison, WI).

Northern Analysis—Northern blots were purchased from (Clontech) and hybridized according to the manufacturer’s specifications with a mouse cDNA radiolabeled with [α-32P]dCTP by primer extension with a Multiprime DNA labeling kit (Amersham). Autoradiograms were prepared by exposing washed membranes to Hyperfilm-MP (Amersham) in the presence of intensifying screens at ~70 °C for 2 days.

Cloning, Expression, and Purification of mPLK—The mPLK cDNA was subcloned into the EcoRI site of pET-28a(+) (Novagen, Madison, WI), which generated a His tag at the NH2 and COOH termini plus an NH2-terminal 7× tag to allow for immunodetection. The resulting fu

*This research was supported in part by National Institutes of Health Grants GM43972 (to M. A. H.) and GM45460 (to M. G. G.) and the Howard Hughes Medical Institute (to T. W. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Scholar of the Leukemia Society of America. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Indiana University, 975 W. Walnut St., Indianapolis, IN 46202-5121. Tel.: 317-274-7527; Fax: 317-274-7592.

‡ This research was supported in part by National Institutes of Health Grants GM43972 (to M. A. H.) and GM45460 (to M. G. G.) and the Howard Hughes Medical Institute (to T. W. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Scholar of the Leukemia Society of America. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Indiana University, 975 W. Walnut St., Indianapolis, IN 46202-5121. Tel.: 317-274-7527; Fax: 317-274-7592.
Cloning of the Mouse Pelle-like Kinase

RESULTS AND DISCUSSION

Given the parallel between the NF-\kappaB/c-Rel and Dorsal pathways, we set out to determine whether mammals contain a homolog of the Drosophila Pelle protein. Degenerate primers corresponding to the sequences GGF GDVY (kinase subdomain I) and DIKPAN (kinase subdomain VIb) located in the catalytic domain of the Pelle protein kinase (see Fig. 1 legend) were used for PCR amplifications using mouse embryonic cDNA as a template. The DNA sequences of five different PCR products revealed similarity to protein kinases. One sequence encoded an open reading frame that shared 44% amino acid sequence identity with a portion of the Pelle kinase catalytic domain. A mouse embryo cDNA library (Clontech Laboratories, Palo Alto, CA) was then screened using the PCR product as a probe leading to the isolation of a clone containing a 2.7-kilobase insert. This cDNA encodes an open reading frame of 677 amino acids corresponding to a protein with a predicted molecular mass of 74 kilodaltons (Fig. 1, panel A). The COOH-terminal portion of mPLK contains identity to the homologous region in Pelle (20). The serine construct was verified by DNA sequencing and used to transform into E. coli strain BL21 (DE-3; Novagen). Induction of mPLK expression and purification of the fusion protein was performed according to the manufacturer’s specifications, except that protein production was induced at 30°C overnight with 0.4 mM isopropyl-1-thio-\beta-D-galactoside. Cells were harvested by centrifugation, and lysed with a French press in a buffer containing 50 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% Triton X-100, and 0.1% sodium dodecyl sulfate. Crude cell extracts were clarified by centrifugation (10,000 g) and treated with DNase I (5 mg/ml) for 15 min. Bacterial sonicates were centrifuged (10,000 g) and supernatants were loaded onto Ni²⁺–NTA-agarose resin (Novagen). His-tagged mPLK was eluted in a buffer containing: 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, and 200 mM imidazole. Fractions were pooled and concentrated with Amicon Centricon columns (Amicon Corp., Beverly, MA). Protein concentrations were determined with the Bio-Rad reagent according to the manufacturer’s specifications.

The identity of mPLK containing fractions was verified by Western analysis with commercially available T7 tag monoclonal antibody, according to the manufacturer’s specifications (Novagen).

In vitro Kinase Assays—Kinase assays were performed in 20 \mu l of kinase buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol) containing 10 \mu M ATP (10 mM MgCl₂) and 2 \mu g of purified His-mPLK in the presence or absence of 2 \mu M of purified His-IκBα at 37°C for 30 min. Individual reactions were terminated by the addition of 2× SDS-sample buffer (0.1 M Tris, pH 6.8, 0.2 M dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol) and boiled 5 min, and proteins were separated on 8% SDS-polyacrylamide gels. Autoradiograms were prepared from dried gels exposed to Hyperfilm-MP (Amersham) film in the presence of intensifying screens at −70°C for 2 days.

mPLK amino acid sequence. Panel B, comparison of the amino acid sequences corresponding to mPLK, IRAK, Pelle, Pto, and Pti-1 catalytic domains. The amino acids corresponding to the mPLK catalytic domain is positioned above the corresponding Pelle, Pto, and Pti-1 amino acid sequences (GenBank accession numbers: L84476, U13923, and U28207, respectively). Amino acid numbering is shown to the right; identical amino acids are indicated by dashes. Kinase domains are identified by the roman numeral directly above the appropriate region; invariant residues common to all kinases are in boldface type (23).
A high level of gene expression in adult tissue. Interestingly, mPLK also expresses a high level of NF-κB. In a cDNA library, we wanted to determine whether the gene expression in 0–3-h embryos and are higher in adult females (20). Since mPLK contains a glutamic acid at residue 30, whereas the human interleukin-1 receptor-associated kinase contains a glycine. A putative death domain also exists in the Drosophila Tube protein, which has been shown genetically and biochemically to interact with Pelle (28, 29). The death domain has been implicated in protein-protein interactions and would suggest that this region of mPLK may be critical for interacting with another protein of its signaling pathway, such as a Tube homolog.

The pelle gene is expressed throughout the Drosophila life cycle. However, levels of expression are transiently increased in 0–3-h embryos and are higher in adult females (20). Since the mPLK cDNA was cloned from a day 17 mouse embryo cDNA library, we wanted to determine whether the mPLK gene expression was present at other times during mouse embryogenesis. Analysis of an mRNA isolated from developing mouse embryos revealed no detectable mPLK transcripts in day 7 embryos and a steady increase in the abundance of mPLK transcripts. The protein band indicated by the top arrow corresponds to mPLK, and the bottom arrow corresponds to IκBα.

The fact that mPLK resembled a protein kinase led us to determine whether it had protein kinase activity. Results of in vitro kinase assays with bacterially expressed His-tagged mPLK revealed that mPLK is a protein kinase, as judged by its ability to autophosphorylate (Fig. 4, lanes 3 and 4). Pelle has been implicated in the control of the Dorsal/Cactus pathway; therefore, we considered the possibility that IκBα, a Cactus homolog, might be a substrate for mPLK in vitro. Recombinant mPLK was able to phosphorylate IκBα (Fig. 4). Autophosphorylation was reduced in the presence of IκBα substrate. As is commonly observed with protein kinases (Fig. 4, compare lanes 3 and 4).

The novel mouse protein kinase mPLK appears to be a mammalian homolog of the Drosophila Pelle protein kinase. A family of plant protein kinases highly related to mPLK include not only Pto, which confers resistance to Pseudomonas syringae in tomatoes (26), and Pti-1, which may act downstream of Pto in a regulatory cascade (26), but also Fen, which confers sensitivity to fenthion in tomatoes (31). The relationship between a kinase(s) potentially associated with a mediator of immune and inflammatory responses in mammals, and plant kinases involved in bacterial resistance, suggests not only a high degree of evolutionary conservation but also a high degree of functional commonality for this growing family of kinases.

We have demonstrated that mPLK is capable of autophosphorylation. Like the mammalian MAP kinases, the plant Pelle-like kinases, Pto and Pti-1, are currently thought to function in a regulatory cascade with Pti-1 serving as a substrate for Pto. It will be interesting to determine if mPLK is a homolog of either Pto or Pti-1 or instead operates at yet another point in the signaling pathway. The nature of the activating signal is of great interest. Based upon the parallels between the Drosophila Dorsal protein and NF-κB, one potential mPLK substrate is IκBα. We demonstrate IκBα phosphorylation in vitro by recombinant mPLK. The functional significance of the IκBα phosphorylation remains to be determined. However, these results coupled with the autophosphorylation of mPLK demonstrate that mPLK can function as a kinase. The role of the putative death domain in the activation of mPLK through its signaling pathway also needs to be evaluated.

The link between mPLK and NF-κB/IκBα is of prime physiological importance. The acute phase response is the body's primary defense against bacterial, viral, and parasitic infection (32). The expression of numerous acute phase response genes, including those encoding interleukin-6 and -8 is modulated by NF-κB/c-Rel family members (33, 34). Consistent with a role for mPLK in the control of genes encoding acute phase proteins,
mPLK gene expression was highest in the liver, the primary site of acute phase protein synthesis in vivo. Intriguingly, massive degeneration of the liver via programmed cell death or apoptosis occurs in mice lacking the NF-κB family member, RelA (35). Developmentally, de novo expression of the mPLK gene occurred near the time that hematopoiesis is initiated in the fetal liver (36). The functional significance of this latter finding is unclear; however, in parallel to the RelA/p65 null site of acute phase protein synthesis, our data suggest that mPLK is expressed in a tissue-specific manner, yet the NF-κB family of transcription factors are ubiquitously expressed. It will be of great interest to determine if mPLK, like the plant kinases Pto, Pti-1, and Fen, is a member of a larger family of kinases in mammals with distinct patterns of tissue distribution. Based upon amino acid homology, the recently identified human interleukin-1 receptor-associated kinase is a highly related family member (24). Therapeutically this class of kinases could be prime targets for the management of acute and chronic inflammatory conditions.

Acknowledgments—We thank J. Blum, A. DePaoli-Roach, P. Roach, and A. Roman for critically reviewing the manuscript.

Note Added in Proof—Based upon sequence comparison with IRAK, it is likely that the DNA sequence encoding the N terminus of mPLK has not been isolated.

REFERENCES
1. Siebenlist, U., Franzoso, G., and Brown, K. (1994) Annu. Rev. Cell Biol. 10, 405–455
2. Baeuerle, P. A., and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179
3. Israël, A. (1995) Trends Genet. 11, 203–205
4. Grimm, S., and Baeuerle, P. A. (1993) Biochem. J. 290, 297–308
5. Bressler, P., Brown, K., Timmer, W., Bours, V., Siebenlist, U., and Faund, A. S. (1993) J. Virol. 67, 288–293
6. Logeat, F. Israël, N. Ten, Blank, V., Kourilsky, P., and Israël, A. (1991) EMBO J. 10, 1827–1832
7. Baeuerle, P. A., and Baltimore, D. (1988) Cell 53, 211–217
8. Baeuerle, P. A., and Baltimore, D. (1988) Science 242, 340–346
9. Baeuerle, P. A., and Baltimore, D. (1989) Genes Dev. 3, 1689–1698
10. Wilk, S., and Baeuerle, P. A. (1995) EMBO J. 14, 2876–2883
11. Broun, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995) Science 267, 1485–1488
12. McElhinny, J. A., Trushin, S. M., Bren, G. D., Chester, N., and Paya, C. V. (1996) Mol. Cell. Biol. 16, 899–906
13. Kunis, K., Ishikawa, Y., Ernst, M. E., Ogata, M., Rice, N. R., Mukaida, N., and Matsushima, K. (1995) J. Biol. Chem. 270, 27914–27919
14. Chen, A., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) Genes Dev. 9, 1586–1597
15. Alkalay, I., Yaron, A., Hatzubai, A., Orian, A., Cicchiano, A., and Ben-Neriah, Y. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10599–10603
16. Baldi, L., Brown, K., Franzoso, G., and Siebenlist, U. (1996) J. Biol. Chem. 271, 376–379
17. Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskill, S., Rosen, C. A., and Baldwin, A. S., Jr. (1992) Genes Dev. 6, 1899–1913
18. Gandhi, P. A., Sun, S.-C., Greene, W. C., and Ballard, D. W. (1992) Mol. Biol. Cell 3, 1339–1352
19. Zabel, U., Henkel, T., dos Santos Silva, M., and Baeuerle, P. A. (1993) EMBO J. 12, 201–211
20. Sheltown, C. A., and Wasserman, S. A. (1993) Cell 72, 515–525
21. Altshul, S. F., Gish, W., Miller, W., Meyers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
22. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8149
23. Hanks, S. K., and Quinn, A. M. (1990) Methods Enzymol. 200, 38–81
24. Cao, Z., Henzel, W. J., and Gao, X. (1996) Science 271, 1128–1131
25. Schneider, D. S., Hudson, K. L., Lin, T.-Y., and Anderson, K. V. (1991) Genes Dev. 5, 787–807
26. Zhou, J., Loh, Y.-T., Bressan, R. A., and Martin, G. B. (1995) Cell 83, 925–935
27. Feinstein, E., and Kimchi, A. (1995) Trends Biochem. Sci. 20, 342–344
28. Wasserman, S. A. (1993) Mol. Biol. Cell 4, 767–771
29. Großhans, J., Bergmann, A., Haffter, P., and Nüsslein-Volhard, C. (1994) Nature 372, 563–566
30. Kohchi, C., Nioguchi, K., Tanabe, Y., Mizuno, D.-I., and Soma, G.-I. (1994) Int. J. Biochem. 26, 111–119
31. Loh, Y.-T., and Martin, G. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4181–4184
32. Blaumann, H., and Gauldie, J. (1994) Immunol. Today 15, 74–80
33. Sanceau, J., Kaisho, T., Hirano, T., and Wietzerbin, J. (1995) J. Biol. Chem. 270, 27920–27931
34. Mukaida, N., Mahe, Y., and Matsushima, K. (1990) J. Biol. Chem. 265, 21128–21133
35. Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., and Baltimore, D. (1995) Nature 376, 167–170
36. Tavassoli, M. (1988) in Ontogeny of Hemopoiesis in Handbook of Human Growth and Developmental Biology (Meisami, E., and Timiras, P. S., eds) pp. 101–111, CRC Press, Boston