Phosphorylation of a novel zinc-finger-like protein, ZPR9, by murine protein serine/threonine kinase 38 (MPK38)

Hyun-A SEONG*, Minchan GIL^{*1}, Kyong-Tai KIM[†], Sung-Jin KIM[‡] and Hyunjung HA^{*2}

^{*}Department of Biochemistry, School of Life Sciences, Research Center for Bioresource and Health, Chungbuk National University, 48 Gaeshin-dong, Cheongju, Chungbuk 361-763, Korea, [†]Department of Life Science, Pohang University of Science and Technology, San 31, Hyoja-dong, Pohang 790-784, Korea and [‡]Department of Pharmacology, School of Dentistry, Kyung Hee University, Haegi-dong, Seoul 130-701, Korea

We have identified previously a new murine protein serine/threonine kinase, MPK38, closely related to the sucrose-non-fermenting protein kinase family [Gil, Yang, Lee, Choi and Ha (1997) *Gene* **195**, 295–301]. Using the C-terminal half of the putative human counterpart of MPK38, HPK38, as a bait in a yeast two-hybrid screen of a human HeLa cDNA library, it was discovered that the zinc-finger-motif-containing protein, termed zinc-finger-like protein 9 (ZPR9), bound both HPK38 and MPK38. In a co-expression assay, ZPR9 associated with MPK38 *in vivo*, and we showed that the ZPR9 is also phosphorylated by MPK38. In addition, ZPR9 physically interacts with itself in mammalian

cells. The ZPR9 cDNA hybridized with a mRNA species of approx. 1.7 kb in Northern-blot analysis. The ZPR9 transcript was detected in all tissues examined, including lung, kidney, spleen, liver and brain. Co-expression of ZPR9 with MPK38 caused the accumulation of ZPR9 in the nucleus. These findings suggest a potentially important role for ZPR9 in MPK38-mediated signal transduction, and that ZPR9 is a physiological substrate of MPK38 *in vivo*.

Key words: association *in vivo*, subcellular localization, substrate *in vivo*, yeast two-hybrid screen.

INTRODUCTION

Murine protein kinase 38 (MPK38), also named Melk, is a member of the sucrose-non-fermenting (SNF1) protein serine/threonine kinase family [1]. MPK38 was originally identified as a murine counterpart for its human homologue, designated human protein kinase 38 (HPK38, and also named hMelk or KIAA175), that may be involved in the proliferation of normal human keratinocytes induced by interleukin (IL)-4 [2]. Recent studies based on the regulation of MPK38 expression have suggested that MPK38 may play a functional role as one of the signal regulators in early T-cell activation and, potentially, certain lineages of haematopoietic cell activation by concanavalin A [3]. The importance of MPK38 during embryonic development was also underscored by the finding that MPK38 was expressed predominantly in the unfertilized egg and pre-implantation embryo [1,4].

A number of different proteins have regions, termed zinc-finger motifs, which fold around a central zinc ion to produce a compact domain. These motifs were first recognized in DNA-binding domains of many different eukaryotic transcription factors, but is now found in proteins that do not bind to DNA [5]. The proposed interaction of zinc with the conserved cysteine and histidine residues was structurally confirmed by X-ray spectroscopy of the purified transcription factor TFIIIA [6]. The importance of zinc-finger proteins in regulating gene expression is underscored by the large number of transcription factors containing zinc-finger motifs present in eukaryotes. In addition, this motif has been identified in a number of proteins that are implicated in transcriptional regulation, DNA repair, DNA recombination and oncogenic function, suggesting that many

members of this family are involved in cellular events such as cell growth, oncogenesis, differentiation and development [7].

To gain more insights about the biological roles of MPK38, we have used the yeast two-hybrid system to identify interacting molecules that associate with MPK38. In the present study, we have described the identification and molecular cloning of a novel zinc-finger-like protein, designated ZPR9, that associated with MPK38, and demonstrated that ZPR9 forms a homodimeric complex and is a substrate for MPK38.

EXPERIMENTAL

Cell culture, plasmids, and reagents

Cells derived from human kidney embryonal fibroblasts containing SV40 T-antigen, 293T, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, U.S.A.), 100 units/ml penicillin/streptomycin and 1 mM glutamine in an atmosphere of 5% CO₂/95% O₂ at 37 °C [8]. A full-length ZPR9 cDNA obtained from a human normal keratinocyte cDNA library and MPK38 were cloned into pFLAG-CMV-2 and pBluescript KS (Stratagene, La Jolla, CA, U.S.A.) to generate the pFLAG-ZPR9 and KS-MPK38 vectors respectively. To express proteins in mammalian cells, green fluorescent protein (GFP)-MPK38 and histidine-tagged (His)-ZPR9 were created by subcloning of a *KpnI*-*Bam*HI fragment from KS-MPK38 and an *Eco*RI-*Xba*I fragment from pFLAG-ZPR9 into pRSGFP-C1 (Clontech, Palo Alto, CA, U.S.A.) and pcDNA3.1/HisC (Invitrogen, San Diego, CA, U.S.A.) respectively. To generate glutathione S-transferase (GST)-MCAT containing the N-terminal catalytic domain of MPK38 (amino acids 7–269), the full-length MPK38 cDNA was

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GST, glutathione S-transferase; HPK38, human protein kinase 38; IL, interleukin; MPK38, murine protein kinase 38; MCAT, N-terminal catalytic domain of MPK38; SNF1, sucrose-non-fermenting protein kinase; ZF1, zinc-finger motif-1; ZPR9, zinc-finger-like protein 9.

¹ Present address: Department of Pathology, Seoul National University Medical School, Yeongun-dong, Seoul 110-799, Korea.

² To whom correspondence should be addressed (e-mail hyunha@cbucc.chungbuk.ac.kr).

The nucleotide sequence data reported will appear in GenBank® Nucleotide Sequence Database under the accession number AY046059.

used as template for the PCR. The amplified PCR products were cut with *EcoRI* plus *Sall*, and cloned into pBluescript KS (Stratagene). GST-MCAT was constructed in several steps. We first cloned the *EcoRI*-*XhoI* fragment of MCAT, cloned in pBluescript KS, into pBacPAK9 (Clontech), and digested with *EcoRI* plus *NotI*, and subcloned into pBluescript KS. Finally, the *ClaI*-*NotI* fragment of the resulting plasmid was cloned into pEBG cut with *ClaI* and *NotI*, yielding pEBG-MCAT (GST-MCAT). The plasmid vector pGEX4T-1 for expression in bacteria was purchased from Amersham Pharmacia Biotech. The anti-GST antibody was as described previously [9]. The anti-FLAG (M2) antibody, isopropyl- β -D-thiogalactopyranoside, dithiothreitol, aprotinin and PMSF were purchased from Sigma (St. Louis, MO, U.S.A.). PVDF membrane was obtained from Millipore (Bedford, MA, U.S.A.). [γ - 32 P]ATP was purchased from NEN Life Science Products (Boston, MA, U.S.A.).

Yeast two-hybrid screen

The yeast genetic screening for the isolation of proteins interacting with the C-terminal region of HPK38, comprising amino acids 514–651 [10], was carried out essentially as described previously [9]. The bait plasmid LexA-HPK38, pEG202-HPK38, was transformed into the yeast strain EGY48 [*Mat α trp1 ura3–52 leu2::pLeu2-lexAop6(Δ UAS leu2)*]. This strain was transformed with the human HeLa cDNA interaction library in the pJG4-5 plasmid. Yeast transformation was performed by the lithium acetate method. Positive clones were selected for the formation of blue colonies on 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside plates with leucine, and the cDNA inserts were recovered and sequenced with dideoxy sequencing in accordance with the manufacturer's instructions (Amersham Pharmacia Biotech). To test whether the library cDNA encoding the ZPR9 protein interacted specifically with the C-terminal region of HPK38, a fish plasmid, pJG4-5 containing ZPR9, was transformed back into EGY48 cells along with either the bait plasmid or other non-specific bait plasmids available in our laboratory [9]. The β -galactosidase activity was used for selection of proteins interacting with the HPK38.

Eukaryotic expression and binding assay *in vivo*

The eukaryotic GST-expression vector (pEBG) and pFLAG-CMV-2 vector with a FLAG epitope were obtained as described previously [8,9]. To express proteins in mammalian cells, the full-length MPK38 and ZPR9 were cloned into pEBG and pFLAG-CMV-2. Cells, 293T, grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum were plated in 6-well flat-bottomed microplates at a concentration of 2×10^5 cells per well the day before transfection. Each plasmid DNA (1–5 μ g) was transfected into 293T cells with the calcium phosphate precipitation method. After transfection (48 h) cells were washed three times with ice-cold PBS and solubilized with 100 μ l of lysis buffer (20 mM Hepes, pH 7.9, 10 mM EDTA, 0.1 M KCl and 0.3 M NaCl) containing 0.1% Nonidet P-40, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 mM NaF, 2 μ g/ml α -1-antitrypsin, 2 mM sodium pyrophosphate, 25 mM sodium β -glycerophosphate, 1 mM sodium orthovanadate and 1 mM PMSF. Detergent insoluble materials were removed by centrifugation at 11000 *g* at 4 °C for 15 min. Approx. 80 μ l of the cleared lysates were mixed with 15 μ l of glutathione-Sepharose beads (Amersham Pharmacia Biotech) and rotated for 2 h at 4 °C. Beads were washed three times with the lysis buffer. The bound proteins were eluted by boiling in SDS sample buffer, subjected to SDS/PAGE, and then transferred to PVDF mem-

branes. The membranes were probed with an anti-FLAG (M2) antibody and then developed using an enhanced chemiluminescence (ECL[®]) detection system (Amersham Pharmacia Biotech).

Purification of recombinant proteins and kinase assay *in vitro*

For production of bacterial fusion proteins, the ZPR9 cDNA was subcloned in frame into pGEX4T-1 at the *EcoRI*-*NotI* site using PCR. The resulting plasmid was transformed into *Escherichia coli* strain BL21(DE3). Transformed BL21(DE3) bacteria were grown in Luria-Bertani broth containing 50 μ g/ml ampicillin at 37 °C to D_{600} of 0.5, and induced with 0.1 mM isopropyl-1-thio- β -D-galactopyranoside at 37 °C for 4 h. Cells were collected by centrifugation and lysed by sonication in a buffer containing 20 mM potassium phosphate, pH 7.0. The bacterial lysates were centrifuged at 11000 *g* for 40 min at 4 °C, and the recombinant GST-ZPR9 fusion protein was purified from the soluble extracts of bacterial lysates by affinity chromatography on glutathione-Sepharose 4B columns (Amersham Pharmacia Biotech).

The transiently transfected cells were washed three times with ice-cold PBS and solubilized with 100 μ l of lysis buffer (20 mM Hepes, pH 7.9, 10 mM EDTA, 0.1 M KCl and 0.3 M NaCl). The cleared lysates were mixed with glutathione-Sepharose beads and rotated for 2 h at 4 °C. After washing the precipitate three times with lysis buffer, then twice with kinase buffer (50 mM Hepes, pH 7.4, 1 mM dithiothreitol and 10 mM $MgCl_2$), the precipitate was incubated with 5 μ Ci of [γ - 32 P]ATP at 37 °C for 15 min in the presence of kinase buffer containing 3 μ g of recombinant ZPR9 (GST-ZPR9), was separated by SDS/PAGE (8% gel), and was transferred to PVDF membranes and the radiolabelled proteins were revealed by autoradiography.

Northern-blot analysis and cDNA cloning

A human RNA Tissue Blot (Bioneer, Cheongwon, Korea) was incubated with a 32 P-labelled ZPR9 cDNA at 42 °C as described below. Total cytoplasmic RNAs were prepared from cell lines using RNazol[™] B (Biotecx Laboratories, Houston, TX, U.S.A.). Approx. 30 μ g/ml total RNA was subjected to electrophoresis on a 1.2% agarose-formaldehyde gel, and transferred to a Genescreen Plus[™] nylon membrane (NEN Research Products). The membranes were hybridized with a 32 P-labelled ZPR9 cDNA probe at 42 °C overnight in 50% formamide, 10% dextran sulphate, 7% SDS, 0.25 M NaHPO₄, 0.25 M NaCl, 1 mM EDTA and 100 μ g/ml denatured salmon sperm DNA. The membranes were washed to a final stringency of $0.25 \times$ SSC (SSC, 0.15 M NaCl and 0.015 M sodium citrate) and 0.2% SDS at 55–60 °C for 20–30 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin mRNA were used as the internal controls.

A human normal keratinocyte cDNA library [11] was screened using the partial ZPR9 cDNA (pJG4-5-ZPR9), obtained from a HeLa cDNA interaction library, as a probe. Positive clones, contained within the pBluescript SK(–) phagemid, were excised *in vivo* from the Uni-ZAP XR vector using ExAssist helper phage system (Stratagene), in accordance with the manufacturer's instructions. The largest clone (approx. 1.7 kb) containing the full-length cDNA for ZPR9 was chosen and sequenced from both strands.

Translation *in vitro*

A clone containing the complete coding region of ZPR9 cDNA was inserted into plasmid pcDNA3 (Invitrogen) and used in a

TNTTM-coupled-reticulocyte-lysate system (Promega, Madison, WI, U.S.A.). Transcription and translation were carried out with T7 RNA polymerase at 30 °C for 90 min in a reticulocyte-lysate system, which contained [³⁵S]methionine. The translation products were analysed by SDS/PAGE (10% gel). Autoradiograms were prepared using Kodak XAR-5 film with intensifying screens at 25 °C for 4 h.

Immunofluorescence and confocal microscopy

293T cells were plated and transfected with GFP-MPK38 and/or FLAG-tagged ZPR9 constructs on sterile coverslips, and placed on ice and washed three times with ice-cold PBS prior to fixation with 4% paraformaldehyde for 10 min at 25 °C. Cells were then washed with PBS, treated with 0.2% Triton X-100, and rewashed with PBS. The mouse monoclonal anti-FLAG (M2) antibody diluted 100-fold in PBS was applied for 2 h at 37 °C. The cells were then washed three times with PBS, and were incubated with 30-fold diluted Texas Red-conjugated anti-mouse secondary antibody (Amersham Pharmacia Biotech) at 37 °C for 1 h. The coverslips were washed three times with PBS and then mounted on glass slides, using Gelvatol. Confocal-laser-scanning-microscopy observations were carried out with a Bio-Rad MRC 1024 (15-mW argon-krypton laser; Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.) mounted on a Zeiss Axioskop (Jena, Germany) equipped with a $\times 63$ (numerical aperture 1.40) oil-immersion objective, using 488- and 568-nm band-pass filters for GFP and Texas Red respectively.

RESULTS

Identification of ZPR9 as a MPK38-interacting protein

We performed the yeast two-hybrid system to screen a human HeLa cDNA library for clones encoding proteins that interact with the C-terminal region of HPK38. From 5×10^6 clones, we identified 35 positive clones that showed galactose-dependent growth on Leu⁻ selective medium and galactose-dependent β -galactosidase activity, and then subdivided these clones into three groups based on sequence analysis. A novel zinc-finger-like protein, designated ZPR9, was encoded by 27 of the clones. From the remaining clones, no identical genes were evident in the databases. The specificity of the interaction between HPK38 and ZPR9 in yeast was tested using several unrelated proteins available in our laboratory. From the transformants, ZPR9 cDNA was only found to interact with the original selection HPK38 and MPK38, a murine homologue, baits, but not with the other baits containing unrelated sequences (results not shown).

A full-length human ZPR9 was obtained by screening a human normal keratinocyte cDNA library. After three rounds of screening, 12 positive clones were isolated from approx. 1×10^7 plaques. The insert of the longest cDNA (TF2 clone) was sequenced completely on both strands using oligonucleotide primers based on newly acquired sequences (Figure 1). Additionally, the other clone (TF1) was also sequenced to confirm the TF2 clone. The total length of the ZPR9 was 1702 nt. There is a 1434-bp open reading frame from nt positions 132–1565. ZPR9, translated *in vitro*, migrates with an apparent molecular mass of 52 kDa on SDS/PAGE (Figure 2). Sequence analysis using Prosite indicates that ZPR9 contains three putative C2H2 zinc-finger motifs (ZFs) [Prosite pattern ZINC_FINGER_C2H2, formulated as C-X_{2,4}-C-X₃-(F,Y,W,C,L,I,V,M)-X₈-H-X_{3,4,5}-H] located at amino acid positions 69–91 (ZF1), 254–275 (ZF2) and 305–330 (ZF3). ZF1 corresponded well when compared with the typical pattern of the zinc-finger motif, but ZF2 and ZF3 have

GGCACGAGGGAAGTCCGCGTGTGAGTGGCTCTTCTGGCTGCTGAGCGGGTAAAGTCT	60
TGAGGGTCTTGTGGGCGACGCGCTGATCACCAGGTGTTTGGCTTGGTGGTCTTATT	120
TCTCCCTGGCAATGGCGACGTACACCTGCATAACTTGCCTGGTGGCTTCCGCGACGCGG	180
M A T Y T C I T C R V A F R D A	16
ACATGCAGCGGGCCACTATAAGACGGACTGGCACCCTACAACCTCGCGCGAAGGTGG	240
D M Q R A H Y K T D W H R Y N L R R K V	36
CCAGCATGGCCCGACTGACCGCGAGGGCTTCCAGGAGCGGCTCGCGCGCAGCGGCGG	300
A S M A P V T A E G F Q E R V R A Q R A	56
TCCGCGAGGAGGAGCAAGGGCTCGGCCACTACTGCACCTTTCGACGAAGAAGTTTG	360
V A E E E S K G S A T Y C T V C S K K F	76
CCTCTTCAACGCTACGAGAACCACCTCAAGTCCCGCGCTCACGTTGAGCTGGAGAAGA	420
A S F N A Y E N H L K S R R H V E L E K	96
AGGCCGTGCAGGAGTGAATCGGAAAGTGGAGATGATGAATGAAAGAACTTGGAGAAG	480
K A V Q A V N R K V E M M N E K N L E K	116
GACTGGCGTGGACAGTGTGGACAAGGATGCCATGAACGCGGCCATCCAGCAGGCCATCA	540
G L G V D S V D K D A M N A A I Q Q A I	136
AGGCCGCGCTGCATGTCTCCCAAGAGGCGCCCGCGCTGCAAGGAGGAGGCCAGGA	600
K A Q P S M S P K K A P P A P A K E A R	156
ATGTCGTGGCGTGGTACTGGTGGCGTGGGACCCACGACCGGAGCCGAGTGAAGAAC	660
N V V A V G T G G R G T H D R A G S E K	176
CACCCCGCTCCAGTGGTTGAACAGCAGGCGAAGAGTGGCAAGCAGCAGGAGGAGG	720
P P R L Q W F E Q Q A K K L A K Q Q E E	196
ACAGCGAGGAGGAGGAGGAGGACCTGGATGGAGCAGATTGGGAAGATATTGATTCTGATG	780
D S E E E E D L D G D W E D I D S D	216
AAGAATTGGAATGTGAGGATCTGAAGCAATGGACGATGTGGTGGAGCAGGATGCAGAGG	840
E E L E C E D T E A M D D V E Q D A E	236
AGGAAGAGGCTGAGGAAGGCCACCCCTTGGTGCCATCCCTATCACGGACTGCTTATTTT	900
E E E A E E G P P L G A I P I T D C L F	256
GTTCCCATCATCCAGCTCGCTGATGAAGAATGTGGCTCACATGACCAAGACCCAGCATG	960
C S H H S S S L M K N V A H M T K D H S	276
TCTTTATCTCTGATATAGATATCTTTGATATTAAGGACTGATTAATACCTTGGGAG	1020
F F I P D I E Y L S D I K G L I K Y L G	296
AGAAAGTTGGTGTGGCAAGATTTGCTTGGTGGCAAGGAGGAGGAGTCTTCTACT	1080
E K V G V G K I C L W C N E K G K S F Y	316
CCACAGAAGCTGTACAGGCACATATGAATGACAAAGCCACTGTAAGCTCTTCACAGATG	1140
S T E A V Q A H M N D K S H C K L F T D	336
GCGATGCTGCTTGGAAATTTGCAGACTCTATGATTTTAGGAGTAGCTATCCAGATCACA	1200
G D A L E F A D F Y D F R S S Y P D H	356
AGGAAGGGGAGGACCCCAATAAGGCTGAGGAGTGTCCCTCAGAAAGAACTTGGAAATG	1260
K E G E D P N K A E E L P S E K N L E Y	376
ATGATGAAACCATGGAATGATTCTGCCTTCTGGTGCCAGAGTGGGTGATCGCTCCTTGA	1320
D D E T M E L I L P S G A R V G H R S L	396
TGAGATCTACAAACAGCGATTGGCTTGTCAAGAGCTGTGGCAGTGGCCAAAATCGGA	1380
M R Y Y K Q R E G L S R A V A V A K N R	416
AGGCCGTGGGCGGAGTACTTCAGCAGTACAGAGCCCTGGGATGGACTGGCAGCACAGGAG	1440
K A V G R V L Q Q Y R A L G W T G S T G	436
CGGCTCTTATCGGAGAGCGAGCATGCAGTATGCCAAGGATGAAATCAAAATGGATGC	1500
A A L M R E R D M Q Y V Q R M K S K W M	456
TGAAGACAGGAATGAAGAACAATGCCACCAAGCAGATGCACCTTCGGTCCCAAGTGAAT	1560
L K T G M K N N A T K Q M H F R V Q V R	476
TCGAGAGTCTGCTGGGATGAGCAATCATCTCCGCGCCCAAGTTCTCTCTGCTCTGAG	1620
F *	477
GACCACTGAAAGACAGATCATAGGAGAGACCCCTTGTGCTACTTCATTGTTCTGACC	1680
TAATAATAAAAGTTAGAACCAT	1702

Figure 1 Nucleotide and deduced amino acid sequences of the full-length human ZPR9 cDNA

The nucleotide sequence is numbered starting with the first base of the human ZPR9 cDNA (TF2 clone). The corresponding amino acid and amino acid sequence positions are indicated below the nucleotide sequence. The cysteine (C) and histidine (H) residues that form the zinc fingers are underlined and in **bold**. Amino acids are identified by the standard single letter code, and the asterisk indicates the termination codon.

some variations against the typical pattern. A consensus sequence of a polyadenylation recognition site (AATAAA) is located 12 bp upstream of the 3' end. A database search revealed that ZPR9 is novel, but shares a significant similarity with a putative zinc-finger protein C47S (accession number AAK97213, 62% amino acid identity) and a yeast finger protein (accession number S32967, 41% amino acid identity).

Expression of ZPR9

To address whether ZPR9 is expressed in human cell lines, we performed Northern-blot analysis using RNAs obtained from various human cell lines. The full-length 1.7 kb mRNA was expressed in all cells examined (Figure 3A). Even loading and integrity of RNA per lane was confirmed by ultraviolet visualization of ethidium bromide staining (results not shown)

Figure 2 Translation of ZPR9 mRNA *in vitro*

pcDNA indicates the transcription and translation with the pcDNA3 vector alone, as a control, and pcDNA-ZPR9 indicates the transcription and translation with the pcDNA3-ZPR9 cDNA clone. Transcription *in vitro* using T7 RNA polymerase and translation in a reticulocyte system was carried out in accordance with the manufacturer's instructions as described in the Experimental section.

Figure 3 Expression of ZPR9 mRNA in various human cell lines and tissues

Total cytoplasmic RNAs were extracted from the indicated human cell lines (A), and the membrane was probed with a 32 P-labelled ZPR9 cDNA insert. The expression of ZPR9 in various human tissues was examined by Northern-blot analysis of polyadenylated mRNA (B). Autoradiographs at 7 days (A) and 3 days (B) exposure are shown. GAPDH (A) and β -actin (B) mRNAs were used as the internal controls.

and hybridization with labelled GAPDH cDNA. In addition, the 1.7 kb ZPR9 mRNA was widely expressed in various human tissues, with the lowest expression level observed in kidney (Figure 3B).

Figure 4 Interaction of MPK38 with ZPR9 *in vivo*

(A and B), GST alone (pEBG), as a control, pEBG-MPK38 (GST-MPK38) and pEBG-MCAT (GST-MCAT) were co-transfected with pFLAG-ZPR9 (FLAG-ZPR9) into 293T cells. After 48 h, cells were extracted in a lysis buffer containing 0.1% Nonidet P-40. GST-fusion proteins were purified on glutathione-Sepharose beads (GST puri.) and analysed by SDS/PAGE, and the complex formation (GST puri.) and the FLAG-tagged ZPR9 of the amount used for the binding assay *in vivo* (Lysate) were determined by anti-FLAG antibody immunoblot. The same blot was stripped and re-probed with an anti-GST antibody to confirm expression of the GST-fusion proteins [GST-MPK38 (A) and GST-MCAT (B)] and the GST control (GST) (A and B). Abbreviations: WB, Western blot; anti-FLAG, anti-FLAG antibody; anti-GST, anti-GST antibody.

ZPR9 associates with MPK38 *in vivo*

To investigate the association *in vivo* between ZPR9 and MPK38, we performed the co-transfection experiments using GST- and FLAG-tagged eukaryotic expression vectors. In these experiments, MPK38 and ZPR9 were co-expressed as a GST-fusion proteins and a FLAG-tagged protein in 293T cells respectively. The interactions of FLAG-tagged ZPR9 proteins with the GST-MPK38 fusion proteins were analysed by immunoblotting with an anti-FLAG antibody. As shown in Figure 4(A), the FLAG-tagged ZPR9 was co-precipitated with GST-MPK38, but not with control GST alone, indicating that the ZPR9

Figure 5 Phosphorylation of ZPR9 by MPK38

The kinase assays were performed as described in the Experimental section. **(A)** For the kinase assay, 3 μg of recombinant ZPR9 was mixed with 10 μM ATP, 5 μCi of [γ - ^{32}P]ATP and 10 mM MgCl_2 in 20 μl of kinase buffer and incubated with the immobilized MPK38 for 15 min at 37 $^\circ\text{C}$ with frequent gentle mixing. The positions and expression levels for GST-tagged constructs (GST-MPK38 and GST-ZPR9) were monitored by the anti-GST antibody (lower panel). **(B)** 293T cells were transiently transfected with the appropriate expression plasmids, and GST-fusion proteins were purified on glutathione-Sepharose beads (GST puri.). The co-precipitates were incubated with [γ - ^{32}P]ATP, separated by SDS/PAGE, and transferred to PVDF membranes and detected by autoradiography. The arrowheads indicate the positions of the phosphorylated MPK38 and ZPR9 proteins.

proteins physically interact with MPK38 proteins in mammalian cells. To determine which regions of MPK38 contribute to ZPR9 binding, a deletion construct (MCAT) harbouring the N-terminal region of MPK38, comprising the catalytic domain (amino acids 7–269), was generated. GST-tagged MCAT and FLAG-tagged ZPR9 were co-expressed in 293T cells, and a co-precipitation experiment was performed. As demonstrated in Figure 4(B), we were able to detect association of FLAG-tagged ZPR9 with the transfected GST-MCAT in cells, indicating that MPK38 has two independent ZPR9-binding domains: the N-terminal catalytic and C-terminal domains.

MPK38 phosphorylates ZPR9

To determine whether the ZPR9 is a substrate for MPK38 *in vitro*, it was necessary to generate a non-phosphorylated form of

Figure 6 Self-association of ZPR9 proteins *in vivo*

(A) 293T cells were co-transfected with pEBG (GST) or pEBG-ZPR9 (GST-ZPR9), together with pFLAG-ZPR9 (FLAG-ZPR9). ZPR9 was co-precipitated from cell lysates using the glutathione-Sepharose beads (GST puri.) and subsequently separated by SDS/PAGE. The binding of ZPR9 was detected by immunoblotting using anti-FLAG antibody. **(B)** His-tagged ZPR9 plasmid (His-ZPR9) was transiently co-transfected into 293T cells with the vector alone (CMV), as a control, or FLAG-ZPR9, and the cell lysates were immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were analysed for the presence of His-ZPR9 by Western blotting using anti-His antibody. Bands representing co-immunoprecipitating His-ZPR9 by anti-FLAG antibody are indicated (upper left panel, IP: α -FLAG). The amount of immunoprecipitated FLAG-ZPR9 was analysed using anti-FLAG antibody (lower panel). Total cell lysates were analysed for His-ZPR9 (upper right panel, Lysate). Abbreviations: WB, Western blot; IP, immunoprecipitation.

the ZPR9 that could be used in the kinase assay. The recombinant ZPR9 protein was expressed in *E. coli*, purified and then used as a substrate for the MPK38 kinase assay. Extracts from 293T cells expressing GST-tagged MPK38 were purified with glutathione-Sepharose beads, and incubated with [γ - ^{32}P]ATP to allow the phosphorylation of the recombinant ZPR9 protein. As shown in Figure 5(A), the phosphorylation of ZPR9 was observed. However, we did not detect its phosphorylation by the ZPR9 alone, as a control (results not shown). To determine whether MPK38 phosphorylates ZPR9 within cells, we performed co-transfection experiments using GST-MPK38 and FLAG-tagged ZPR9. Transfected cells were extracted and purified with glutathione-Sepharose beads, and the co-precipitates were incubated with [γ - ^{32}P]ATP. The ^{32}P -labelled proteins were resolved by SDS/

Figure 7 Subcellular localization of ZPR9

293T cells were transfected with either FLAG-tagged ZPR9 alone (**A**), as a control, or FLAG-tagged ZPR9 and GFP-MPK38 (**B**). Cells were immunostained with the anti-FLAG (M2) monoclonal antibody, followed by Texas Red-conjugated anti-mouse secondary antibody, and analysed by confocal microscopy.

PAGE. As shown in Figure 5(B), GST-MPK38 phosphorylated FLAG-ZPR9, whereas the GST alone, as a control, did not phosphorylate FLAG-ZPR9.

Self-association of ZPR9 *in vivo*

To verify the interaction specificity of ZPR9, the ZPR9 library plasmid was rescued from the galactose-dependent *Leu⁺/lacZ⁺* yeast and re-introduced into the original selection HPK38 bait strain, as well as the other strains containing different baits available, including ZPR9 bait. From the transformants, ZPR9 cDNA was found to interact with the HPK38, as well as the ZPR9 (results not shown). This finding raised the question of a potential homodimeric interaction of ZPR9. To determine

whether the self-association of ZPR9 could occur *in vivo*, GST- and FLAG-tagged ZPR9 were expressed in 293T cells. GST-ZPR9 was purified with glutathione-Sepharose beads, and co-precipitation of FLAG-ZPR9 was observed by Western blotting using a monoclonal antibody against the FLAG-tag. As shown in Figure 6(A), the FLAG-tagged ZPR9 was co-precipitated with GST-ZPR9, but not with control GST alone. To further verify the self-interaction of ZPR9 *in vivo*, we performed co-immunoprecipitation experiments using 293T cells transiently co-transfected with the vector alone or FLAG-ZPR9, together with His-tagged ZPR9. FLAG-ZPR9 was immunoprecipitated from cell lysates, and Western-blot analysis showed that FLAG-ZPR9 was precipitated (Figure 6B). The binding of His-ZPR9 was subsequently analysed using Western blotting with an anti-His antibody and His-ZPR9 was present in the FLAG-ZPR9 immunoprecipitate (Figure 6B). These results suggest that ZPR9 proteins physically interact with each other in mammalian cells.

Redistribution of ZPR9 from cytoplasm to nucleus by MPK38 expression

To test the hypothesis that MPK38 could modify ZPR9 movement, 293T cells were transfected with FLAG-tagged ZPR9 alone or together with GFP-MPK38. Cells expressing ZPR9 exhibit both cytosolic and nuclear staining, but the ZPR9 protein was predominantly distributed in the cytoplasm of 293T cells (> 90 % of positively stained cells). Co-expression of ZPR9 with MPK38 resulted in nuclear localization of the ZPR9 protein, with an average increase of 1.8-fold in the four experiments (Figure 7). However, MPK38 proteins were only detected in the cytoplasm in the presence or absence of ZPR9 (results not shown). Taken together, these results demonstrate that MPK38 expression redirects ZPR9 from the cytoplasm to the nucleus.

DISCUSSION

In the present study, we have identified a novel zinc-finger-like protein, ZPR9, which interacts with MPK38, a new member of the SNF1 kinase family, and demonstrated the association *in vivo* of ZPR9 with MPK38, in addition to the self-association of ZPR9. Furthermore, we show that ZPR9 moves to the nucleus following its co-expression with MPK38, implying that the phosphorylation of ZPR9 by MPK38 might be associated with translocation of ZPR9.

Analysis of the amino acid sequence revealed that the N-terminus of MPK38 contains all the sequence motifs typical of protein kinases [12] and the HRDLKPEN¹³⁷ motif for the protein serine/threonine kinase family, rather than the protein tyrosine kinase family-associated sequence [2]. In addition, a leucine-zipper motif consisting of a periodic repetition of leucine residues at every seventh residue was found within the N-terminal catalytic domain [1]. This motif has been observed in numerous DNA-binding proteins and is thought to be involved in protein-DNA interactions, and potentially in protein-protein interactions [13–15]. It has been shown that the N-terminus of bovine cGMP-dependent protein kinase contains a leucine-isoleucine-zipper motif and the active form of this kinase is dimeric [15]. Thus the presence of the leucine-zipper motif in MPK38 raises the intriguing possibility that MPK38 represents a novel serine/threonine protein kinase, which might be involved in the formation of homo- and heterodimeric complexes. This concept is supported by the finding that the MCAT construct, harbouring the N-terminal catalytic domain of MPK38, con-

taining the leucine-zipper motif, could associate with the ZPR9 (Figure 4B), together with the presence of a homodimer formation in the bovine cGMP-dependent protein kinase containing a leucine-isoleucine-zipper motif [15]. In addition, based on the observed result (Figure 4A) and identification of several proteins that interact with the C-terminal region of MPK38 (results not shown), it is possible that the C-terminal region of MPK38, lacking the leucine-zipper motif, is also involved in the interaction of MPK38 and other cellular proteins. However, further studies will be necessary to determine whether this complex formation can affect the catalytic activity.

We isolated and characterized a cDNA clone for a new member of the zinc-finger family of proteins, which we named ZPR9. A zinc-finger motif is a sequence-specific DNA-binding motif that is generally involved in the specific activation or repression of a downstream target gene. Zinc-finger motifs of the Cys₂-His₂ type have been found in numerous transcription factors, such as human Wilms tumour WT1 that is involved in sex differentiation of the gonads [16], a novel nucleoplasmic protein [17] and RIN ZF protein (where RIN refers to a rat insulinoma cell line) [18]. In addition, this zinc-finger motif has been recognized in several proteins that may modulate receptor signalling, such as ZPR1 [19], tumour necrosis factor receptor-associated factor [20–22], CD40 receptor-associated factor [21–23], enigma [24] and LMP1-associated protein [25]. Recently, the specific expression of genes encoding zinc-finger proteins during spermatogenesis and the potential for the regulation of transcription by these proteins have also been reported [26]. Formation of homo- and heterodimerization in many transcription factors results in an increased diversity and specificity in the regulation of gene expression. In this respect, the self-association of ZPR9, containing zinc-finger motifs (Figure 6), and the interaction of ZPR9 with MCAT (Figure 4B), a deletion mutant consisting of kinase catalytic domain of MPK38, provided an interesting aspect to the regulation of this factor. Thus in addition to the homodimer formation of ZPR9, it is possible to hypothesize that heterodimers will also form, allowing the production of dimeric factors with novel DNA-binding specificities or affinities.

Many transcription factors are modified by phosphorylation of specific residues in transcription factors [27,28]. The most direct example of such an effect is seen in the case of gene activation by several cytokines such as interferons, IL-2, IL-4, IL-6, IL-10 and IL-12. The binding of interferon to its receptor resulted in activation of an associated tyrosine kinase ('JAK') activity, leading to phosphorylation of the STAT (signal transduction and activators of transcription) transcription factor, allowing it to move to the nucleus and stimulate interferon-responsive genes [29,30]. In a similar fashion, cytosolic Smad transcription factors are activated by receptor serine/threonine kinases that bind growth factors in the transforming growth factor- β superfamily [31]. By virtue of its ability to associate with MPK38, ZPR9 defines a novel class of putative signal transducers. Although a putative nuclear localization sequence with significant matches in the PROSITE database is not found, the cytoplasmic ZPR9 proteins accumulate in the nuclei of the cell co-expressing MPK38 (Figure 7), suggesting that these proteins may participate in signal transduction to the nucleus. These results strongly indicate a possible role for phosphorylation of ZPR9 proteins in their translocation to the nucleus since MPK38 proteins were only found to be cytosolic after co-expression of ZPR9 (results not shown), but do not rule out the possibility that other proteins that interacted with ZPR9 are also involved in the translocation, because we are able to detect a significant stimulation in the nuclear translocation of ZPR9 after transfection of

other putative ZPR9-interacting proteins (results not shown). However, their functional role and mechanism of action in MPK38-mediated signalling are presently unresolved. Furthermore, the potential interaction of ZPR9 with other cellular proteins remains to be investigated. Thus extensive efforts to define the complex formation of ZPR9 with MPK38, and further identification and characterization of the downstream targets, will be required to clarify the mechanism of MPK38-signal transduction, as well as the biological functions of ZPR9.

This work was supported by Korea Research Foundation Grant (KRF-1998-019-D00147) and by a grant (00-J-LF-01-B-76) from the Ministry of Science and Technology.

REFERENCES

- Heyer, B. S., Warsowe, J., Solter, D., Knowles, B. B. and Ackerman, S. L. (1997) New member of the Snf1/AMPK kinase family, Melk, is expressed in the mouse egg and preimplantation embryo. *Mol. Reprod. Dev.* **47**, 148–156
- Gil, M., Yang, Y., Lee, Y., Choi, I. and Ha, H. (1997) Cloning and expression of a cDNA encoding a novel protein serine/threonine kinase predominantly expressed in hematopoietic cells. *Gene* **195**, 295–301
- Gil, M., Yang, Y. and Ha, H. (1998) MPK38 expression is upregulated in immature T cells activated by concanavalin A. *Immunol. Lett.* **64**, 79–83
- Heyer, B. S., Kochanowski, H. and Solter, D. (1999) Expression of Melk, a new protein kinase, during early mouse development. *Dev. Dyn.* **215**, 344–351
- Evans, R. M. and Hollenberg, S. M. (1988) Zinc fingers: guilt by association. *Cell (Cambridge, Mass.)* **52**, 1–3
- Diakun, G. P., Gairall, L. and Klug, A. (1986) EXAFS study of the zinc-binding sites in the protein transcription factor IIIA. *Nature (London)* **324**, 698–699
- Freemont, P. S. (1993) The RING finger. A novel protein sequence motif related to the zinc finger. *Ann. NY Acad. Sci.* **684**, 174–192
- Jung, H., Kim, T., Chae, H. Z., Kim, K.-T. and Ha, H. (2001) Regulation of macrophage migration inhibitory factor and thiol-specific antioxidant protein PAG by direct interaction. *J. Biol. Chem.* **276**, 15504–15510
- Kim, T., Jung, H., Min, S., Kim, K.-T. and Ha, H. (1999) B-myc proto-oncogene products interact *in vivo* with each other via the carboxy-terminal conserved region. *FEBS Lett.* **460**, 363–368
- Nagase, T., Seki, N., Tanaka, A., Ishikawa, K. I. and Nomura, N. (1996) Prediction of the coding sequences of unidentified human genes: The coding sequences of 40 new genes (KIAA0161–KIAA0200) deduced by analysis of cDNA clones from human cell line KG1. *DNA Res.* **3**, 17–24
- Yang, Y., Gil, M., Choi, E. Y., Park, S. H., Pyun, K. H. and Ha, H. (1997) Molecular cloning and chromosomal localization of a human gene homologous to the murine R-PTP-kappa, a receptor-type protein tyrosine phosphatase. *Gene* **186**, 77–82
- Hanks, S. K., Quinn, A. M. and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science (Washington, D.C.)* **241**, 42–52
- Landschulz, W. H., Johnson, P. F. and McKnight, S. L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science (Washington, D.C.)* **240**, 1759–1764
- Forman, B. M., Yang, C. R., Au, M., Casanova, J., Ghysdael, J. and Samuels, H. H. (1989) A domain containing leucine-zipper-like motifs mediate novel *in vivo* interactions between the thyroid hormone and retinoic acid receptors. *Mol. Endocrinol.* **3**, 1610–1626
- Atkinson, R. A., Saudek, V., Huggins, J. P. and Pelton, J. T. (1991) 1H NMR and circular dichroism studies of the N-terminal domain of cyclic GMP dependent protein kinase: a leucine/isoleucine zipper. *Biochemistry* **30**, 9387–9395
- Gessler, M., Poustka, A., Cavenee, W., Neve, R. L., Orkin, S. H. and Bruns, G. A. (1990) Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature (London)* **343**, 774–778
- Inagaki, H., Matsushima, Y., Nakamura, K., Ohshima, M., Kadowaki, T. and Kitagawa, Y. (1996) A large DNA-binding nuclear protein with RNA recognition motif and serine/arginine-rich domain. *J. Biol. Chem.* **271**, 12525–12531
- Tillotson, L. G. (1999) RIN ZF, a novel zinc finger gene, encodes proteins that bind to the CACC element of the gastrin promoter. *J. Biol. Chem.* **274**, 8123–8128

- 19 Galcheva-Gargova, Z., Konstantinov, K. N., Wu, I.-H., Klier, F. G., Barrett, T. and Davis, R. J. (1996) Binding of zinc finger protein ZPR1 to the epidermal growth factor receptor. *Science* (Washington, D.C.) **272**, 1797–1802
- 20 Rothe, M., Wong, S. C., Henzel, W. J. and Goeddel, D. V. (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* (Cambridge, Mass.) **78**, 681–692
- 21 Hu, H. M., O'Rourke, K., Boguski, M. S. and Dixit, V. M. (1994) A novel RING finger protein interacts with the cytoplasmic domain of CD40. *J. Biol. Chem.* **269**, 30069–30072
- 22 Cheng, G., Cleary, A. M., Ye, Z. S., Hong, D. I., Lederman, S. and Baltimore, D. (1995) Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science* (Washington, D.C.) **267**, 1494–1498
- 23 Ishida, T., Tojo, T., Aoki, T., Kobayashi, N., Ohishi, T., Watanabe, T., Yamamoto, T. and Inoue, J. (1996) TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 9437–9442
- 24 Wu, R.-Y. and Gill, G. N. (1994) LIM domain recognition of a tyrosine-containing tight turn. *J. Biol. Chem.* **269**, 25085–25090
- 25 Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C. and Kieff, E. (1995) The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* (Cambridge, Mass.) **80**, 389–399
- 26 Inoue, A., Ishiji, A., Kasagi, S., Ishizuka, M., Hirose, S., Baba, T. and Hagiwara, H. (2000) The transcript for a novel protein with a zinc finger motif is expressed at specific stages of mouse spermatogenesis. *Biochem. Biophys. Res. Commun.* **273**, 398–403
- 27 Hunter, T. and Karin, M. (1992) The regulation of transcription by phosphorylation. *Cell* (Cambridge, Mass.) **70**, 375–387
- 28 Hill, C. S. and Treisman, R. (1995) Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* (Cambridge, Mass.) **80**, 199–211
- 29 Darnell, Jr, J. E., Kerr, I. M. and Stark, G. R. (1994) Jak–STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* (Washington, D.C.) **264**, 1415–1421
- 30 Ihle, J. N. and Kerr, I. M. (1995) Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends Genetics* **11**, 69–74
- 31 Massague, J. (1998) TGF- β signal transduction. *Annu. Rev. Biochem.* **67**, 753–791

Received 25 July 2001/15 October 2001; accepted 12 November 2001