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Analysis of the function of protein kinase PKN under heat stress

Kitagawa, Michinori

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博士論文

Analysis of the function of protein kinase PKN under heat stress

熱ストレス下における蛋白質燐酸化酵素 PKN の機能解析

平成 11 年 1 月

神戸大学大学院自然科学研究科

北川 道憲

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ABBREVIATIONS

ATP	adenosine triphosphate
MAPK	mitogen-activated protein kinase
ERK	extracellular signal regulated kinase
MEK	MAPK /ERK kinase
MEKK	MEK kinase
HSE	heat shock response element
HSF	heat shock transcription factor
HSP	heat shock protein
PKC	protein kinase C
GST	glutathione S-transferase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
DTT	dithiothreitol
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
PBS	phosphate-buffered saline
PVDF	polyvinylidene difluoride
NDGA	nordihydroguaiaretic acid

INTRODUCTION

1. Protein kinases

Protein kinases are defined as enzymes that transfer a phosphate group from a phosphate donor, ATP, onto an acceptor amino acid, such as serine, threonine, and tyrosine in a substrate protein. The transference of a phosphate group, so-called phosphorylation, is known as an important step for the regulation of the substrate protein (Fig. 1), playing crucial roles in signal transduction pathways of metabolism, gene expression, cell growth, cell motility, cell differentiation, and cell division (Hunter and Cooper, 1986; Edelman et al., 1987). To elucidate these signal transduction pathways and how the transducers are regulated, it is necessary to know the physiological substrates for individual protein kinases. One factor involved in substrate selection is substrate structure, a substrate specificity well investigated *in vitro*. However, these studies also demonstrate that nonphysiological substrates can be modified, suggesting that additional mechanisms are needed to restrict protein kinase substrates in *vivo*. One of the additional mechanisms is the restricted subcellular distribution of the protein kinase activity. Recent studies show that protein kinases are targeted to specific compartments of cells and are translocated dynamically during many cellular events (Inagaki et al., 1994). Thus, to understand how the protein kinase of interest phosphorylates the physiological substrate, we have to know about subcellular distribution of the protein kinase besides regulation of the protein kinase activity.

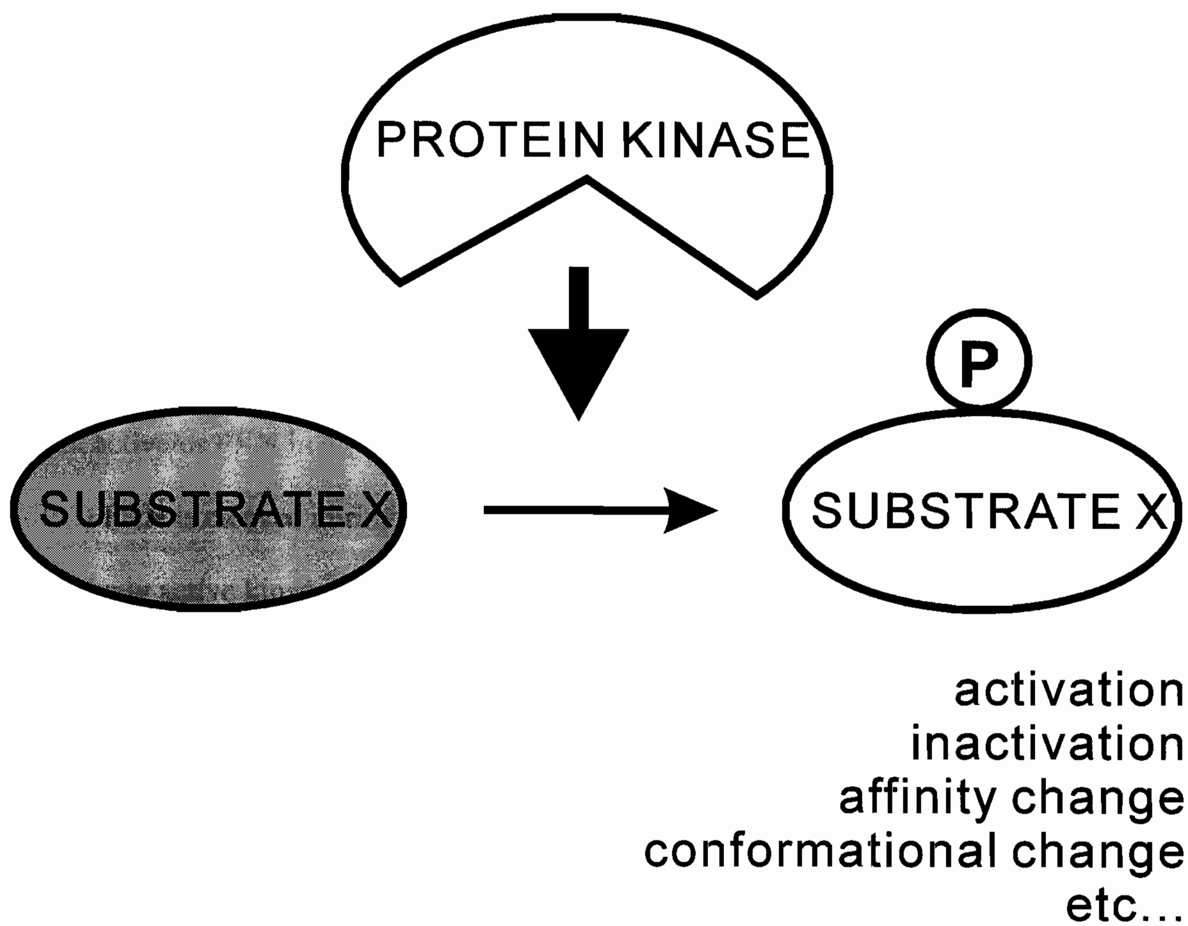


Fig. 1. Schematic diagram of protein phosphorylation. Protein kinases are regulated by a variety of signals which lead to activation, inactivation, affinity change, or conformational change of the target substrate.

2. PKN

PKN is a serine/threonine protein kinase, having a catalytic domain homologous to PKC family in the carboxyl terminus and a unique regulatory region containing repeats of leucine-zipper like sequence in the amino terminus (Mukai and Ono, 1994; Fig. 2) and widely distributed in tissues and organs of mammals and other organisms (Mukai et al., 1995; Kitagawa et al., 1995). The amino-terminal region plays a role in the regulation of PKN and the suppressive effect of the amino-terminal region is suggested by the following observations: 1) truncation of the amino-terminal region by limited proteolysis results in the activation of PKN (Mukai et al., 1994); 2) the amino-terminal region contains the binding site for a small GTPase Rho that activates PKN in a GTP-dependent manner (Amano et al., 1996; Watanabe et al., 1996); 3) PKN is cleaved during apoptosis presumably by caspase-3, which generates a constitutively active kinase fragment (Takahashi et al., 1998). This suppression is also supposed to be cancelled or reduced by unsaturated fatty acids such as arachidonic acid (Mukai et al., 1994; Kitagawa et al., 1995), detergents (Kitagawa et al., 1995), and phosphoinositides such as phosphatidylinositol 4, 5-bisphosphate (Palmer et al., 1995). Although the knowledge for the potential regulators of PKN is accumulating, little is known about its physiological target proteins in most tissues. We have reported that PKN associates with and phosphorylates intermediate filament proteins such as neurofilament and vimentin, and proposed that PKN selects its substrates or localization with the N-terminal unique sequence of the enzyme (Mukai et al., 1996; Matsuzawa et al., 1997). Moreover, PKN associates with α-actinin, an actin cross-linking protein, in a phosphatidylinositol 4,5-bisphosphate-dependent manner *in vitro* (Mukai et al., 1996). Together with that RhoA and phosphoinositides are thought to be primarily involved in the organization of actin cytoskeleton, some target proteins of PKN may reside in the cytoskeletal components (Fig. 3). On the other hand, immunostaining with

fibroblasts revealed that the translocation of PKN from the cytosol to the nucleus was induced by stress such as heat shock, sodium arsenite, and serum starvation (Mukai et al., 1996; Fig. 4). This observation raises the possibility that PKN mediates signals responding to these stresses to the nucleus, and that PKN has a target protein also in the nucleus as well as MAPK cascade, a well established signal pathway mediating several growth factors and stresses from plasma membrane to nucleus (Cano and Mahadevan, 1995; Fig. 5).

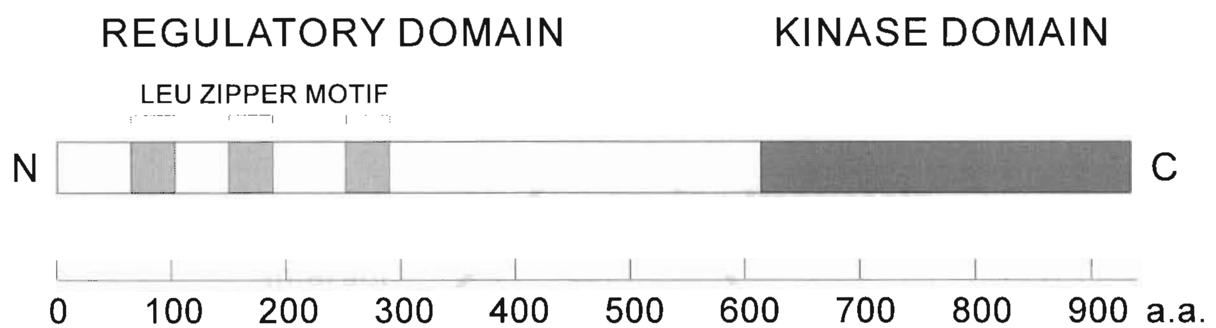


Fig. 2. Structure of PKN. PKN is a serine/threonine protein kinase, having a catalytic domain in the carboxyl terminus and a unique regulatory region containing repeats of leucine-zipper like sequence in the amino terminus

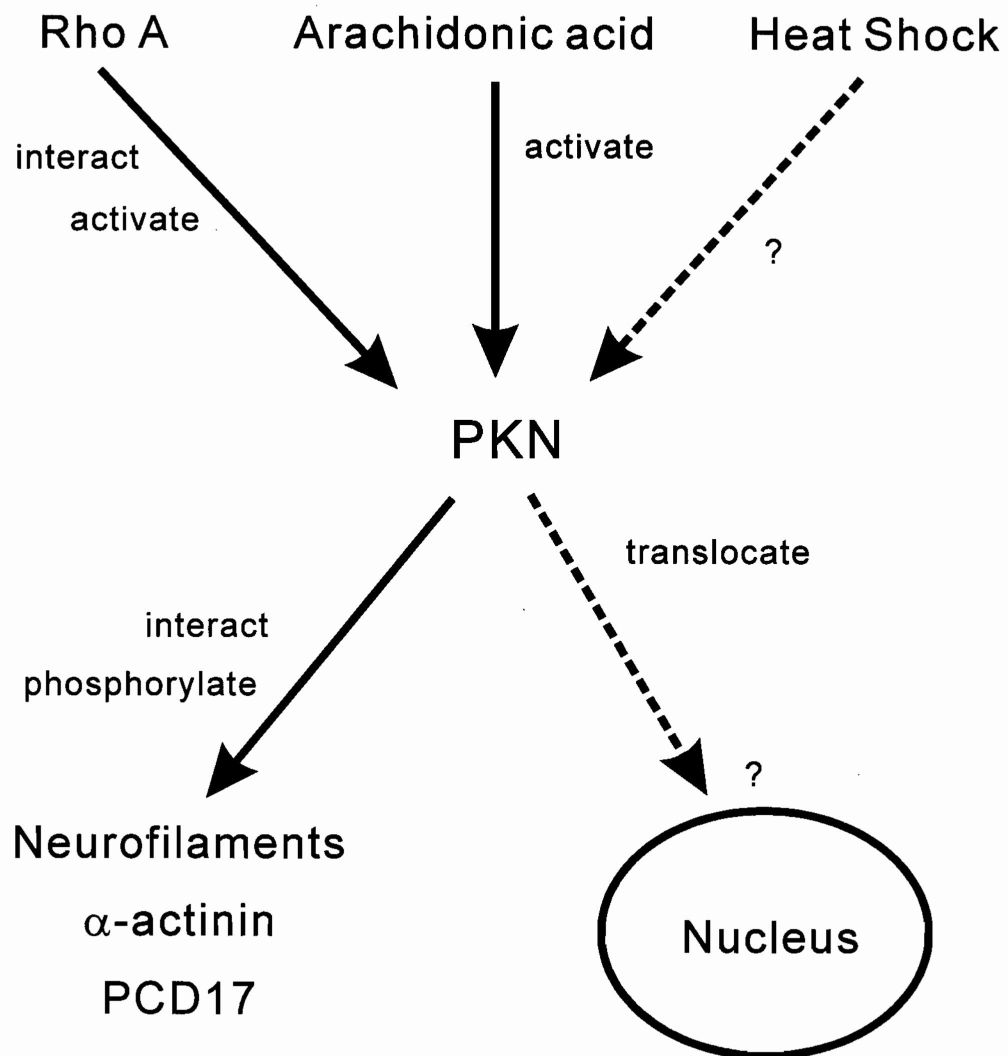
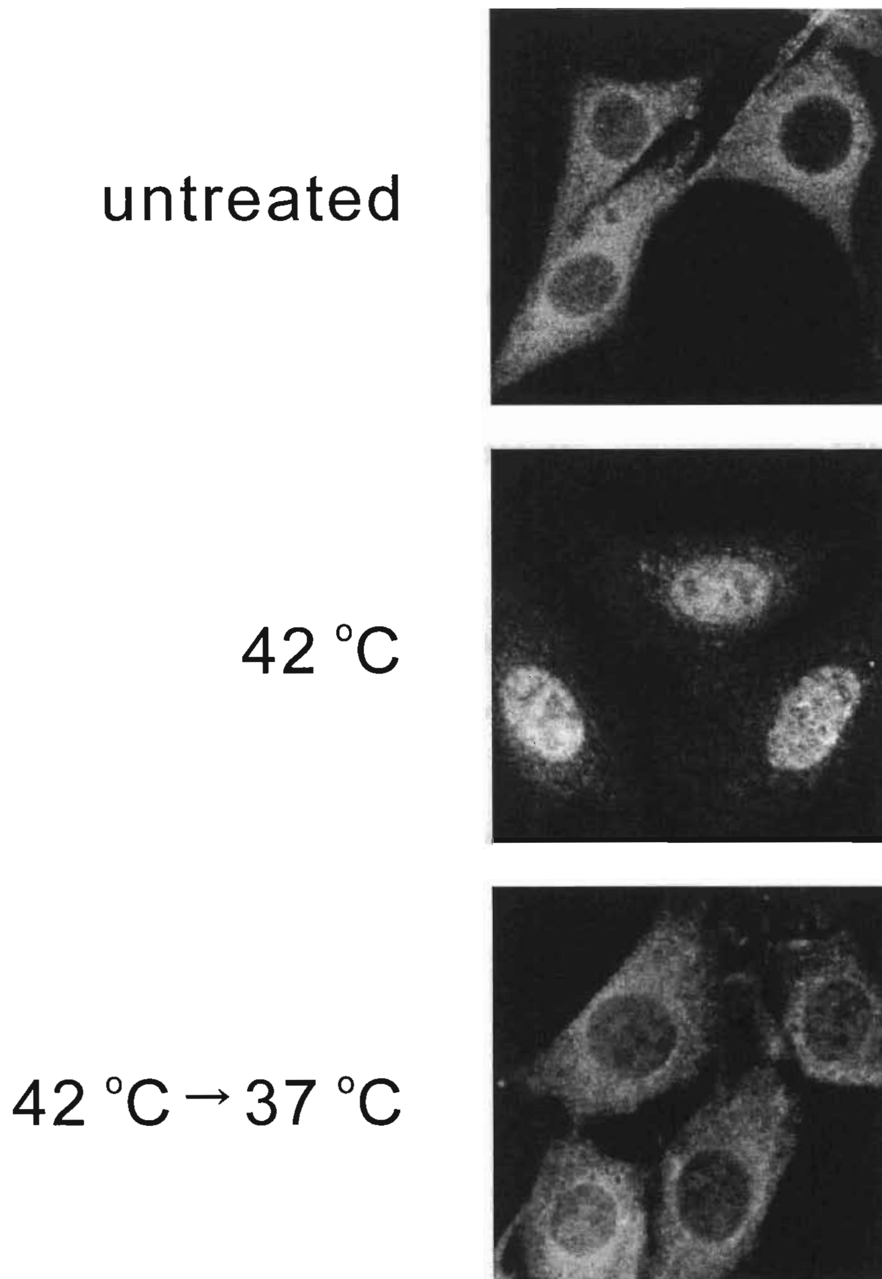


Fig. 3. Activators and targets of PKN.



Mukai et al. (1996) *Proc. Natl. Acad. Sci. USA*

Fig. 4. Translocation of PKN by heat stress. Immunofluorescence staining of PKN. NIH/3T3 cells in normal condition (untreated) or incubated at 42 °C for 90 min (42 °C) or incubated at 37 °C for 240 min following 90 min-heat shock (42 °C→37 °C) were immunostained by anti PKN antiserum.

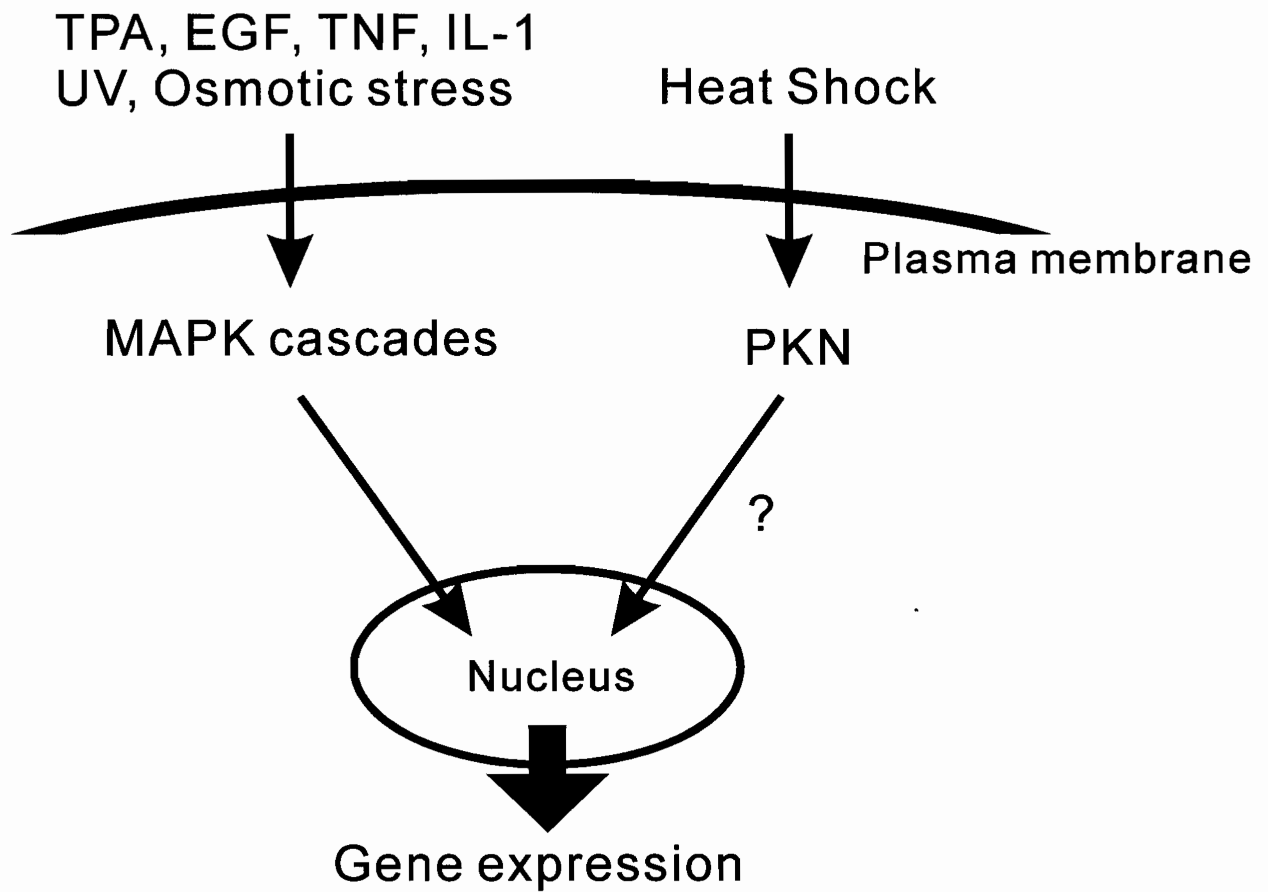


Fig. 5. Signal transduction of stress. MAPK cascades are known to mediate stress and cytokine-induced signal transduction from the cell surface to the nucleus.

3. Heat shock

Exposure of cells to heat shock or to chemical and physiological stresses leads to the heat shock response in which the induction of a cohort of heat shock proteins (HSPs) is accompanied by the expression of stress resistance (Lindquist and Craig, 1988; Georgopolis and Welch, 1993). In mammalian cells, heat shock genes are regulated at the transcriptional level by heat shock factor 1 (HSF1), a sequence-specific transcription factor that binds to heat shock elements (HSE) in their promoters, requiring at least two distinct regulatory steps for its activation (Fig. 6). In the first step, cytoplasmic, non-DNA-binding HSF1 is converted to a homotrimer that is DNA-binding. This homotrimeric factor translocates to the nucleus and binds to HSE sequences but is incapable of enhancing transcription. In a second step, the inactive HSF1 trimer is converted to the active transcription factor. Support for this activation step comes from the following observations: (1) exposure to sodium salicylate triggers only the first steps (Jurivich et al., 1992); (2) the overexpression of HSF1 bypasses the first step of the activation and the overexpressed HSF1 is present in a transcriptionally inert, oligomeric form in the nuclear fraction (Xia et al., 1995). It is also well known that HSF1 becomes hyperphosphorylated after heat shock or when cells are exposed to one of chemical stresses (Wu, 1995). Inhibitors of Ser/Thr kinases partially inhibit heat-induced hyperphosphorylation of HSF1 and prevent increased HSPs expression but do not affect HSF1 trimerization and HSE-binding activity (Xia and Voellmy, 1997), suggesting that one or more kinases involved in HSPs expression and induced phosphorylation is required for the activation of HSF1. Although several kinases, such as PKCα, GSK3α (Chu et al., 1998), MAPK, GSK3β (Chu et al., 1996), and ERK1/ERK2 (Knauf et al., 1997), have been shown to phosphorylate HSF1 directly, the phosphorylation repressed its transcriptional activity and it is still unclear what kinase phosphorylates and activates HSF1.

As PKN translocates to the nucleus in response to heat shock, I have examined the potential role of PKN in regulation of HSPs expression via HSF1. The results indicate that catalytically active PKN and HSF1 cooperatively induce the expression of αB-crystallin and the transcriptional activation is mediated by the promoter region of αB-crystallin.

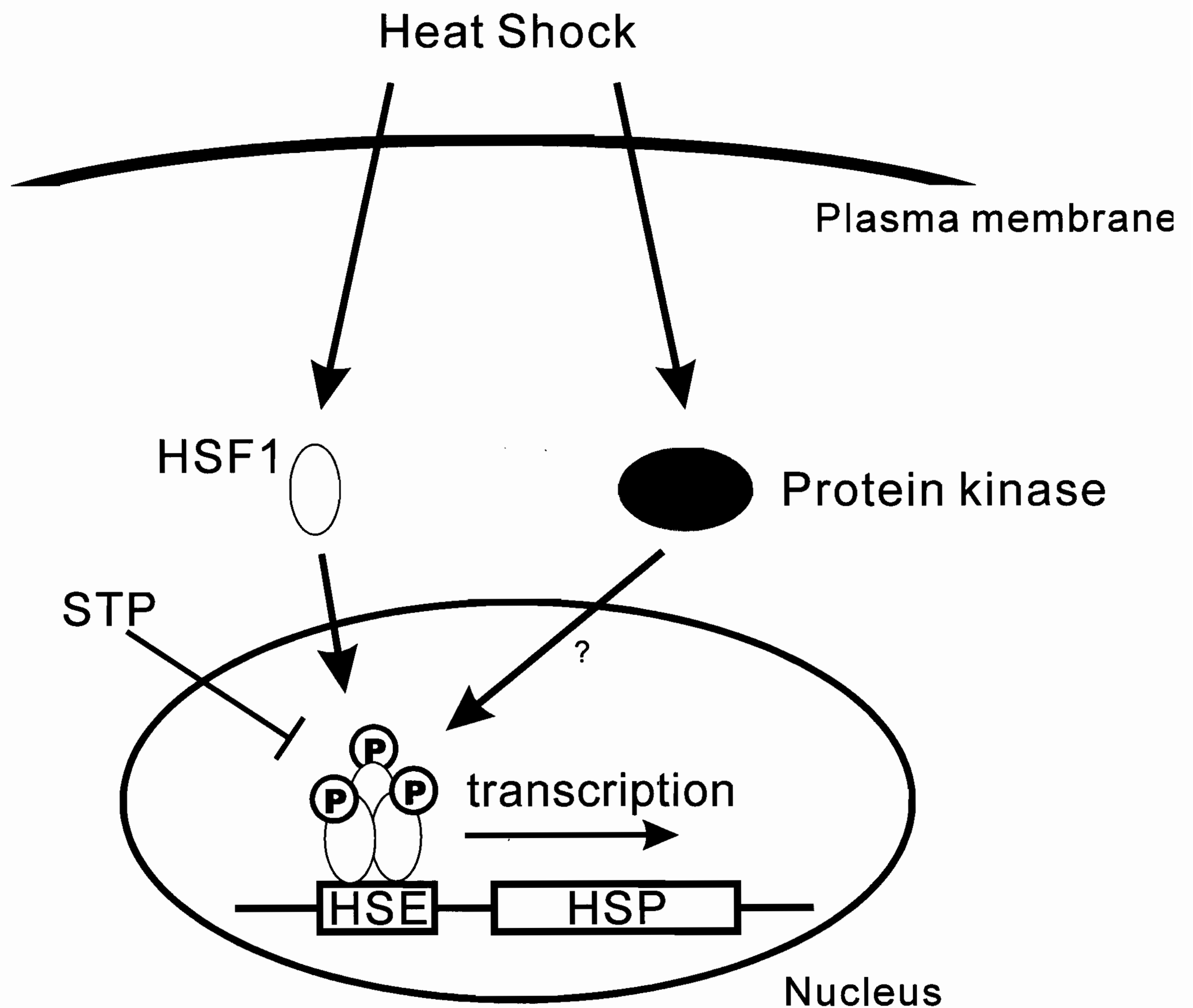


Fig. 6. Scheme of HSF1-mediated signal transduction in response to heat stress. Heat stress induces nuclear translocation, trimerization, HSE-binding and transcriptional activation of HSF1 accompanied by its phosphorylation.

EXPERIMENTAL PROCEDURES

Materials

Anti-FLAG (M2) monoclonal antibody was purchased from Kodak Co., USA. Anti-HSP70 (C92F3A-5) and anti-HSP27 (G3.1) monoclonal antibodies, and anti-αB-crystallin polyclonal antibody were purchased from Stressgen Biotechnologies Corp., Canada Anti-human HSF1 antiserum was kindly provided by Dr. A. Nakai (Kyoto University, Japan).

Plasmid constructions

Plasmid pTB701/HSF1 to express human HSF1 in mammalian cells was constructed by inserting the *Eco*RI fragment of pGEM-Aki5 (Nakai et al., 1997) into pTB701 (Ono et al., 1988). Mammalian expression plasmids pRc/CMV/PKN/FL and pRc/CMV/PKN/AF3/FL encoding FLAG-tagged full length of PKN and the catalytically active fragment of PKN (AF3, amino acids 561-942), respectively, were constructed as described previously (Takahashi et al., 1998). An expression plasmid of the protein kinase-negative mutant of AF3 (pRc/CMV/AF3(K644E)/FL) was constructed by substituting Glu for Lys^{644} using QuickChange site-directed mutagenesis kit (Stratagene, USA). Tetracyclin-regulated mammalian expression plasmids pTRE/PKN/AF3/FL and pTRE/PKN/AF3(K644E)/FL were constructed as follows. The plasmids pRc/CMV/PKN/AF3/FL and pRc/CMV/PKN/AF3(K644E)/FL were digested with *Hind*III, blunted, and ligated with *Xba*I linker, then digested with *Xba*I. The resultant fragment was inserted into the XbaI site of pTRE, containing a tetracyclin-responsive promoter (CLONTECH, USA). pCry-Luc, a reporter plasmid for αB-crystallin promoter (- 540 to + 45), was constructed as follows. Human αB-crystallin promoter region (Dubin et al., 1989) was amplified by PCR from human placenta genomic DNA using the primers: 5'-ACGCGTTTGCACGTTTCCACACCTCATT-3' and 5'-AGATCTAGGTGAGTGTGAGGGGTCAGCT-3'. This fragment was digested with *Bam*HI

and blunted, then digested with *Mlu*I. The resultant fragment was cloned into the *Sma*I/*Mlu*I site of pGVB containing the firefly luciferase gene (Wako Pure Chemical, Japan). pHB-Luc (Taira et al., 1997), possessing the human HSP70 upstream region linked to the firefly luciferase gene, was kindly provided by Dr. H. Ariga, Hokkaido University. Several deletion mutants of the human αB-crystallin promoter region were generated using a Kilo-Sequencing Deletion Kit (TAKARA-shuzo, Japan).

Cell cultures, heat shock treatment, transfections, and luciferase assay

HeLa S3 cells and C6 glioma cells (obtained from RIKEN Cell Bank, Japan) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin, in a humid 37 °C chamber containing 5 % CO_2. HeLa Tet-Off cells (purchased from CLONTECH, USA) were cultured in the medium additionally supplemented with 1 μg/ml tetracyclin. For heat shock treatment, monolayers of the cells were heated at 43 °C for 60 min, then incubated at 37 °C for 8 hours. For immunoblotting, the cells were plated in 35 mm petri dish at 3×10^5 cells/dish and allowed to recover for 1 day, and then co-transfected with 1 μg of the protein kinase expression constructs and 1 μg of the pTB701/HSF1 using Trans-IT (TAKARA-shuzo, Japan) as described in the manufacture's protocol. For luciferase assay, the cells were plated as described above, and then co-transfected with 1 μg of the firefly luciferase reporter constructs, 1 μg of the protein kinase expression constructs, 1 μg of the pTB701/HSF1, and 10 ng of pRL-SV40 plasmid (which contains an SV40 promoter upstream of the renilla luciferase gene, Promega, USA). Cells were harvested after incubation at 37 °C for 24 hours, lysed, and each luciferase activity for the firefly and for the renilla luciferase reporter was measured with the Dual Luciferase Kit of Promega, using Lumat LB9507 luminometer (Berthold Australia). Firefly luciferase values were standardized to renilla values.

Immunoblotting

Samples were separated by SDS-PAGE, and the proteins were transferred to a PVDF membrane. In the case of immunoblotting against HSF1, the proteins were transferred to a nitrocellulose membrane. The membrane was incubated with the antiserum, and the immunoreactive bands were visualized with an Enhanced Chemiluminescence immunoblotting detection kit (Amersham, UK).

Expression and purification of recombinant PKN

The expression and purification of recombinant GST-PKN/AF3 was made as described (Yoshinaga et al., submitted). In brief, the recombinant baculovirus was generated from pBlueBacHis/GST/PKN/AF3 and the recombinant protein was purified from Sf9 cells expressing recombinant GST-PKN/AF3 using glutathione Sepharose 4B (Pharmacia).

Kinase assay

Purified GST-PKN/AF3 (~10 ng) was incubated for 5 min at 30 °C in a reaction mixture (final volume 200 μl) containing 20 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 20 μM ATP, 18.5 kBq of [γ^{32}P]ATP, 100 μM δPKC peptide as a phosphate acceptor, and staurosporine as indicated in each experiment. Reaction was started by addition of the enzyme and terminated by spotting onto Whatman P81 paper. The paper was washed in 75 mM phosphate five times. Incorporation of ^{32}P phosphate into phosphate acceptors was assessed by scintillation counting.

Gel shift assay

The gel shift assay of HSF1 was made as following. HeLa S3 cells were extracted in a buffer containing 20 mM HEPES, pH 7.9, 25 % glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2

mM EDTA, 0.5 mM PMSF and 0.5 mM DTT and centrifuged at 100,000 x g for 5 min. The protein concentration of the supernatant was estimated with Protein Assay Kit (Bio-Rad Laboratories, USA). Labeling of HSE oligonucleotide was made as described by Mosser et al. Ten μg of extract was mixed 0.1 ng of a ^{32}P labeled HSE oligonucleotide and 0.5 μg of poly (dI-dC) in a binding buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 5 % glycerol in a final volume of 25 μl. Binding reactions were incubated for 20 min at 25 °C, and directly loaded onto a 4 % polyacrylamide gel. Gels were fun at room temperature for 2.5 hr at 100 V, dried, and the gel shift was visualized with an imaging analyzer (Fuji BAS1000).

Immunofluorescence

For immunofluorescence localization of overexpressed PKN and PKN/AF3, COS7 cells grown in 35-mm plastic tissue culture dishes were washed twice with phosphate-buffered saline (PBS), fixed for 1 hr at 4 °C in 4 % paraformaldehyde, rinsed with PBS, and then blocked for 1 hr in PBS-T (PBS containing 0.05 % Triton X-100) containing 5 % normal goat serum. After washing with PBS, cells were incubated overnight at 4°C with each antiserum diluted with PBS-T at ~10 μg/ml. Coverslips were rinsed with PBS-T, and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti rabbit IgG (Medical and biological laboratories, Co.Ltd.) for 60 min. Coverslips were rinsed with PBS-T followed by PBS, mounted with glycerol-containing 0.1 % 1,4-diazabicyclo(2,2,2)octane (DABCO), viewed on a Zeiss laser scan microscope.

RESULTS

Overexpression of HSF1 and the catalytically active fragment of PKN leads to the accumulation of αB-crystallin.

The content of HSP70, HSP27, and αB-crystallin in HeLa S3 cells was analyzed by immunoblotting with the antiserum against each HSP (Fig. 8). Exposure to heat stress increased HSPs, and this increase was blocked by staurosporine (Fig. 8, lane 1-3), an inhibitor of protein kinase (Tamaoki, 1991), as previously described in other cell lines (Kim et al., 1993), suggesting the involvement of protein kinases in regulation of the content of these HSPs after heat shock. To examine whether PKN regulates the content of HSPs *in vivo* (Fig. 7), HeLa S3 cells were transfected with an expression vector for full length of PKN or its catalytically active carboxyl-terminal region (PKN/AF3; Takahashi et al., 1998), and the cell lysates were analyzed. However, there was no detectable change in the content of HSPs in the cells overexpressing PKN or PKN/AF3 (Fig. 8, lane 4, 5). It is known that the endogenous HSF1 in unstressed cells exists as a cytoplasmic, non-DNA-binding, and monomeric protein and translocates from the cytoplasm to the nucleus in response to heat stress (Georgopolis et al., 1993), and that overexpressed recombinant HSF1 exists as a nucleic, DNA-binding, and homotrimeric protein (Xia et al., 1997). Therefore, we examined the effect of overexpression of HSF1 and PKN on the accumulation of HSPs (Fig. 9). The overexpression of HSF1 alone and HSF1 with full length of PKN did not affect the contents of any HSPs, whereas co-expression of HSF1 and PKN/AF3 induced the accumulation of αB-crystallin (Fig. 10). The accumulation of αB-crystallin was not observed in cells co-tansfected with HSF1 and the kinase-negative mutant of PKN/AF3, which was constructed by conversion of the conserved lysine at ATP-binding site to an glutamic acid residue (PKN/AF3(K644E), Fig. 10, lane 4), suggesting that the protein kinase activity of PKN/AF3 plays an important role in the accumulation of αB-crystallin. There was no change in the content of HSP70 and HSP27. Similar results were obtained by using C6

glioma cells and HeLa Tet-off Expression System (Fig. 11). To confirm that the accumulation of αB-crystallin is dependent on the kinase activity of PKN/AF3, we utilized staurosporine, a known inhibitor of various protein kinases (Tamaoki, 1991). The presence of staurosporine inhibited the accumulation in cells co-transfected with HSF1 and the catalytically active fragment of PKN in a dose-dependent manner (Fig. 12), and the presence of 10 nM staurosporine completely suppressed induction of the synthesis of αB-crystallin. This effect correlated completely with the *in vitro* kinase activity of PKN/AF3 in the presence of staurosporine. To investigate the effect of the kinase activity of PKN/AF3 on DNA-binding activity of HSF1, we examined the interaction of HSE and HSF1 by using gel shift assay (Fig. 13). Heat treatment and the overexpression of HSF1 increased the DNA-binding activity as described by Sarge et al. On the other hand, the overexpression of PKN/AF3 had no effect on the DNA-binding activity of endogenous and overexpressed HSF1, suggesting the kinase activity of PKN/AF3 is concerned with not so much the DNA-binding activity as the transcriptional activity of HSF1.

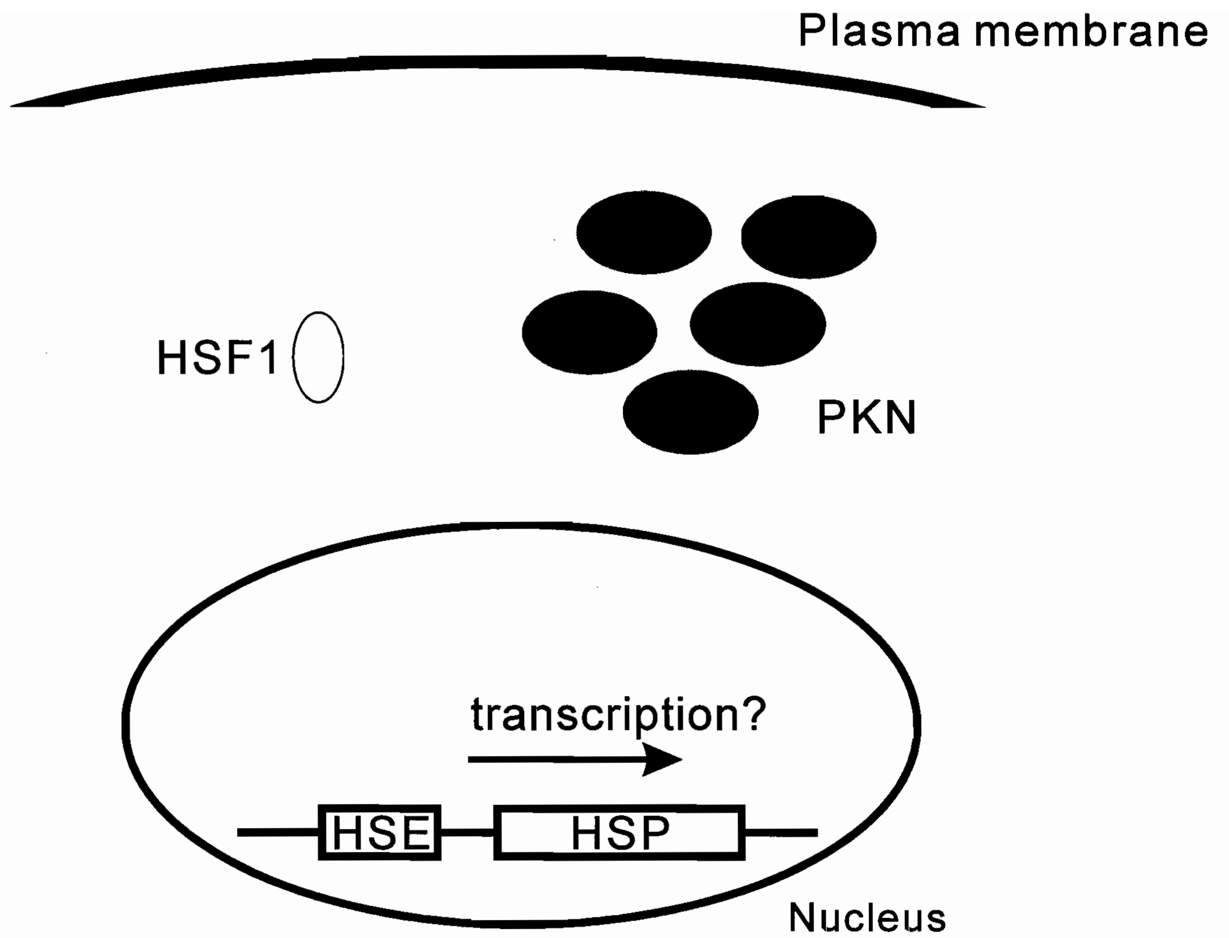


Fig. 7. Schematic representation of overexpression of PKN. I examined the effect of overexpression of PKN on the expression of HSPs via endogenous HSF1.

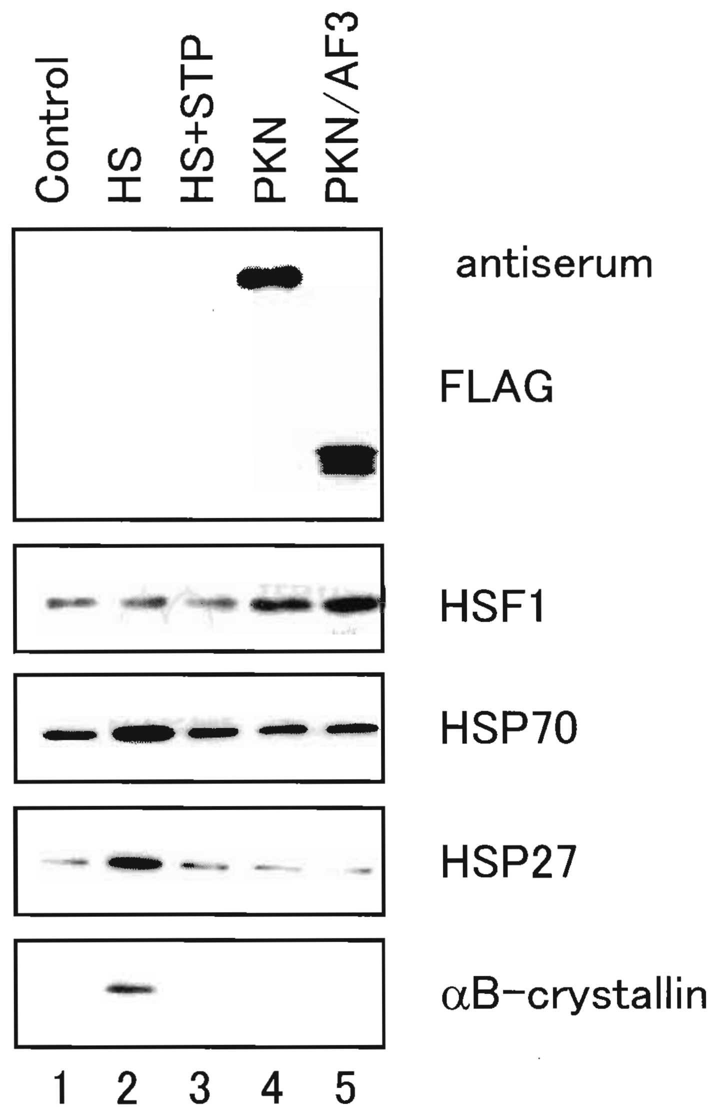


Fig. 8. Immunoblot analysis of HSPs content in HeLa S3 cells overexpressing PKN. HeLa S3 cells were transfected with either empty pRc/CMV expression vector or the indicated PKN expression vectors, and incubated at 37 °C. Lane 2, HS, cells were treated with heat shock. Lane 3, HS+STP, cells were exposed for 1 h to 0.5 μM staurosporine, and treated with heat shock. After being washed with PBS, cells were directly lysed in Laemmli's sample buffer by sonication. For each lane, 10 μg of protein of crude lysate was loaded and subjected to SDS-PAGE, with subsequent immunoblotting with antibodies against FLAG, HSF1, HSP70, HSP27, and αB-crystallin indicated at right column.

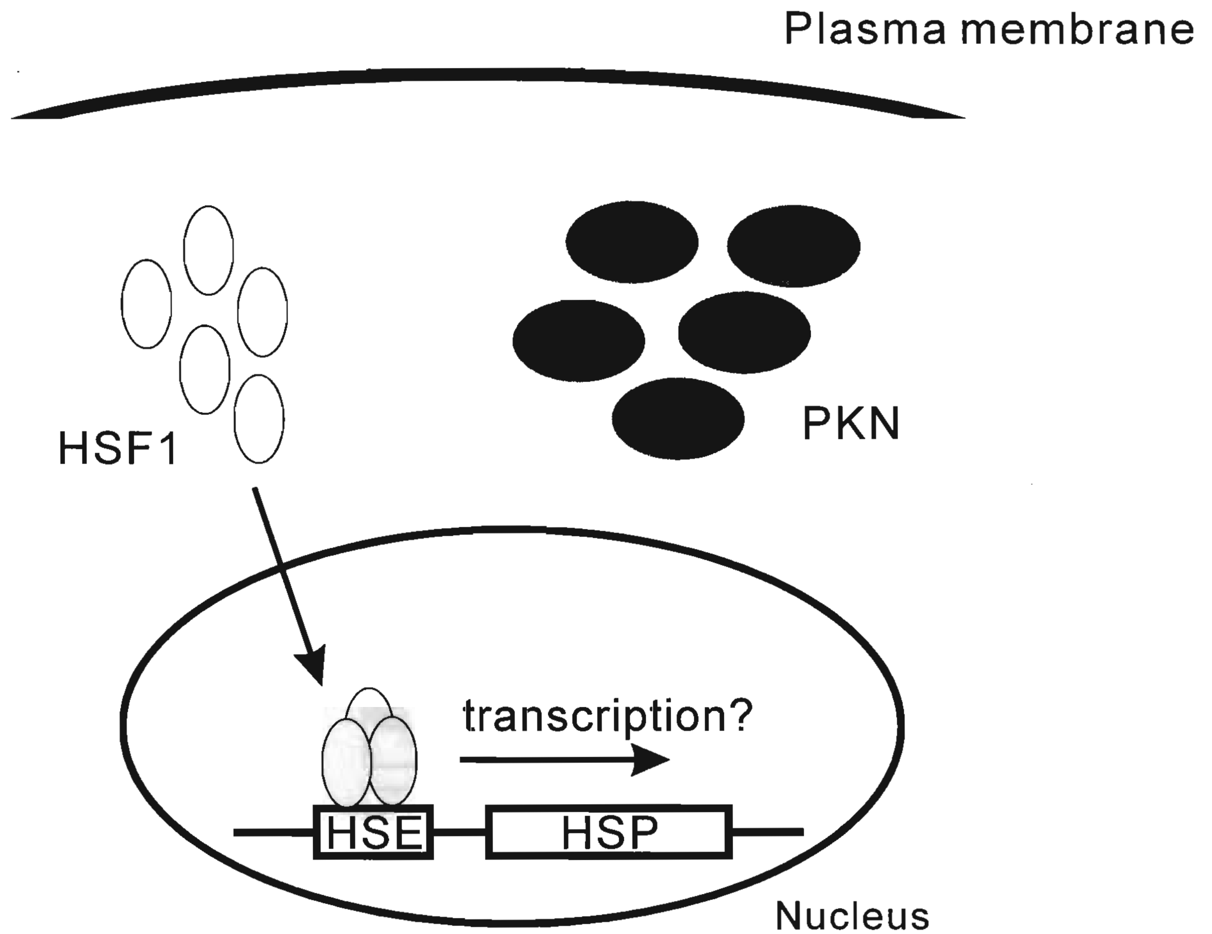


Fig. 9. Schematic representation of overexpression of PKN and HSF1. I examined the effect of overexpression of PKN on the expression of HSPs via nuclear-translocated overexpressed HSF1.

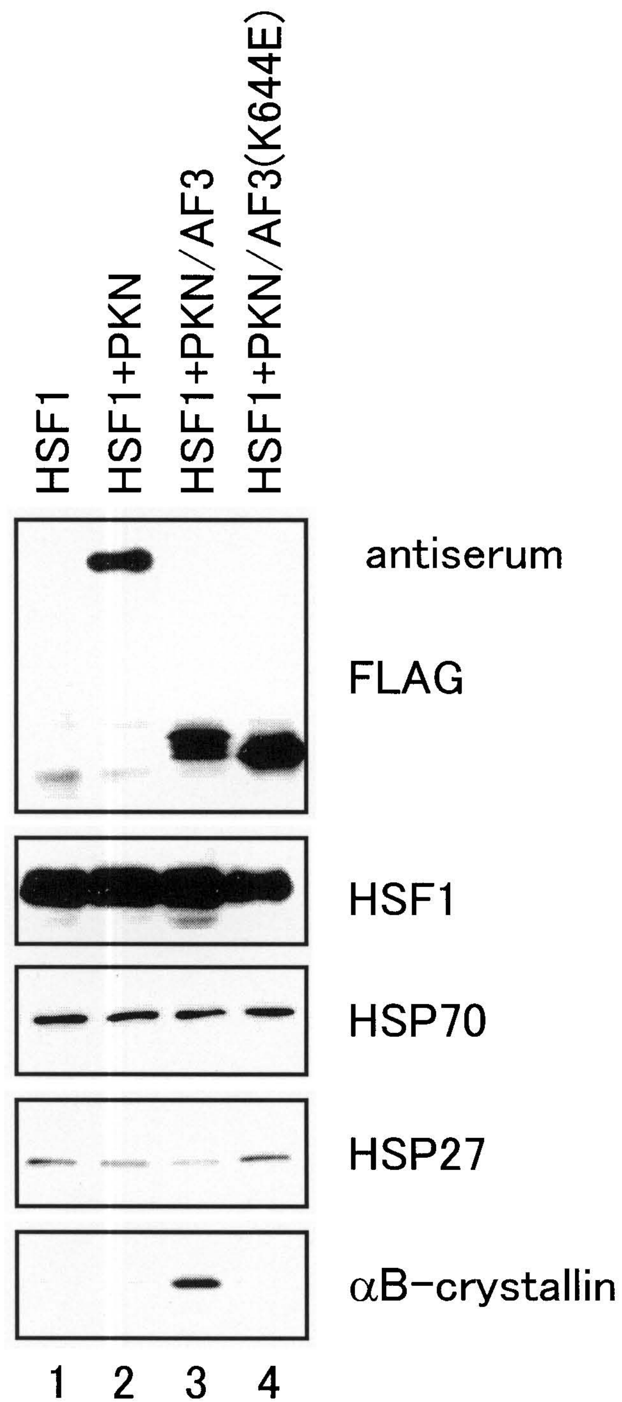


Fig. 10. Immunoblot analysis of HSPs content in HeLa S3 cells overexpressing PKN and HSF1. HeLa S3 cells were transfected with pTB701/HSF1 (HSF) and either empty pRc/CMV expression vector or the indicated PKN expression vectors, and incubated at 37 °C. Cells were subjected to immunoblotting as described in Fig. 8.

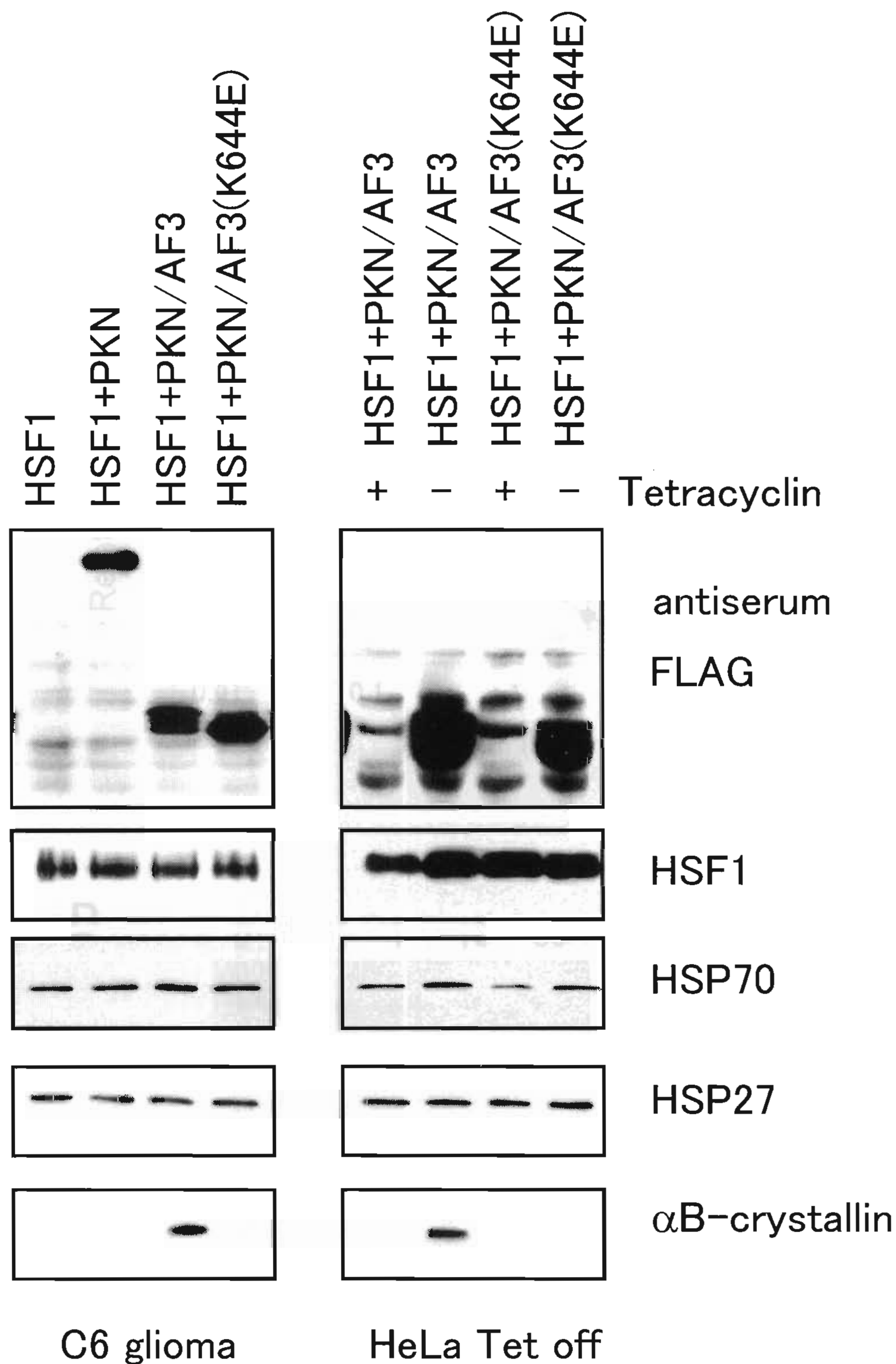


Fig. 11. Immunoblot analysis of HSPs content in other cell lines. C6 glioma cells were transfected with pTB701/HSF1 (HSF) and either empty pRc/CMV expression vector or the indicated PKN expression vectors, and incubated at 37 °C. HeLa Tet-off cells were transfected with pTB701/HSF1 (HSF) and either tetracyclin-regulated expression vector pTRE/PKN/AF3/FL or pTRE/PKN/AF3(K644E)/FL, and incubated in the presence or absence of tetracyclin at 37 °C. Cells were subjected to immunoblotting as described in Fig. 8.

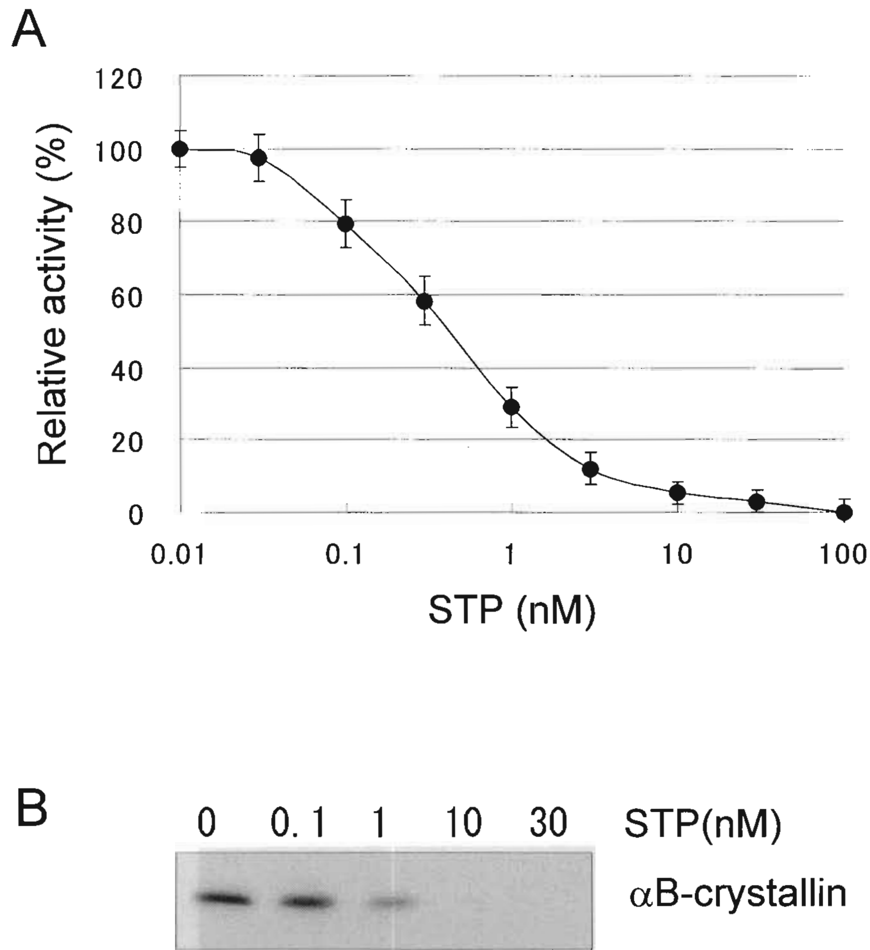


Fig. 12. The effect of staurosporine on the protein kinase activity of PKN/AF3 and the accumulation of αB-crystallin. A. The protein kinase activity of GST-PKN/AF3 was measured with δPKC peptide as substrate and staurosporine (STP) as inhibitor at the indicated concentrations. The results are means ± S. E. from three independent experiments performed in duplicate. B. HeLa S3 cells were transfected with pTB701/HSF1 and pRc/CMV/PKN/AF3, and exposed for 24 h to staurosporine (STP) at the indicated concentrations. Cells were subjected to immunoblotting with antibody against αB-crystallin as described in Fig. 8.

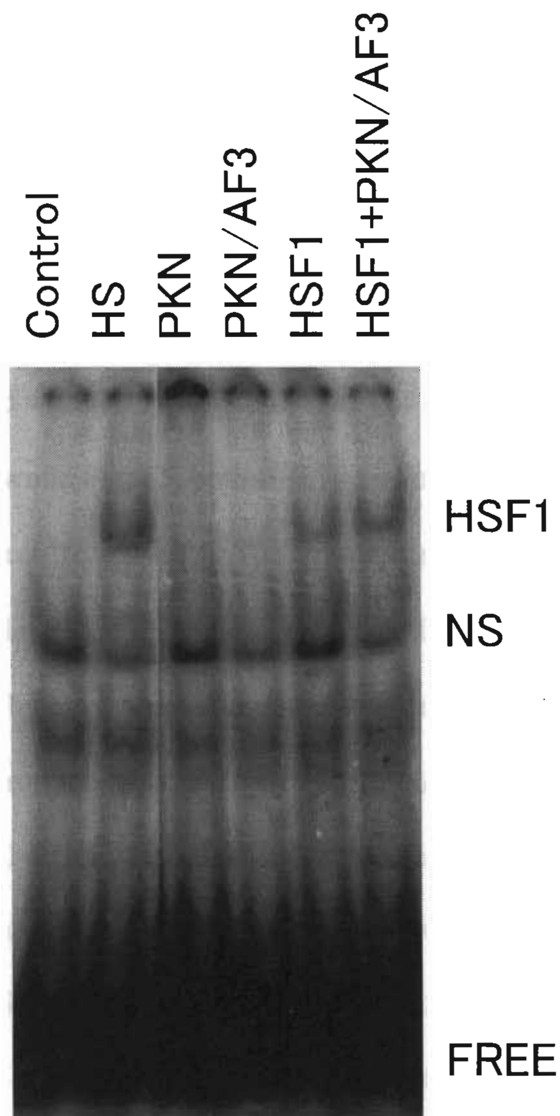


Fig. 13. Gel shift analysis of HSF1. HeLa S3 cells were co-transfected with either empty pRc/CMV expression vector or the indicated PKN expression vectors. Lane HS, cells were treated with heat shock. Extract from HeLa S3 cells were subjected to gel shift analysis with ^{32}P-labeled HSE probe. Position of HSF-DNA binding complex (HSF), nonspecific protein-DNA interactions (NS), and free probe (FREE) are shown.

αB-crystallin promoter region is specifically transactivated by co-expression of HSF1 and the catalytically acitive fragment of PKN.

To address whether the accumulation of αB-crystallin is regulated at the transcriptional level, we examined the effect of HSF1 and PKN on transcriptional activity of the promoter region for each HSP gene (Fig. 14; Mathur et al., 1994; Klemenz et al., 1991). HSP70 or αB-crystallin promoter region containing the HSEs was fused to a firefly luciferase gene, and each construct was co-transfected with the expression vectors of HSF1 and various PKN mutants into HeLa S3 cells. As shown in Fig. 15, both HSP70 and αB-crystallin promoter region showed transactivation in heat-treated cells. The overexpression of PKN/AF3 activated the αB-crystallin promoter, while PKN/AF3(K644E) had no effect. On the other hand, HSP70 promoter was not activated by overexpression of these recombinant PKN. To investigate whether the αB-crystallin promoter was acvtivated by PKN/AF3 without HSF1, we also examined the transcriptional activity in the absence or presence of exogenous recombinant HSF1 (Fig. 16). In normal cells the transcriptional activity was slightly enhanced by endogenously expressed HSF1, while there was no difference in functional response to heat shock between the presence and absence of the HSF1. Overexpression of PKN/AF3 stimulated the transcriptional activity about 3-fold, though the activation level did not reached that of heat-treated cells. Moreover, co-expression of HSF1 and PKN/AF3 activated transcription to the same level as that of heat-treated cells. On the other hand, the expression of PKN/AF3(K644E) showed no effect on transcription either in the presence or absence of exogenous HSF1. It is known that the promoter region of αB-crystallin contains two HSE (Klemenz et al., 1991). In order to narrow down the responsive region for HSF1 and PKN/AF3, we shortened the αB-crystallin promoter region fused to the luciferase reporter gene and tested the transcriptional activation by heat treatment or co-expression of HSF1 and PKN/AF3 (Fig.

17). Removal of the region containing the distal HSE decreased the extent of transactivation by heat treatment or by co-expression of HSF1 and PKN/AF3 to ~ 40 %. Deletion of both the proximal and the distal HSE revealed no transcriptional activation. Responsive region for heat treatment seems to be similar to that for HSF1 and PKN/AF3, suggesting that co-expression of these two proteins stimulates transactivation of αB-crystallin gene by using a similar mechanism to that of heat shock response.

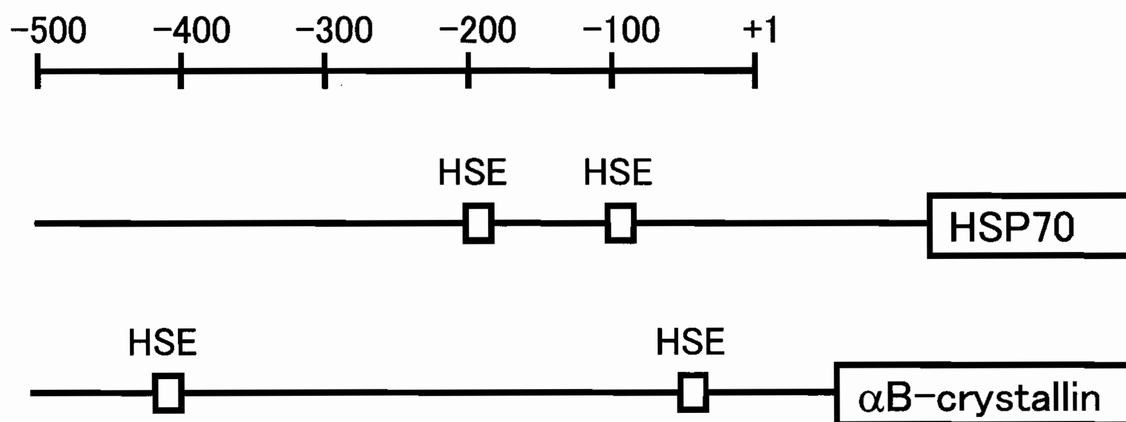


Fig. 14. Scheme of the promoter region of HSP70 and αB-crystallin. Schematic representation of the HSP70 and αB-crystallin promoter (Mathur et al., 1994; Klemenz et al., 1991), nucleotides –500 from the transcriptional start site (+1). Heat shock response elements (HSE) are indicated by boxes.

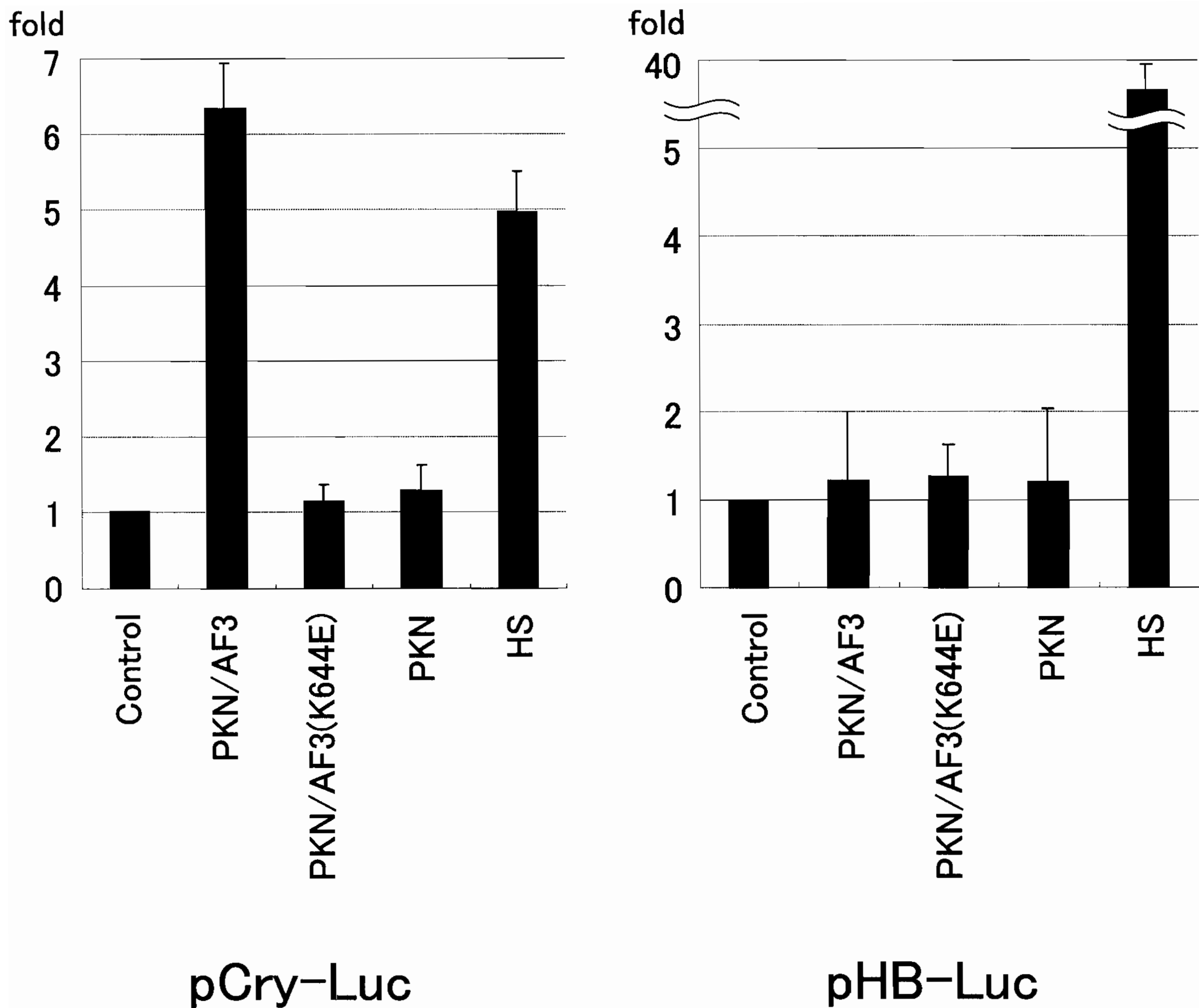


Fig. 15. Transactivation of HSP70 and αB-crystallin promoter region. HeLa S3 cells were co-transfected with either empty pRc/CMV expression vector or the indicated PKN expression vectors, pTB701/HSF1 and luciferase reporter constructs containing αB-crystallin (pCry-Luc) or HSP70 (pHB-Luc) promoter region. Bars indicate the relative luciferase activities compared to the value obtained with the empty pRc/CMV expression vector and the reporter constructs (Control), which was set at 1. Lane HS, cells were treated with heat shock. The results are means ± S. E. from three independent experiments performed in duplicate.

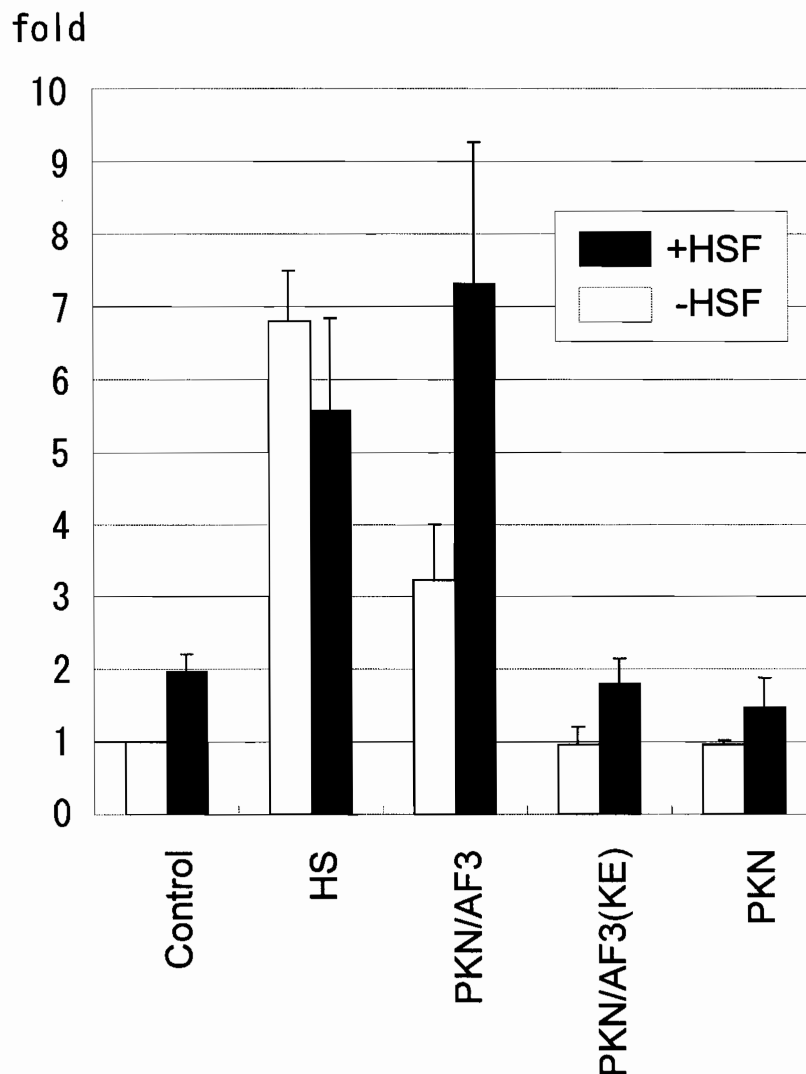


Fig. 16. Transactivation of αB-crystallin promoter region in the presence or absence of overexpressed HSF1. HeLa S3 cells were co-transfected with luciferase reporter constructs containing αB-crystallin (pCry-Luc), either empty pRc/CMV expression vector or the indicated PKN expression vectors and either empty pTB701 (white bar) expression vector or pTB701/HSF1 (black bar). Bars indicate the relative luciferase activities compared to the value obtained with the empty pRc/CMV expression vector and the reporter constructs (Control), which was set at 1. Lane HS, cells were treated with heat shock. The results are means ± S. E. from three independent experiments performed in duplicate.

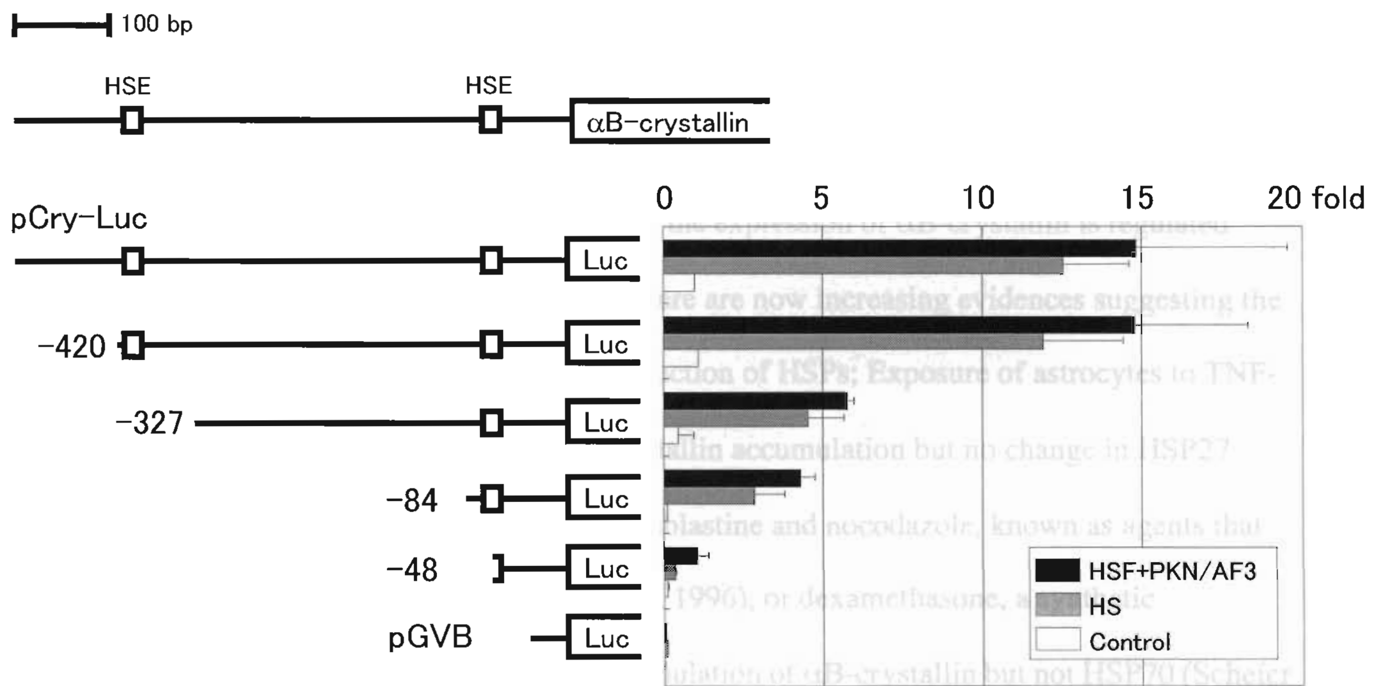


Fig. 17. Deletion analysis of the αB-crystallin promoter region. HeLa S3 cells were transfected with the luciferase reporter construct containing αB-crystallin promoter region (pCry-Luc) and its deletion mutants (shown in left panel). Cells were incubated at 37 °C (white bars) or treated with heat shock (shaded bars) or co-transfected with pTB701-HSF1 and pRc/CMV/PKN/AF3/FL (black bars). Bars indicate the relative luciferase activities compared to the value obtained with the pCry-Luc reporter constructs at 37 °C, which was set at 1.

DISCUSSION

In this report we have examined the potential roles of PKN in the regulation of HSPs expression via HSF1. A marked accumulation of αB-crystallin was observed in HeLa S3 cells overexpressing HSF1 and the catalytically acitive fragment of PKN. Reporter gene analysis showed that the accumulation of αB-crystallin was regulated at the transcriptional level and the transactivation was directed by the promoter region of αB-crystallin. However, there was no change in the expression of HSP27 and HSP70, though the promoter region of these HSPs gene also possesses HSEs. Our results suggest that the expression of αB-crystallin is regulated differently from that of HSP70 or HSP27. There are now increasing evidences suggesting the divergent factors and pathways mediating induction of HSPs; Exposure of astrocytes to TNF-α and hypertonic conditions result in αB-crystallin accumulation but no change in HSP27 (Head et al., 1994). Colcemid, colchicine, vinblastine and nocodazole, known as agents that promote microtubule disassembly (Kato et al., 1996), or dexamethasone, a synthetic glucocorticoid, induce the synthesis and accumulation of αB-crystallin but not HSP70 (Scheier et al., 1996; Zafarullah et al., 1993). These observations and our results suggest that there might be a difference between the regulation of αB-crystallin and other HSPs or the expression of other HSPs may require any additional factors. Although αB-crystallin is one of the major proteins of the lens, it is induced the expression not only in lens but in a variety of other organs as one of heat-inducible HSPs. It is also known that αB-crystallin in the brain is present at low levels, but it accumulates to high levels in astrocytes in Alexander's disease, a leukodystrophy of unknown etiology (Iwaki et al., 1989). Further examination of the relationship between the accumulation of αB-crystallin and PKN may contribute to understanding the pathogenesis of this disease. In addition, several reports suggest a link between the regulation of αB-crystallin expression and arachidonic acid. Modulators of the arachidonic acid cascade, indomethacin,

NDGA (Ito et al., 1996), and mastoparan (Kato et al., 1995) enhance stress-induced synthesis of αB-crystallin in C6 glioma cells. Moreover, it is well known that heat shock stimulates the release of arachidonic acid (Calderwood et al., 1989). As PKN is activated by a low, possibly physiological, concentration of arachidonic acid (Kitagawa et al., 1995), these reports raise the possibility that PKN may be a mediator of stress-induced signal transduction pathway.

We also showed here that the accumulation of αB-crystallin was completely suppressed in the presence of 1-10 nM staurosporine, known as a potent though non specific PKC inhibitor, suggesting a protein kinase activity is involved in the signal transduction cascade of the induction of αB-crystallin. PKN also exhibited a substrate specificity similar to that of PKC in our previous study (Kitagawa et al., 1995). These observations raise a possibility that not only PKN but also PKC subspecies may be a candidate mediator of the signal transduction. However, there is no report of the translocation of PKC to the nucleus induced by heat stress. Moreover, it has been reported that the accumulation of αB-crystallin was not induced by exposure of cells to PMA or forskolin, known as an activator of PKC or PKA, respectively (Kato et al., 1996), suggesting the possibility that these kinases were not involved.

The combination of overexpressed HSF1 and PKN/AF3 induced the accumulation of αB-crystallin, while the combination of endogenous HSF1and PKN/AF3 was insufficient. Since the overexpression of HSF1 is essential, it is assumed that some of the following conditions are the prerequisite for the induction of αB-crystallin by PKN/AF3, and is not induced by PKN/AF3: i) nuclear localization of HSF1; ii) trimerization of HSF1; iii) acquiring DNA binding activity of HSF1. The combination of overexpression of HSF1 and full length of PKN could not induce the accumulation of αB-crystallin (Fig. 10), although full length of PKN has low but significant protein kinase activity even in the absence of modifiers (Kitagawa et al., 1995). Immunocytochemical analysis indicates that full length of PKN localized in the cytoplasmic region, whereas PKN/AF3 were diffusely distributed in the cells (Fig. 18;

unpublished data). If i) is a prerequisite state and colocalization of HSF1 and PKN in the nucleus is important, full length of PKN, predominantly distributed in the cytosol, could not induce essential phosphorylation event in the nucleus. Xia et al. reported that inhibitors of serine/threonine protein kinases prevent transactivation of HSF1 without affecting trimerization and HSE-binding activity (Xia and Voellmy, 1997), suggesting the phosphorylation event is not prerequisite for ii) and iii). If ii) and iii) are prerequisite states for induction of αB-crystallin by PKN/AF3, PKN might selectively phosphorylate such conformation of HSF1 complex and stimulate the transactivation activity.

It is also known that heat treatment transiently induces phosphorylation-dependent mobility shift of HSF1 in gel electrophoresis (Cotto et al., 1996). PKN could phosphorylate recombinant HSF1 *in vitro* (data not shown), though there was no obvious mobility shift of HSF1 in HeLa S3 cells overexpressing PKN/AF3 (Fig. 8 and 10). Further investigation will be required to clarify whether PKN directly phosphorylates endogenous HSF1 *in vivo* and modulates its transcriptional activity.

Anyway, the finding of αB-crystallin expression by HSF1 and PKN/AF3 is intriguing but not surprising, since many previous studies suggest that the regulation of HSPs may require separate, function-specific pathways. Although the exact place of the molecular mechanisms in the regulation of the αB-crystallin is still uncertain, my results raise PKN to a candidate regulating the expression of αB-crystallin *in vivo*. I believe that this study will be helpful for the further analysis of the physiological function of PKN.

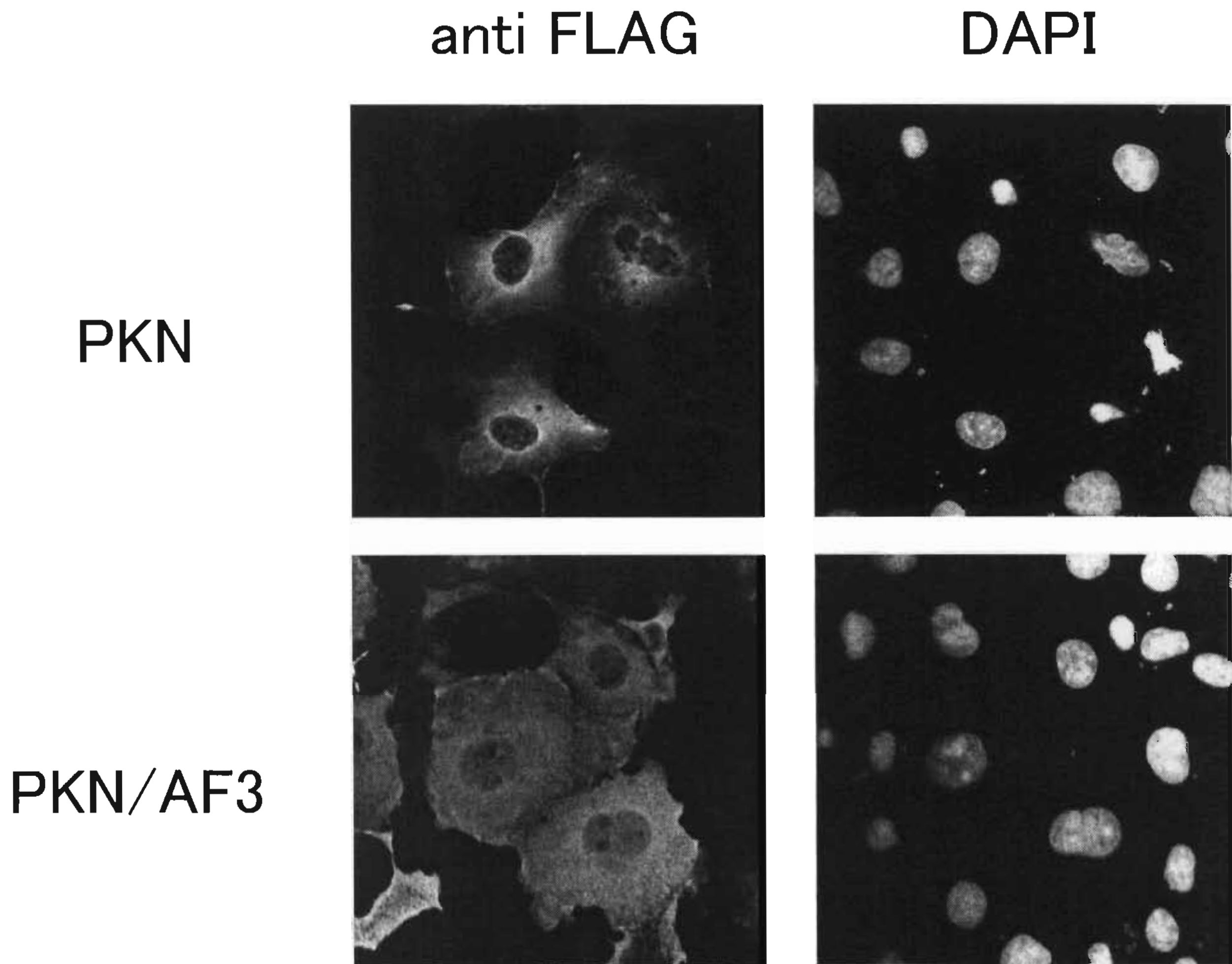


Fig. 18. Immunofluorescence analysis of the localization of PKN and PKN/AF3. COS7 cells were transfected with pRc/CMV/PKN/FL or pRc/CMV/PKN/AF3/FL, incubated with anti-FLAG monoclonal antibody, and then viewed by fluorescence microscopy with an FITC filter.

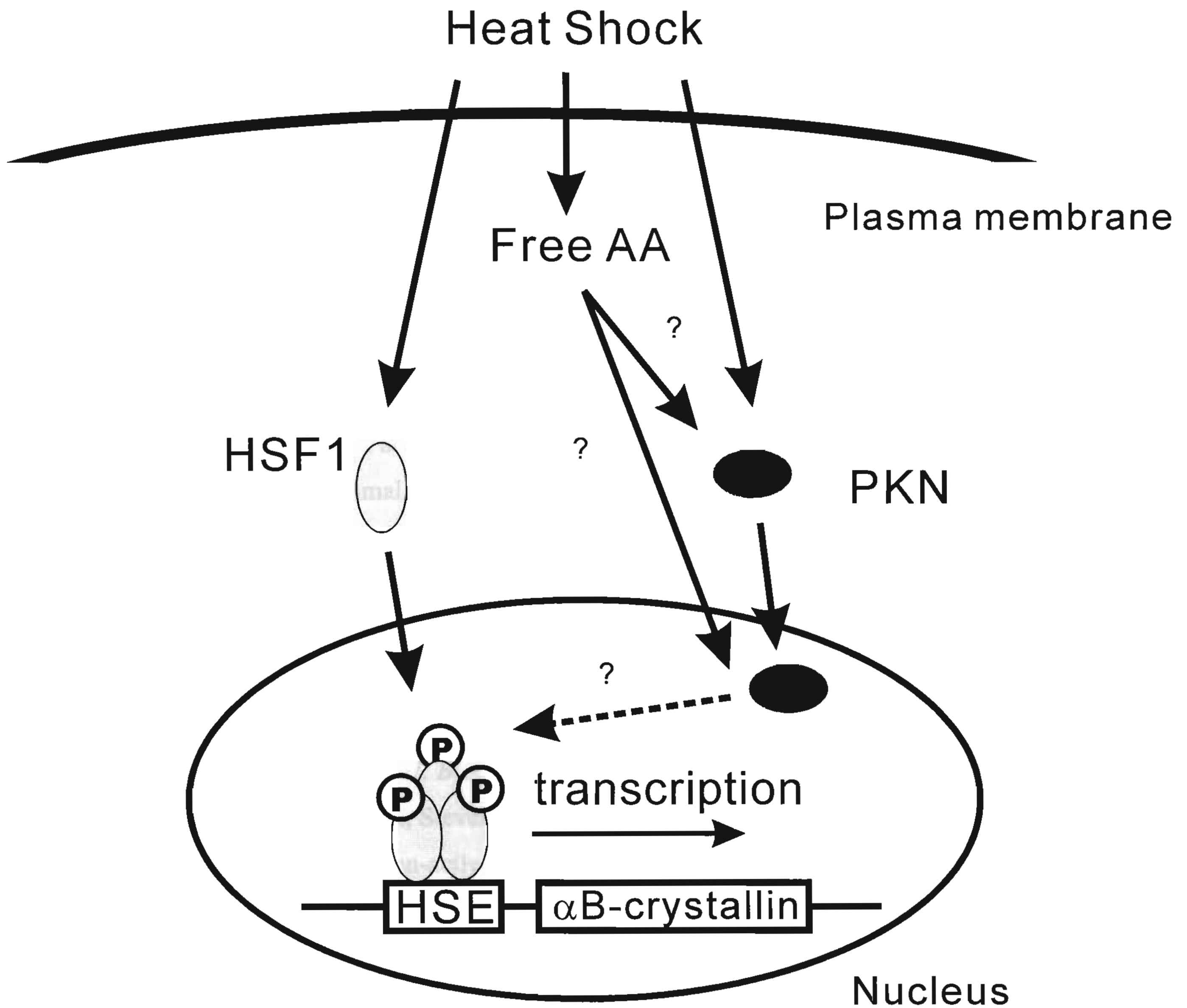


Fig. 19. A possible model for the PKN-mediated signaling pathway under heat stress. Heat shock induces release of free arachidonic acid from plasma membrane. Accumulation of free arachidonic acid may lead to activation of PKN. As a result, catalytically active PKN may lead to transcriptional activation of HSF1 directly or indirectly and induce the transcription of αB-crystallin.

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The Role of PKN in the Regulation of αB-Crystallin Expression via Heat Shock Transcription Factor 1

Michinori Kitagawa,* Hideyuki Mukai,* Mikiko Takahashi,† and Yoshitaka Ono*,†,[1]
Graduate School of Science and Technology, and †Department of Biology, Faculty of Science, Kobe University, Kobe 657-8501, Japan

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We previously reported that PKN, a fatty acid-activated serine/threonine protein kinase, translocates from the cytosol to the nucleus by stresses such as heat shock, sodium arsenite, and serum starvation. To clarify the role of PKN under heat stress, we examined whether PKN regulates the expression of heat shock proteins. Co-expression of heat shock transcription factor 1 (HSF1) and the catalytically active fragment of PKN induced the accumulation of αB-crystallin but not HSP27 and HSP70 in HeLa S3 cells. The expression of the reporter gene for αB-crystallin promoter was activated by co-expression of HSF1 and the catalytically active fragment of PKN, and this activation was dependent on the protein kinase activity of PKN. Deletion analysis of the αB-crystallin promoter region revealed that both the proximal and the distal heat shock elements were necessary for the transactivation. These results raise the possibility that there is a signal transduction pathway mediating stress signals for the accumulation of αB-crystallin by HSF1 and PKN. © 1998 Academic Press

Exposure of cells to heat shock or to chemical and physiological stresses leads to the heat shock response in which the induction of heat shock proteins (HSPs) is accompanied by the expression of stress resistance (1, 2). In mammalian cells, HSP genes are thought to be primarily regulated at the transcriptional level by heat shock transcription factor 1 (HSF1), a sequence-specific transcription factor that binds to heat shock elements (HSEs) in their promoter. HSF1 becomes hyperphosphorylated on serine residues after heat shock or chemical stresses (3). Inhibitors of serine/threonine protein kinases prevent the development of thermotolerance and increased HSPs transcription (4), suggesting that the phosphorylation of HSF1 by one or more protein kinases is required for the expression of HSPs. It is reported that several protein kinases, such as PKCα, GSK3α (5), GSK3β (6), and ERK1/ERK2 (7), have phosphorylated HSF1 directly *in vitro,* and the phosphorylation repressed its transcriptional activity (5-7). It is still unknown, however, what protein kinase phosphorylates HSF1 *in vivo* and induces transactivation of HSPs.

PKN is a serine/threonine protein kinase, having a catalytic domain homologous to that of PKC family in the carboxyl terminus and a regulatory region containing repeats of leucine-zipper like sequence in the amino terminus (8) and widely distributed in tissues and organs of mammals and other organisms (9, 10). The protein kinase activity of PKN is stimulated by fatty acids such as arachidonic acid (9, 11), and by small GTPase RhoA (12, 13). We previously reported that the translocation of PKN from the cytosol to the nucleus was induced by stresses such as heat shock, sodium arsenite, and serum starvation in fibroblasts (14). This observation raises the possibility that PKN could mediates stress signals to the nucleus, and that PKN could have target proteins in the nucleus.

In this paper, we have examined whether PKN is implicated in the regulation of gene expression of HSPs via HSF1. We showed here that HSF1 and the catalytically active PKN induced the gene expression of αB-crystallin cooperatively.

MATERIALS AND METHODS

Materials. Anti-FLAG (M2) monoclonal antibody was purchased from Kodak Co., USA. Anti-HSP70 (C92F3A-5) and anti-HSP27 (G3.1) monoclonal antibodies, and anti-αB-crystallin polyclonal antibody were purchased from Stressgen Biotechnologies Corp., Canada. Anti-human HSF1 antiserum was kindly provided by Dr. A. Nakai (Kyoto University, Japan).

Plasmid constructions. Plasmid pTB701/HSF1 to express human HSF1 in mammalian cells was constructed by inserting the *Eco*RI fragment of pGEM-Aki5 (15) into pTB701 (16). Mammalian expression plasmids pRc/CMV/PKN/FL and pRc/CMV/PKN/AF3/FL encod-

[1] To whom correspondence should be addressed: Department of Biology, Faculty of Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan. Fax: 81-78-803-0322. E-mail: yonodayo@kobe-u.ac.jp.

Abbreviations: PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; NDGA, nordihydroguaiaretic acid.

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ing FLAG-tagged full length of PKN and the catalytically active fragment of PKN (AF3, amino acids 561-942), respectively, were constructed as described previously (17). An expression plasmid of the protein kinase-negative mutant of AF3 (pRc/CMV/AF3(K644E)/FL) was constructed by substituting Glu for Lys^{644} using Quick-Change site-directed mutagenesis kit (Stratagene, USA). pCry-Luc, a reporter plasmid for αB-crystallin promoter (− 540 to + 45), was constructed as follows. Human αB-crystallin promoter region (18) was amplified by PCR from human placenta genomic DNA using the primers: 5′-ACGCGTTTGCACGTTTCCACACCTCATT-3′ and 5′-AGATCTAGGTGAGTGTGAGGGGTCAGCT-3′. This fragment was digested with *Bam*HI and blunted, then digested with *Mlu*I. The resultant fragment was cloned into the *Sma*I/*Mlu*I site of pGVB containing the firefly luciferase gene (Wako Pure Chemical, Japan). pHB-Luc (19), possessing the human HSP70 upstream region linked to the firefly luciferase gene, was kindly provided by Dr. H. Ariga, Hokkaido University. Several deletion mutants of the human αB-crystallin promoter region were generated using a Kilo-Sequencing Deletion Kit (TAKARA-shuzo, Japan).

Cell cultures, heat shock treatment, transfections, and luciferase assay. HeLa S3 cells (obtained from RIKEN Cell Bank, Japan) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin, in a humid 37 °C chamber containing 5 % CO_2. For heat shock treatment, monolayers of the cells were heated at 43 °C for 60 min, then incubated at 37 °C for 8 hours. For immunoblotting, the cells were plated in 35 mm petri dish at 3×10^5 cells/dish and allowed to recover for 1 day, and then co-transfected with 1 μg of the protein kinase expression constructs and 1 μg of the pTB701/HSF1 using Trans-IT (TAKARA-shuzo, Japan) as described in the manufacture's protocol. For luciferase assay, the cells were plated as described above, and then co-transfected with 1 μg of the firefly luciferase reporter constructs, 1 μg of the protein kinase expression constructs, 1 μg of the pTB701/HSF1, and 10 ng of pRL-SV40 plasmid (which contains an SV40 promoter upstream of the renilla luciferase gene, Promega, USA). Cells were harvested after incubation at 37 °C for 24 hours, lysed, and each luciferase activity for the firefly and for the renilla luciferase reporter was measured with the Dual Luciferase Kit of Promega, using Lumat LB9507 luminometer (Berthold Australia). Firefly luciferase values were standardized to renilla values.

Immunoblotting. Samples were separated by SDS-PAGE, and the proteins were transferred to a PVDF membrane. In the case of immunoblotting against HSF1, the proteins were transferred to a nitrocellulose membrane. The membrane was incubated with the antiserum, and the immunoreactive bands were visualized with an Enhanced Chemiluminescence immunoblotting detection kit (Amersham, UK).

RESULTS

Overexpression of HSF1 and the catalytically active fragment of PKN leads to the accumulation of αB-crystallin. The content of HSP70, HSP27, and αB-crystallin in HeLa S3 cells was analyzed by immunoblotting with the antiserum against each HSP (Fig. 1). Exposure to heat stress increased HSPs, and this increase was blocked by staurosporine (Fig. 1, lanes 1-3), an inhibitor of protein kinase (20), as previously described in other cell lines (4), suggesting the involvement of protein kinases in regulation of the content of these HSPs after heat shock. To examine whether PKN regulates the content of HSPs *in vivo,* HeLa S3 cells were transfected with an expression vector for full length of PKN or its catalytically active carboxyl-

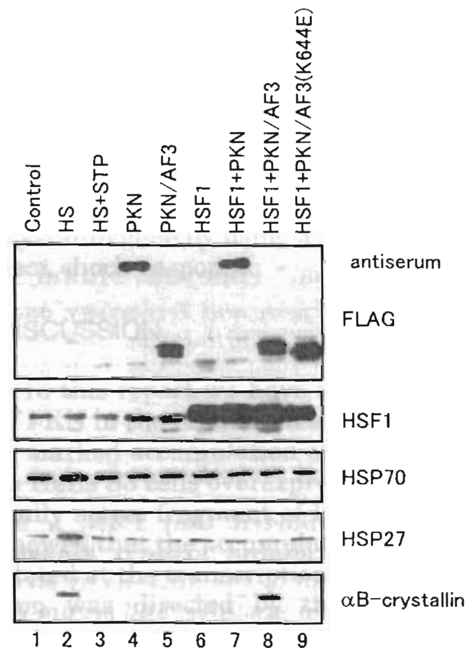

FIG. 1. Immunoblot analysis of HSPs content in HeLa S3 cells. HeLa S3 cells were transfected with either empty pTB701 expression vector or pTB701/HSF1 (HSF) together with either empty pRc/CMV expression vector or the indicated PKN expression vectors, and incubated at 37 °C. Lane 2, HS, cells were treated with heat shock. Lane 3, HS+STP, cells were exposed for 1 h to 0.5 μM staurosporine, and treated with heat shock. After being washed with PBS, cells were directly lysed in Laemmli's sample buffer by sonication. For each lane, 10 μg of protein of crude lysate was loaded and subjected to SDS-PAGE, with subsequent immunoblotting with antibodies against FLAG, HSF1, HSP70, HSP27, and αB-crystallin indicated at right column.

terminal region (PKN/AF3; ref 17), and the cell lysates were analyzed. However, there was no detectable change in the content of HSPs in the cells overexpressing PKN or PKN/AF3 (Fig. 1, lanes 4, 5). It is known that the endogenous HSF1 in unstressed cells exists as a cytoplasmic, non-DNA-binding, and monomeric protein and translocates from the cytoplasm to the nucleus in response to heat stress (2), and that overexpressed recombinant HSF1 exists as a nucleic, DNA-binding, and homotrimeric protein (21). Therefore, we examined the effect of overexpression of HSF1 and PKN on the accumulation of HSPs. The overexpression of HSF1 alone and HSF1 with full length of PKN did not affect the contents of any HSPs, whereas co-expression of HSF1 and PKN/AF3 induced the accumulation of αB-crystallin (Fig. 1, lanes 6-8). The accumulation of αB-crystallin was not observed in cells co-transfected with HSF1 and the kinase-negative mutant of PKN/AF3, which was constructed by conversion of the conserved lysine at ATP-binding site to an glutamic acid residue (PKN/AF3(K644E), Fig. 1, lane 9), suggesting that the protein kinase activity of PKN/AF3 plays an important role in the accumulation of αB-

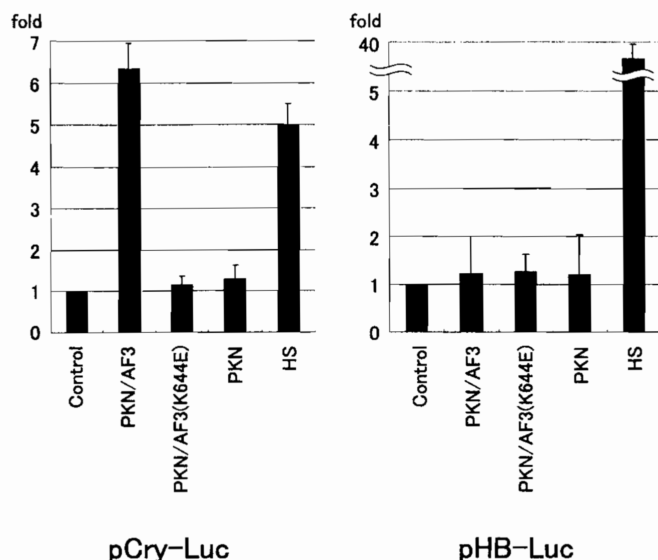

FIG. 2. Transactivation of αB-crystallin promoter region by HSF1 and the catalytically active fragment of PKN. HeLa S3 cells were co-transfected with either empty pRc/CMV expression vector or the indicated PKN expression vectors, pTB701/HSF1 and luciferase reporter constructs containing αB-crystallin (pCry-Luc) or HSP70 (pHB-Luc) promoter region. Bars indicate the relative luciferase activities compared to the value obtained with the empty pRc/CMV expression vector and the reporter constructs (Control), which was set at 1. Lane HS, cells were treated with heat shock. The results are means±S. E. from three independent experiments performed in duplicate.

crystallin. There was no change in the content of HSP70 and HSP27. Similar results were obtained by using C6 glioma cells (data not shown).

αB-Crystallin promoter region is specifically transactivated by co-expression of HSF1 and the catalytically active fragment of PKN. To address whether the accumulation of αB-crystallin is regulated at the transcriptional level, we examined the effect of HSF1 and PKN on transcriptional activity of the promoter region for each HSP gene. HSP70 or αB-crystallin promoter region containing the HSEs was fused to a firefly luciferase gene, and each construct was co-transfected with the expression vectors of HSF1 and various PKN mutants into HeLa S3 cells. As shown in Fig. 2, both HSP70 and αB-crystallin promoter region showed transactivation in heat-treated cells. The overexpression of PKN/AF3 activated the αB-crystallin promoter, while PKN/AF3(K644E) had no effect. On the other hand, HSP70 promoter was not activated by overexpression of these recombinant PKN.

αB-crystallin promoter region contains two HSEs (22). In order to narrow down the responsive region for HSF1 and PKN/AF3, we shortened the αB-crystallin promoter region fused to the luciferase reporter gene and tested the transcriptional activation by heat treatment or co-expression of HSF1 and PKN/AF3 (Fig. 3). Removal of the region containing the distal HSE decreased the extent of transactivation by heat treatment or by co-expression of HSF1 and PKN/AF3 to ~ 40 %. Deletion of both the proximal and the distal HSE revealed no transcriptional activation. Responsive region for heat treatment seems to be similar to that for HSF1 and PKN/AF3, suggesting that co-expression of these two proteins stimulates transactivation of αB-crystallin gene by using a similar mechanism to that of heat shock response.

DISCUSSION

In this report we have examined the potential roles of PKN in the regulation of HSPs expression via HSF1. A marked accumulation of αB-crystallin was observed in HeLa S3 cells overexpressing HSF1 and the catalytically active fragment of PKN. Reporter gene analysis showed that the accumulation of αB-crystallin was regulated at the transcriptional level and the transactivation was directed by the promoter region of αB-crystallin. However, there was no change in the expression of HSP27 and HSP70, though the promoter region of these HSPs gene also possesses HSEs. Our results suggest that the expression of αB-crystallin is regulated differently from that of HSP70 or HSP27. Recent reports supporting our suggestion are as follows: i) exposure of astrocytes to TNF-α and hypertonic conditions result in αB-crystallin accumulation but no change in HSP27 (23); ii) colcemid, colchicine, vinblastine, and nocodazole, known as agents that promote microtubule disassembly (24), or dexamethasone, a synthetic glucocorticoid, induce the synthesis and accumulation of αB-crystallin but not HSP70 (25, 26). In addition, several reports suggest a link between the regulation of αB-crystallin expression and arachidonic acid. Modulators of the arachidonic acid cascade, indomethacin, NDGA (27), and mastoparan (28) enhance stress-induced synthesis of αB-crystallin in C6 glioma cells. As PKN is activated by a low, possibly physiological, concentration of arachidonic acid (9), these reports raise the possibility that PKN may be a mediator of stress-induced signal transduction pathway.

The combination of overexpressed HSF1 and PKN/AF3 induced the accumulation of αB-crystallin, while the combination of endogenous HSF1 and PKN/AF3 was insufficient. Since the overexpression of HSF1 is essential, it is assumed that some of the following conditions are the prerequisite for the induction of αB-crystallin by PKN/AF3, and is not induced by PKN/AF3: i) nuclear localization of HSF1; ii) trimerization of HSF1; iii) acquiring DNA binding activity of HSF1. The combination of overexpression of HSF1 and full length of PKN could not induce the accumulation of αB-crystallin (Fig. 1), although full length of PKN has low but significant protein kinase activity even in the absence of modifiers (9). Immunocytochemical analysis indicates that full length of PKN localized in the cyto-

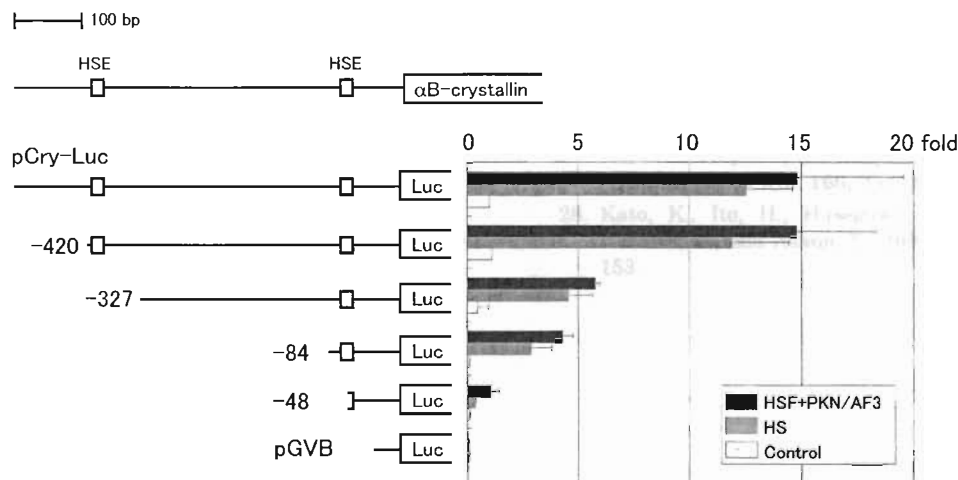

FIG. 3. Deletion analysis of the αB-crystallin promoter. HeLa S3 cells were transfected with the luciferase reporter construct containing αB-crystallin promoter region (pCry-Luc) and its deletion mutants (shown in left panel). Cells were incubated at 37 °C (white bars) or treated with heat shock (shaded bars) or co-transfected with pTB701-HSF1 and pRc/CMV/PKN/AF3/FL (black bars). Bars indicate the relative luciferase activities compared to the value obtained with the pCry-Luc reporter constructs at 37 °C, which was set at 1.

plasmic region, whereas PKN/AF3 were diffusely distributed in the cells (data not shown). If i) is a prerequisite state and colocalization of HSF1 and PKN in the nucleus is important, full length of PKN, predominantly distributed in the cytosol, could not induce essential phosphorylation event in the nucleus. Xia et al. reported that inhibitors of serine/threonine protein kinases prevent transactivation of HSF1 without affecting trimerization and HSE-binding activity (21), suggesting the phosphorylation event is not prerequisite for ii) and iii). If ii) and iii) are prerequisite states for induction of αB-crystallin by PKN/AF3, PKN might selectively phosphorylate such conformation of HSF1 complex and stimulate the transactivation activity.

It is also known that heat treatment transiently induces phosphorylation-dependent mobility shift of HSF1 in gel electrophoresis (3). PKN could phosphorylate recombinant HSF1 *in vitro* (data not shown), though there was no obvious mobility shift of HSF1 in HeLa S3 cells overexpressing PKN/AF3 (Fig. 1). Further investigation will be required to clarify whether PKN directly phosphorylates endogenous HSF1 *in vivo* and modulates its transcriptional activity.

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We thank Dr. Y. Nishizuka for encouragement. We thank Dr. A. Nakai for giving us anti-human HSF1 antiserum and Dr. H. Ariga for an expression vector, pHB-Luc. This work was supported in part by research grants from the Ministry of Education, Science, Sports, and Culture, Japan, research fellowships of the Japan Society for the Promotion of Science for Young Scientists, "Research for the Future" program, the Japan Society for the Promotion of Science.

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ARTICLE NO. 0515

The Role of the Unique Motifs in the Amino-Terminal Region of PKN on Its Enzymatic Activity

Michinori Kitagawa, Hideki Shibata, Masanao Toshimori, Hideyuki Mukai, and Yoshitaka Ono[1]

Department of Biology, Faculty of Science, Kobe University, Kobe 657, Japan

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The yeast two-hybrid system and *in vitro* binding assay were carried out to characterize the interaction between the amino-terminal and carboxyl-terminal region of PKN. It was revealed that the amino-terminal region containing the regulatory domain associated with the carboxyl-terminal catalytic region. A synthetic peptide, corresponding to the amino acid residues of PKN from 39 to 53, with substitution of isoleucine^{46} with serine was shown to become a potent substrate for PKN, and its wild type synthetic peptide inhibited the phosphorylation by PKN. These results suggest that the amino-terminal region of PKN contains the pseudo-substrate sequence and acts as an autoinhibitory region. © 1996 Academic Press, Inc.

PKN is a serine/threonine protein kinase with the catalytic domain highly homologous to that of PKC; and its kinase activity is enhanced by unsaturated fatty acids such as arachidonic acid (1,2). Recently we have reported that a small GTP-binding protein Rho binds to the amino-terminal region of PKN and activates PKN in a GTP-dependent manner, suggesting that PKN functions the downstream of Rho in a signal transduction pathway (3,4). The amino-terminal regulatory region of PKN contains the leucine zipper-like sequences and a basic region located immediately amino-terminal to the first leucine zipper-like motif, which are highly conserved through evolution in vertebrates (5) and among the various isoforms of PKN (6). It is suggested, therefore, that this region is critical for regulating the kinase activity of this enzyme. The precise mechanism of the regulation of its activity, however, is currently unclear.

In this paper, we show that the amino-terminal region of PKN interacts directly to the carboxyl-terminal catalytic region, and that the pseudosubstrate-like sequence in the amino-terminal region might act as an autoinhibitory sequence to regulate the protein kinase activity of PKN.

MATERIALS AND METHODS

Materials. The following oligopeptides were synthesized according to the amino acid sequences of rat δPKC (7) and human PKN (8), respectively, with an automated peptide synthesizer (Applied Biosystems, model 430A): δPKC peptide (corresponds to the amino acid 137–153 of δPKC, substituting Ser for Ala; AMFPTMNRRGSIKQAKI); PKN (39–53) peptide (corresponds to the amino acid 39–53 of human PKN; RERLRREIRKELKLK); PKN (54–73) peptide (corresponds to the amino acid 54–73 of human PKN; EGAENLKKATTDLGKSLGPV); [Ser^{46}]PKN(39–53) peptide (corresponds to the amino acid 39–53 of PKN, substituting Ser for Ile; RERLRRESRKELKLK). Other chemicals were obtained from commercial sources.

Construct for two-hybrid system. The plasmid pBTM/PKN-N, for expression of the fusion protein of DNA binding domain of LexA and the amino-terminal region of PKN (amino acid 1–540), were constructed by subcloning an *Eco*RI/*Bam*HI fragment of human PKN into pBTM116 (3). The plasmid pVP/PKN-N for expression of the fusion protein of transcription activation domain pVP16 and the amino-terminal region of PKN. pBTM/PKN-C and pVP/PKN-C were constructed by subcloning a *Cla*I/*Eco*RI fragment of human PKN encoding its carboxyl-terminal region (amino acid 511–942) into pBTM116 and pVP16, respectively.

In vitro transcription and translation. Truncated human PKN was made as follows; expression vector pPKN-N for

[1] To whom correspondence should be addressed at Department of Biology, Faculty of Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657, Japan. Fax: 81-78-803-0322. E-mail: yonodayo@icluna.kobe-u.ac.jp.

Abbreviations used: PKC, protein kinase C; GST, glutathione *S*-transferase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase.

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amino-terminal region of PKN was made by digesting phPKN-H4 (human PKN cDNA in pBluescript II SK) (8), with *Bst*EII, and filling-in the ends with T4 DNA polymerase, and self-ligation. The resulting plasmid encodes for amino acid residues of PKN from 1 to 474. cDNA insert from this plasmid was cloned into pRc/CMV (Invitrogen Corp.). Fragment encoding for the amino acid 614–942 containing the catalytic domain of human PKN was made by PCR amplification. Expression vector pPKN-C for the carboxyl-terminal region of PKN was made by subcloning this fragment into pRc/CMV. These plasmids were linealized by cutting with *Xba*I, and cRNA was transcribed using T7 RNA polymerase. cRNA was translated in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine.

Preparation of GST fusion proteins. pGST/PKN-N for expression of the fusion protein of GST and the amino-terminal region of PKN (amino acid 1–540) was made by subcloning a *Bam*HI insert of the phPKN-H4 into pGEX4T vector (Pharmacia Biotech Inc.). pGST/PKN-C for expression of the fusion protein of GST and the carboxyl-terminal region (amino acid 634–942) was made by subcloning an *Eco*RI insert of the phPKN-H4 into pGEX4T vector. Expression of GST or GST fusion proteins was induced with 0.1 mM IPTG. Cells were centrifuged at 5,000 × g for 5 min, and the resultant pellet resuspended in GST lysis buffer [50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1 μg/ml leupeptin, 1 mM DTT, 1 mM PMSF] containing 1% Triton X-100. The cells were lysed by sonication. Cell debris was removed by centrifugation at 30,000 × g for 10 min, and the supernatant was added to glutathione-Sepharose 4B (Pharmacia Biotech Inc.). The resin was washed with 40 column volumes of GST lysis buffer. The GST and GST fusion proteins were eluted with GST elution buffer [100 mM Tris/HCl (pH 8.0), 20 mM glutathione, 120 mM NaCl, 1 mM EDTA, 1 μg/ml leupeptin, 1 mM DTT]. The eluate was dialyzed overnight against 10 mM Tris/HCl (pH 8.0) containing 1 mM EDTA, 1 mM DTT, and 0.1 μg/ml leupeptin.

In vitro binding assay. For *in vitro* binding experiment, 2.5 μl of *in vitro* translated PKN-N or PKN-C was mixed with 5 μg of each GST-PKN-N or GST-PKN-C fusion protein or 25 μg of GST alone in 400 μl of GST binding buffer [20 mM Tris/HCl (pH 7.5), 0.5 mM DTT, 150 mM NaCl, 0.05% Triton X-100, 1 mM EDTA, 1 μg/ml leupeptin] and incubated for 1 hr at 4°C. Twenty five μl of glutathione-Sepharose 4B pretreated with 10 mg/ml of *E. coli* extract to block nonspecific binding, was then added, and the binding mixtures were then rotated for an additional 30 min at 4°C. The glutathione-Sepharose 4B was then washed three times in GST wash buffer [20 mM Tris/HCl (pH 7.5), 0.5 mM DTT, 1 mM EDTA, 1 μg/ml leupeptin] containing 0.5 M NaCl and 0.5% Triton X-100 and further washed with GST wash buffer. Bound proteins were eluted with GST elution buffer and subjected to 10% SDS-PAGE. Quantification of the binding reactions was carried out by a BAS 1000 bio-imaging analyzer (FUJI).

Kinase assay. The purified PKN (1 ng) from rat testis cytosol (2) was incubated for 5 min at 30°C in a reaction mixture (final volume 25 μl) containing 50 mM Tris/HCl (pH 7.5), 8 mM $MgCl_2$, 20 μM ATP, 18.5 kBq of [γ-^{32}P]ATP, 40 μM arachidonic acid, phosphate acceptors and inhibitors as indicated in each experiment. The kinase activity of the catalytic subunit of bovine heart PKA (9), was measured by using Kemptide (10) as a substrate under the same conditions without arachidonic acid. Reactions were started by the addition of the enzyme source and terminated by spotting them onto Whatman P81 papers and submersion in 75 mM phosphate, and followed by three times of 10 min washes. Incorporation of ^{32}P phosphate into phosphate acceptors was assessed by liquid scintillation counting.

RESULTS AND DISCUSSION

In the previous study, we demonstrated that truncations of the amino-terminal region of PKN by limited proteolysis resulted in formation of the catalytically active fragments, and suggesting that the amino-terminal region may serves as a regulatory region (1). To study a possibility that the catalytic region is masked with the regulatory region, we performed the yeast two-hybrid assay. As shown in Fig. 1, yeast cells transfected with the combinations of the expression vectors for the

1) pBTM116 + pVP16

2) pBTM116 + pVP/PKN-N

3) pBTM116 + pVP/PKN-C

4) pBTM/PKN-N + pVP/PKN-C

5) pBTM/PKN-C + pVP/PKN-N

FIG. 1. Interaction of the amino-terminal region of PKN with the carboxyl-terminal region in the two-hybrid system. Yeast L40 cells were cotransfected with the expression plasmids encoding the LexA DNA binding domain- and VP16 transcription activation domain-fusion protein. Interaction was examined using a filter lift assay for β-galactosidase activity (15).

amino-terminal and the carboxyl-terminal region of PKN showed a clear expression of β-galactosidase. This interaction was confirmed by *in vitro* binding assay using GST fusion proteins and ^{35}S-labelled proteins produced by *in vitro* translation as shown in Fig. 2. These results indicate that the amino-terminal regulatory region directly binds to the carboxyl-terminal catalytic region.

It is well known that the activation of the serine/threonine protein kinases such as PKA or PKC is associated with conformational change of the enzyme from an inactive to catalytically active form, and that the inactive conformation is stabilized by an autoinhibitory sequence (pseudosubstrate) that masks the catalytic site (11). The catalytic region of PKN shows a high homology with the corresponding regions of PKC family (8), and the PKC pseudosubstrate sequence-derived peptides have been shown to be excellent substrates for PKN (1,2). Thus, a possibility is raised that PKN contains the autoinhibitory sequence in the amino-terminal region.

From sequence alignment of the amino-terminal region of PKN and a consensus phosphorylation site sequence of PKC (XR/KXXS/TXR/KX)(12), a potent pseudosubstrate-like sequence was found in the residues from 39 to 53 (RERLRREIRKELKLK). If the residues from 39 to 53 act as a pseudosubstrate site, a synthetic peptide corresponding to this domain with a Ser substitution of the Ile^{46} as a phosphate acceptor, should be efficiently phosphorylated by PKN. As shown in Fig. 3, the peptide [Ser^{46}]PKN(39–53) was found to act as a potent substrate with a *Km* of approximately 25 μM and a *Vmax* of 400 nmoles/min/mg of protein. The synthetic peptide of the sequence corresponding to the residues from 39 to 53 inhibited the δPKC peptide substrate phosphorylation in the concentration-dependent manner with an IC_{50} (an inhibitor concentration required to give 50% inhibition of the peptide substrate phosphorylation at the *Km* concentration) of approximately 80 μM (Fig. 4). In contrast, another synthetic peptide corresponding to the residues from 54 to 73 was found to be a poor inhibitor (Fig. 4). The specificity of the peptide was confirmed with another basic amino acid-requiring protein kinase. The peptide PKN(39–53) was found to be a poor inhibitor of the PKA with the IC_{50} of >1,000 μM (Fig. 4). Kinetic assays were performed with purified PKN, a fixed ATP concentration of 100 μM, and various concentrations of δPKC peptide (10–80 μM). The results, performed in the presence of 40–80 μM PKN(39–53) peptide, are shown in double-reciprocal plots (Fig. 4). The inhibition was competitive-noncompetitive and gave a linear secondary plot of *Km/Vmax* (a concentration required to give a half-maximal velocity/maximal velocity) versus inhibitor peptide concentrations (Fig. 5A). The apparent inhibitory constant (*Ki*) obtained from double-reciprocal plots and secondary plots of *Km/Vmax* versus inhibitor peptide gave a value of 41 μM (Fig. 5B). The inhibitor peptide gave an uncompetitive inhibition plot with varying ATP concentration, indicating that inhibition was not occurred at the ATP-

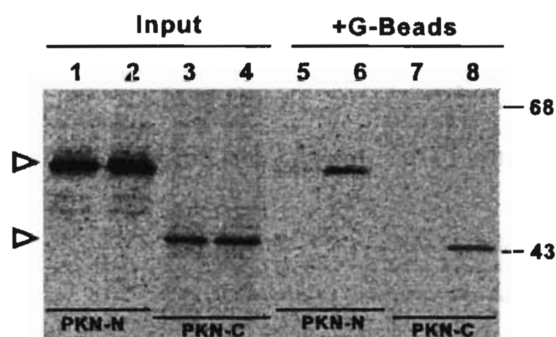

FIG. 2. *In vitro* association between the portions of PKN. S-labeled *in vitro* translated amino-terminal region (lanes 1, 2, 5, 6) or carboxyl-terminal region (lanes 3, 4, 7, 8) were incubated with bacterially synthesized GST (lane 1, 3, 5, 7) or GST fused to the amino-terminal region of PKN (lane 4 and 8) or GST fused to the carboxyl-terminal region of PKN (lane 2 and 6). Proteins were collected with glutathione-Sepharose beads (G-beads), analyzed by SDS-PAGE, and followed by autoradiography as described in Materials and Methods. Input shows 10 μl of the initial binding reaction that was removed before precipitation. The positions of labeled protein are indicated by arrowhead. For reference, the positions of protein markers are shown at the right (kDa). Figure is a typical result from three independent experiments.

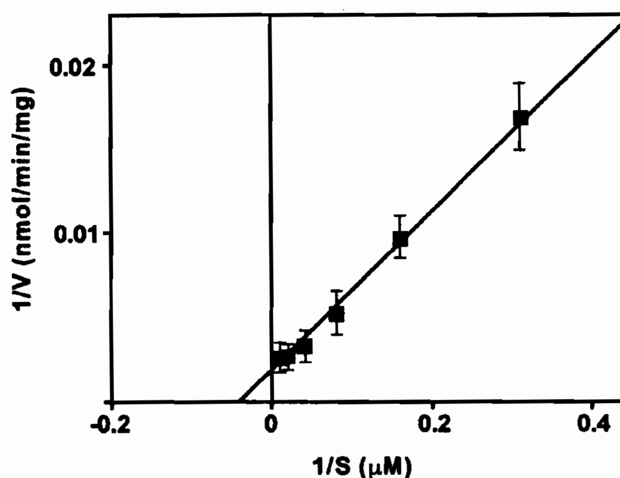

FIG. 3. Phosphorylation of the [Ser^{46}]PKN(39-53) peptide by PKN. The [Ser^{46}]PKN(39-53) peptide was phosphorylated by the purified PKN as described in Materials and Methods. A double-reciprocal plot of the data is shown. The results are means ± S.E. from three independent experiments performed in duplicate.

binding site (data not shown). These inhibitory properties of the sequence suggest that the residues from 39 to 53 act as an autoinhibitory sequence of the enzyme.

Our previous study showed that the small GTP-binding protein Rho associates and activates PKN (3,4), analogous to the situations of Ras-Raf (13) or Rac-PAK (14). However, the activation mechanisms of those GTP-binding protein-regulated serine/threonine kinases are still unclear. In this paper, we presented the possibility that the catalytic domain of PKN is masked by the direct interaction with the amino-terminal regulatory region. The association of inter- and intra-molecule of PKN could contribute to the regulation of the activity of PKN.

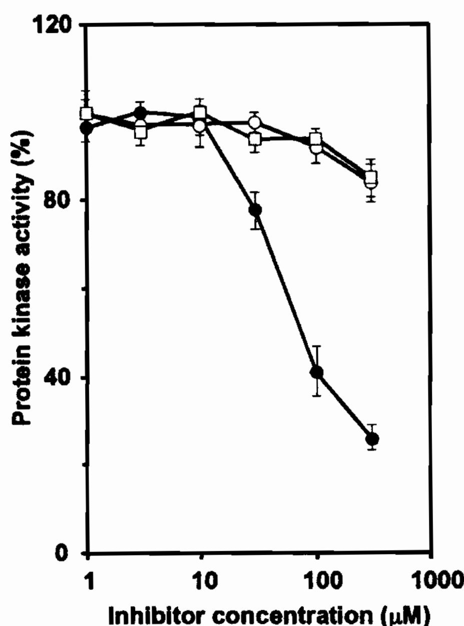

FIG. 4. Inhibition of protein kinases with synthetic peptides. The phosphorylated peptide substrates were used at the respective *Km* concentrations (10 μM for δPKC peptide, 16 μM for Kemptide) in the presence of increasing concentrations of PKN(39-53) or PKN(54-73). PKA was measured by using Kemptide as substrate and PKN(39-53) as inhibitor (□). PKN was measured with δPKC peptide as substrate and with PKN(39-53)(●) or PKN(54-73)(○) as inhibitor. The kinase activity was determined as described in Materials and Methods. The results are means ± S.E. from three independent experiments performed in duplicate.

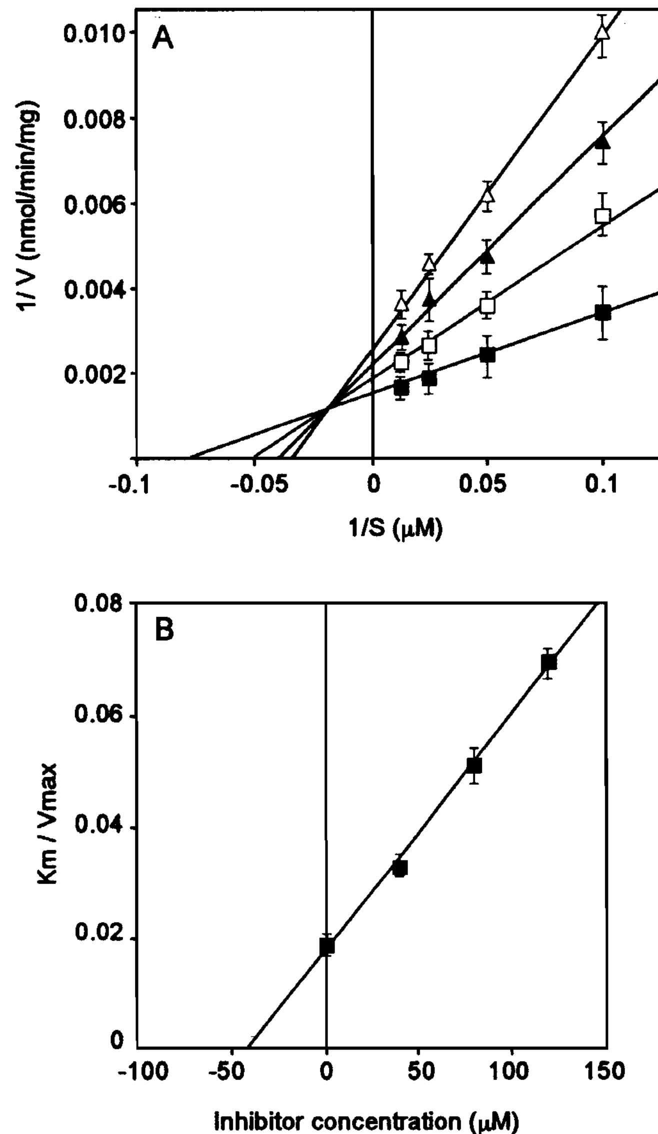

FIG. 5. Effect of PKN(39-53) on peptide substrate phosphorylation by PKN. (A) Double-reciprocal plots at varying PKN(39-53) concentration. Concentration of the peptide, PKN(39-53) was 0 (■), 40 (□), 80 (▲), and 120 (△) μM, respectively. Concentration of the δPKC peptide was 10, 20, 40, and 80 μM, respectively. The kinase activity was determined as described in Materials and Methods. (B) Secondary plot of apparent *Km/Vmax* versus inhibitor concentration. The results are means ± S.E. from three independent experiments performed in duplicate.

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Molecular Cloning and Characterization of a Novel Mitochondrial Phosphoprotein, MIPP65, from Rat Liver

Michinori Kitagawa,* Hideyuki Mukai,† and Yoshitaka Ono*,‡,[1]

Graduate School of Science and Technology, †Radioisotope Research Center, and ‡Department of Biology, Faculty of Science, Kobe University, Kobe, 657, Japan

A novel 65-kDa protein (designated MIPP65), which was phosphorylated by PKN *in vitro* in a manner highly dependent on arachidonic acid, was partially purified from the heat-stable proteins extracted from a 30,000*g* precipitate of rat liver. The cDNA clones were obtained by polymerase chain reaction using oligonucleotides based on partial amino acid sequences. The complete amino acid sequence deduced from the cDNAs contained two homologous regions with the mitochondrial NADH-ubiquinone oxidoreductase 9-kDa subunit precursor at the amino- and carboxyl-termini, whereas the central region was not related to any known proteins and contained a serine cluster. Northern blotting and immunoblotting analyses indicated that MIPP65 was expressed ubiquitously in rat tissues. Immunofluorescence analysis of the endogenous MIPP65 using polyclonal antiserum against MIPP65 showed a predominantly mitochondrial localization in C6 glioma cells. The recombinant MIPP65 expressed in COS7 cells showed a similar pattern of localization to that in C6 glioma cells. On the other hand, deletion of the amino-terminal region of MIPP65 abrogated such localization, indicating that the amino-terminal region contained a mitochondrial-targeting signal. From [^{32}P]orthophosphate-labeled C6 glioma cells, the endogenous MIPP65 could be immunoprecipitated as a phosphoprotein with antiserum against MIPP65. These results suggest that MIPP65 is a novel mitochondrial phosphoprotein that is a candidate substrate for PKN. © 1997 Academic Press

INTRODUCTION

Many protein kinases and kinase-catalyzed reactions play major roles in the regulation of a great variety of cellular processes. Several well-studied protein kinases such as protein kinase C (PKC), cAMP-dependent protein kinase (PKA), Ca^{2+}/CaM-dependent protein kinases, casein kinases, MAP kinases, and ligand receptor-associated tyrosine kinases mediate the responses of eukaryotic cells to external stimuli by phosphorylating specific protein substrates [1, 2]. However, there are only limited methods available for identification of the specific protein substrates relevant to these kinases. Recently, the yeast two-hybrid system, a genetic method for detecting protein interactions *in vivo,* has been used to identify protein substrates for several protein kinases [3–5]. We previously reported a serine/threonine protein kinase PKN with a catalytic domain homologous to the PKC family and a regulatory domain with a unique structure [6, 7] and have identified neurofilament proteins as candidate substrates for PKN using the two-hybrid system [8]. In the case of the second messenger-dependent protein kinases, the target substrates for the various kinases have been identified as proteins which can be phosphorylated in an activator-dependent manner [9–12]. PKN is activated by the small GTP-binding protein Rho in a GTP-dependent manner [13–15] and is also activated by several unsaturated fatty acids, such as arachidonic acid, at relatively low concentrations [7, 16], suggesting that these modifiers are *in vivo* modulators of PKN acting as messengers. To identify additional candidate substrates for PKN, we screened for proteins from fractions extracted from rat liver with the extent of phosphorylation by PKN *in vitro* as an indicator. We have partially purified a 65-kDa protein which was effectively phosphorylated by PKN *in vitro* in a manner highly dependent on arachidonic acid. In this report, we will discuss: (i) the cDNA cloning of this protein, designated MIPP65 (65-kDa mitochondrial phosphoprotein); (ii) the expression of MIPP65 in various tissues and cultured cells; (iii) the intracellular localization of MIPP65 in cultured cells; and (iv) the phosphorylation of MIPP65 in the cells.

[1] To whom correspondence and reprint requests should be addressed. Telephone: (81-78)-803-1245. Fax: (81-78)-803-0322. E-mail: yonodayo@kobe-u.ac.jp.

MATERIALS AND METHODS

Materials. PVDF (polyvinylidene difluoride) membrane (Immobilon) and an ECL (Enhanced Chemiluminescence) immunoblotting

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detection kit were purchased from Millipore and Amersham, respectively. Blue Sepharose, Protein A–Sepharose, and Sephasil C18 SC 2.1/10 were purchased from Pharmacia LKB Biotechnology Inc. MitoTracker Red CMXRos was purchased from Molecular Probes, Inc. Other chemicals were obtained from commercial sources.

Cell lines and cell culture. C6, COS7, NG108-15, and Rat1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. PC12 cells were grown in DMEM containing 10% fetal calf serum and 5% horse serum. Each cell line was cultured as described [17].

Isolation and peptide sequence of MIPP65 from rat liver. All procedures were carried out at 0–4°C. Rat liver (80 g wet wt) was added to 240 ml of buffer A (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 μg/ml leupeptin, and 1 mM PMSF (phenylmethylsulfonyl fluoride)) and homogenized for 5 min with Polytron (KINEMATICA). The resulting crude homogenate was then centrifuged at 30,000g for 20 min. The precipitate was rehomogenized with 160 ml of buffer A and centrifuged at 30,000g for 20 min. This step was repeated eight times, and the final precipitate was homogenized with 120 ml of buffer A and subjected to heat treatment at 100°C for 15 min. The heated protein solution was kept in ice until the temperature dropped to 4° C and the suspension was then centrifuged at 30,000g for 20 min. The supernatant was loaded onto a 1/50 vol of a Blue Sepharose column equilibrated in buffer A. The column was then washed with a 10 column volume of buffer A and eluted with 2 column volumes of buffer A containing 1 M NaCl. The eluate was combined with 7/100 vol of 100% (w/v) trichloroacetic acid and 1/100 vol of 1.5% (w/v) sodium deoxycholate, and after standing at 4°C for 10 min, the resultant suspension was centrifuged at 30,000g for 20 min. The precipitate was suspended in 160 μl of SDS–polyacrylamide gel electrophoresis (PAGE) buffer and subjected to 8% SDS–PAGE. The 65-kDa polypeptide was cut from the gel and digested with endoproteinase Lys-C as described [18], and the peptides were partially purified by chromatography on a Sephasil C18 SC 2.1/10 reverse-phase column of the SMART system (Pharmacia LKB Biotechnology Inc.) The peptide sequences were determined using a gas-phase protein sequencer with an on-line PTH amino acid analyzer (Applied Biosystems Model 490).

Kinase assays. The phosphorylation of the heat-stable proteins extracted from the 30,000g precipitate of rat liver or the 1 M NaCl fraction of Blue Sepharose was carried out at 30°C in an assay mixture containing 20 mM Tris–HCl at pH 7.5, 8 mM $MgCl_2$, 100 μM ATP, 185 kBq of [γ-^{32}P]ATP, phosphate acceptors, 20 ng/ml of PKN purified from rat testis [16], and with or without 40 μM arachidonic acid as indicated in each experiment. After incubation for 5 min, the reaction was terminated by the addition of an equal volume of Laemmli's sample buffer and separated on SDS–polyacrylamide gels. The gels were dried under vacuum and the phosphorylation was visualized with an imaging analyzer (Fuji BAS1000).

Isolation and sequencing of cDNA clones for MIPP65. cDNAs for MIPP65 were amplified from rat brain cDNA by PCR using a pair of synthetic oligonucleotides based on the amino acid sequence of the isolated protein. The forward primer, 5′-AA(CT) CAN TT(CT) GGN GA(AG) (CA)GN GC-3′ (where N is A/C/G/T), corresponding to the sequence of NTFGERA (amino acid residues 370–376), and the reverse primer, 5′-GG (CT)TC (CT)TG NGT (AG)TC NCC NGG-3′, corresponding to the sequence of PGDTQEP (amino acid residues 403–409), were used to produce a cDNA of the expected length of 120 bp from rat brain cDNA. The PCR product was directly cloned into the pT7Blue (Novagen) vector and the nucleotide sequence was determined. cDNAs encoding the 5′- or 3′-coding region for MIPP65 were generated by the rapid amplification of cDNA end method (RACE) [19] using a Marathon-Ready cDNA kit from Clontech Laboratories. For the 5′-RACE, a first round of PCR was performed using AP1 primer (supplied with the kit) and an antisense MIPP65-specific primer; 5′ GGCTTCGCCGGCTGGCGCCTGGAACGT (residues 1165–1191). A second round of PCR used AP2 primer (supplied with the kit) and an antisense primer; 5′ TTTCGTCACTTTCTGGACATG-CCGCCGA (residues 408–435). For the 3′-RACE, a first round of PCR was performed using AP1 primer and a sense primer; 5′ GGG-AGATATTCGAAGACAGTTCCTGTTG (residues 1090–1117). A second round of PCR used AP2 primer and a sense primer; 5′ TTTGGA-GAGAGAGCTGGACCACAACTGG (residues 1132–1159). The secondary PCR products were directly cloned into pT7Blue vector and sequenced.

Northern blot analysis. A rat multiple tissue Northern blot of poly(A)$^+$ RNA was purchased from Clontech Laboratories. The membrane was hybridized with a ^{32}P-labeled *SphI/NarI*-digested 0.8-kb cDNA fragment (nucleotides 381–1178 in Fig. 3) as a probe.

Preparation of antiserum against MIPP65. Antiserum against MIPP65 was obtained by immunizing rabbits with the bacterial synthesized fragment of amino acids 1–140 of rat MIPP65.

Preparation of crude homogenates of rat tissues and various cultured cells. The crude homogenates of rat tissues were prepared as described [16]. The crude homogenates of various cultured cells were prepared by homogenizing the cells in 10 vol of 50 mM Tris–HCl at pH 7.5 containing 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 10 μg/ml leupeptin, 1 mM PMSF, 150 mM NaCl, 1% NP-40, with 30 strokes of a Dounce homogenizer. These samples were subjected to SDS–PAGE and immunoblotting.

Immunoblotting. Samples were separated by SDS–PAGE, and the proteins were transferred to a PVDF membrane. The membrane was incubated with antiserum, and the immunoreactive bands were visualized with an Enhanced Chemiluminescence immunoblotting detection kit (Amersham).

Construction of expression plasmids. A vector for the expression of rat MIPP65 in COS7 cells was constructed as follows: The fragment containing the entire coding region for rat MIPP65 was obtained by PCR from a cDNA library of rat brain using the specific primer set for MIPP65. The resulting PCR product was directly cloned into the pT7Blue vector (pT7-MIPP). After the confirmation of the nucleotide sequence of the insert, the 1.4-kb *NcoI/Bam*HI-digested fragment was inserted into the *Eco*RI site of pTB701 [20] by addition of *Eco*RI linker (pTB-MIPP). A vector for the expression of amino-terminal-deleted MIPP65 was constructed as follows: The 1.3-kb *XhoI/Bam*HI-digested fragment of pT7-MIPP was ligated with *Nco*I linker and digested with *Nco*I. Then, the fragment was blunted and inserted into the blunted *Eco*RI site of pTB701 (pTB-MIPP-ΔN). A vector for the expression of carboxyl-terminal-deleted MIPP65 was constructed by inserting the 0.4-kb *NcoI/Sph*I-digested fragment of pT7-MIPP into the *Eco*RI site of pTB701 by addition of *Eco*RI linker (pTB-MIPP-ΔC). These constructs were transfected into COS7 cells (2×10^5 cells/35-mm dish) with 2 μg of each DNA by electroporation.

Immunofluorescence. For immunofluorescence localization of MIPP65, C6 and COS7 cells grown in 35-mm plastic tissue culture dishes were washed twice with phosphate-buffered saline (PBS), fixed for 1 h at 4°C in 4% paraformaldehyde, rinsed with PBS, and then blocked for 1 h in PBS-T (PBS containing 0.05% Triton X-100) containing 5% normal goat serum. After washing with PBS, cells were incubated overnight at 4°C with each antiserum diluted with PBS-T at ~10 μg/ml. Coverslips were rinsed with PBS-T and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Medical and Biological Laboratories, Co. Ltd.) for 60 min. Coverslips were rinsed with PBS-T followed by PBS, mounted with glycerol-containing 0.1% 1,4-diazabicyclo(2,2,2)octane (DABCO), and viewed on a Zeiss laser scan microscope. For the staining of mitochondria, the medium was replaced with fresh medium containing 25 nM MitoTracker (Molecular Probes, Inc.) prior to the fixation. After 30 min, the cells were washed with PBS and then fixed. Nonspecific fluorescence, determined by incubation without primary antiserum, was negligible.

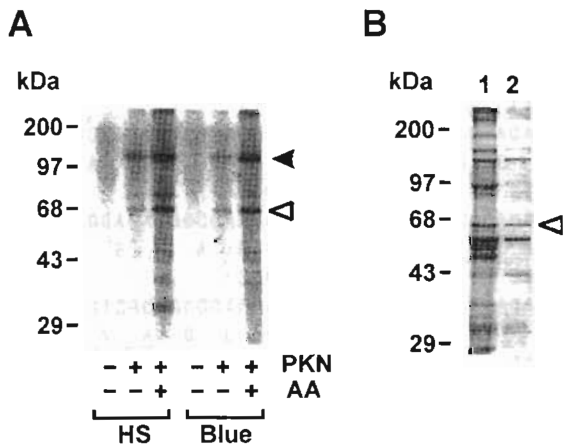

FIG. 1. Phosphorylation of partially purified MIPP65 by PKN. The position of MIPP65 is indicated by a white arrowhead. Molecular mass markers are indicated at left. (A) Autoradiographs of partially purified MIPP65 phosphorylated by PKN. The heat-stable proteins extracted from the 30,000*g* precipitate of rat liver (indicated by HS) and 1 *M* NaCl fraction of Blue Sepharose (indicated by Blue) were incubated with purified rat PKN (indicated by PKN) for 5 min at 30°C with or without 40 μM arachidonic acid (indicated by AA). The position of autophosphorylation of PKN is indicated by a black arrowhead. (B) Coomassie brilliant blue staining of heat-stable proteins extracted from the 30,000*g* precipitate of rat liver (lane 1) and 1 *M* NaCl fraction of Blue Sepharose (lane 2).

Phosphorylation of MIPP65 in vivo. Phosphorylation of MIPP65 *in vivo* was examined using C6 cells incubated in [^{32}P]-orthophosphate. C6 cells were grown in DMEM medium containing 10% fetal calf serum in 100-mm dishes. After reaching ~70% confluence, cells were washed three times with phosphate-free DMEM medium and incubated with 37 MBq carrier-free [^{32}P]orthophosphate in 2 ml of the same medium. After 3 h of incubation, cells were washed three times with PBS and suspended in 500 μl of 20 m*M* Tris–HCl, pH 7.5, containing 1 m*M* EDTA, 1 m*M* EGTA, 150 m*M* NaCl, 1 m*M* DTT, 50 μM sodium vanadate, 5 m*M* NaF, 1 μg/ml leupeptin, and 1 m*M* PMSF. The suspension was subjected to heat treatment at 100°C for 5 min in an Eppendorf tube. The heated solution was kept on ice until the temperature dropped to 4°C and the suspension was then centrifuged at 15,000*g* for 10 min. Then, antiserum (3 μl) raised against MIPP65 was added to the supernatant and rotated at 4°C for 1 h. Protein A–Sepharose (20 μl) was added to the mixture and rotation was continued at 4°C for 30 min. The beads were washed four times with cell lysis buffer and then heated with 20 μl of SDS–PAGE sample buffer at 98°C for 5 min. The supernatant was subjected to SDS–PAGE.

RESULTS AND DISCUSSION

Isolation of Rat Liver MIPP65

To identify potential substrates of PKN, we prepared heat-stable proteins to eliminate the endogenous protein kinase activity. It was previously reported that PKN was abundantly expressed in rat liver [16]. We first utilized rat liver extract as a substrate source, roughly separated by centrifugation at 30,000*g*. The protein band, which was significantly phosphorylated by PKN, was not observed in the heat-stable supernatant, whereas the heat-stable proteins extracted from the precipitate contained several proteins phosphorylated by PKN. As shown in Fig. 1A, a major band with an apparent molecular mass of 65 kDa was effectively phosphorylated in the presence of arachidonic acid. The 65-kDa protein was further separated by Blue Sepharose column chromatography and SDS–PAGE (Fig. 1B). The polypeptide band was excised from SDS–polyacrylamide gels and subjected to *in situ* digestion with endoproteinase Lys-C. The resultant peptides were separated by reverse-phase column chromatography, and their partial amino acid sequences were determined (Fig. 2).

Isolation and Characterization of cDNA Encoding MIPP65

As the peptide sequences of MIPP65 did not correspond to any known protein sequences in the protein sequence databank, cDNA cloning was carried out to deduce the complete protein sequence. First, we prepared a 120-bp cDNA fragment encoding a sequenced peptide from rat brain cDNA. The deduced amino acid sequence of the 120-bp fragment matched the determined sequence of the peptide. To determine the entire

1 S S P P P S S Y P S V E N T G G A V A

2 G P E L E W K

3 N T F G E R A G P Q L E G T F Q A P A G E A P P T D A G P P Q E A P G D T Q E P T L V

4 T E S V F K

5 L L D T H T A A A L S K

6 E S T E L F E A E G I L P G H R K

FIG. 2. Amino acid sequence of peptides derived from the endoproteinase Lys-C treated MIPP65. Protease-treated sample was applied to reverse-phase chromatography and separated peptides were sequenced from the amino-terminus by a gas-phase sequencer.

```
                    20                   40                   60                   80                  100
CGCCCGGGCAGGTGTACCATGGCGGTCTCTCTCCTGCTGCGGGGAGGACGAATCCGGGCGCTGAAGGCTGCCCTTCTCGAGGCAAAGGTGTTCCGAGGAG
                   M  A  V  S  L  L  L  R  G  G  R  I  R  A  L  K  A  A  L  L  E  A  K  V  F  R  G
                   120                  140                  160                  180                  200
AACTGGCTTCTACAGTGCCCCTGTCTACAGAGTCCGAGAATAATAAAAAGGCGGCAGGACCAACTAGCAAGACGGAAAGTGTGTTCAAAGATGTGGTGGG
E  L  A  S  T  V  P  L  S  T  E  S  E  N  N  K  K  A  A  G  P  T  S  K  T  E  S  V  F  K  D  V  V  G
                   220                  240                  260                  280                  300
AGCAGAGGAGAGGGCCAAGCTCCTAGACACTCACACAGCAGCGGCACTGTCCAAAAGCTCCCCTCCACCCAGTTCTTACCCATCAGTGGAGAACACAGGT
  A  E  E  R  A  K  L  L  D  T  H  T  A  A  A  L  S  K  S  S  P  P  P  S  S  Y  P  S  V  E  N  T  G
                   320                  340                  360                  380                  400
GGGGCTGTCGCAGGCCTGCCGCTCACAGACTTGTCAAGAAAGACTTTGGTAGCGTTTCCTCAGAAAGTTACGCCTCCACCGCATGCCTGGGCTTCTGACT
 G  A  V  A  G  L  P  L  T  D  L  S  R  K  T  L  V  A  F  P  Q  K  V  T  P  P  P  H  A  W  A  S  D
                   420                  440                  460                  480                  500
CGGAGGCTCGGCGGCATGTCCAGAAAGTGACGAAAGATTCATCTTCATCTTCCTCATCCAGCTCCTCAGATTCAGACTCTGATGGGGAGGAACATGGCTC
S  E  A  R  R  H  V  Q  K  V  T  K  D  S  S  S  S  S  S  S  S  S  S  D  S  D  S  D  G  E  E  H  G  S
                   520                  540                  560                  580                  600
CGACATTGGTCCCCGAGTGGCAAGCAAAAGCAAGGCAGGGTTTTCCAAACCTGAGGCTTCTCGTCCCTCGAAGAACGGAGCCCCTAAGATCACAGTTTTT
  D  I  G  P  R  V  A  S  K  S  K  A  G  F  S  K  P  E  A  S  R  P  S  K  N  G  A  P  K  I  T  V  F
                   620                  640                  660                  680                  700
GCAAAAGAGAAGGCCAAGGTGCAGAAGCCACACACAGATGTCACCTACCCAGAGAAGCCCCTGCAGCCTAAGAAGAAAGGGACTTTCACCAAGCCTGTAG
 A  K  E  K  A  K  V  Q  K  P  H  T  D  V  T  Y  P  E  K  P  L  Q  P  K  K  K  G  T  F  T  K  P  V
                   720                  740                  760                  780                  800
AAGATAGTAAAGAAATCAGGTCCAAGCTCATGACATCCAAGCCACAGTCCAGTGAGGTTTTGGAGCAAAAAATGAAGGAAAAACAGCACCAGGGAAAACG
E  D  S  K  E  I  R  S  K  L  M  T  S  K  P  Q  S  S  E  V  L  E  Q  K  M  K  E  K  Q  H  Q  G  K  R
                   820                  840                  860                  880                  900
GAGACCAGACAAGACAGGAAAGGAAAGCACAGAGCTGTTTGAAGCTGAAGGAATTTTACCCGGCCACAGGAAAGCCAGAGTTTCCACACAGCCCACAACC
  R  P  D  K  T  G  K  E  S  T  E  L  F  E  A  E  G  I  L  P  G  H  R  K  A  R  V  S  T  Q  P  T  T
                   920                  940                  960                  980                 1000
GGAACACAAGAGGCCAGCGCAGAACTCCGGCCAGCCGCTGCCCCCGAGTCGGGAGCTAGACAGGAAGCCAAAGGGCCAGAGCTCGAATGGAAGACCGCTT
 G  T  Q  E  A  S  A  E  L  R  P  A  A  A  P  E  S  G  A  R  Q  E  A  K  G  P  E  L  E  W  K  T  A
                  1020                 1040                 1060                 1080                 1100
CTCCCCTGGTCAGAAAAGAAAGCCTGGAGAAGCAGGTGCCAGAAGGGAGCTTCCAGGCGGAGGAGGAGACCTCAGGAGATCAGACACTAGGGAGATATTC
S  P  L  V  R  K  E  S  L  E  K  Q  V  P  E  G  S  F  Q  A  E  E  E  T  S  G  D  Q  T  L  G  R  Y  S
                  1120                 1140                 1160                 1180                 1200
GAAGACAGTTCCTGTTGAGGAGAAAAATACCTTTGGAGAGAGAGCTGGACCACAACTGGAGGGGACGTTCCAGGCGCCAGCCGGCGAAGCCCCGCCCACT
  K  T  V  P  V  E  E  K  N  T  F  G  E  R  A  G  P  Q  L  E  G  T  F  Q  A  P  A  G  E  A  P  P  T
                  1220                 1240                 1260                 1280                 1300
GATGCAGGCCCACCCCAGGAAGCCCCAGGCGACACACAGGAGCCCACACTGGTTCCCGAGTCGAGTGACACCACCACCTACAAGAACCTGCAGCATCATG
 D  A  G  P  P  Q  E  A  P  G  D  T  Q  E  P  T  L  V  P  E  S  S  D  T  T  T  Y  K  N  L  Q  H  H
                  1320                 1340                 1360                 1380                 1400
AGTACAACGCATTCACCTTCCTAGACCTGAACCTGGACCTCTCCAAGTTCAGGCTGCCACAGCCGTCTTCGGGACGAGAGTCGCCCCGGCACTGAACTCA
E  Y  N  A  F  T  F  L  D  L  N  L  D  L  S  K  F  R  L  P  Q  P  S  S  G  R  E  S  P  R  H
                  1420                 1440                 1460                 1480                 1500
ACTCAGGGTAGACCCCCGTGTGGTCCCTTGAATTCTTGCCTGTGAGAATAAAAGTCAGAGGCACAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

FIG. 3. Nucleotide and deduced amino acid sequence of the MIPP65. The deduced amino acid sequence of an open reading frame is given in single-letter code below the nucleotide sequence. Amino acid sequences derived from purified peptides are underlined. The initiation and termination codons are in boldface. The poly(A) addition signal sequence is double-underlined. DDBJ, EMBL, and GenBank Accession No. AB000098.

sequence of MIPP65 cDNA, 5′ and 3′ RACE methods were employed. As shown in Fig. 3, the combined cDNA clone has approximately 1.5 kb, with a 5′ noncoding region of 18 bp, a 3′ noncoding region of 105 bp, and a coding region of 1377 bp, encoding a polypeptide of 458 amino acids. The deduced amino acid sequence of the cDNA contained all of the sequenced peptides. To verify that the cDNA encoded MIPP65 from rat liver, antiserum was raised against the bacterially expressed amino-terminal region of the cDNA. The antiserum specifically reacted with the partially purified rat liver MIPP65 (data not shown). Analysis of the amino acid sequence revealed that the amino- and carboxyl-terminal regions of MIPP65 were highly homologous to the 9-kDa subunit of NADH-ubiquinone oxidoreductase (Complex I) [21]. The amino acid alignment of the homologous regions of MIPP65 with the 9-kDa subunit showed 65% sequence identity and 89% homology (Fig.

```
MIPP     1   MAVSLLLRGGRIRALKAALLEAKVFRGELASTVPLSTESENNKKAAGPTSKTESVFKDVVGAE  63
             **·***** **  ***··**** **** ·*··*·**·**··*·*·  *··*··*  *· *·*
NUO9     1   MAASLLLRQGRAGALKTVLLEAGVFRG-VAPAVSLSAESGKNEKGLPPNPKKQSPPKKPVSAA  62


MIPP   412   VPESSDTTTYKNLQHHEYNAFTFLDLNLDLSKFRLPQPSSGRESPRH  458
              ·*· *·*********·*···*************·************
NUO9    63   PTEPFDNTTYKNLQHHDYSTYTFLDLNLDLSKFRMPQPSSGRESPRH  109
```

FIG. 4. Amino acid sequence comparison of the MIPP65 versus NADH-ubiquinone oxidoreductase 9-kDa subunit precursor. Sequence alignment was done using GENETYX (Software Development Co., Ltd., Tokyo, Japan). The deduced amino acid sequences are shown in one-letter code. The amino acid sequence of the NADH-ubiquinone oxidoreductase 9-kDa subunit precursor is from the report by Skehel *et al.* [21]. The matched residues are connected by asterisks. Conservative substitutions are indicated by dots. A gap introduced to maximize homology is indicated by a dash. MIPP, MIPP65; NUO9, NADH-ubiquinone oxidoreductase 9-kDa subunit precursor.

4). A sequence analysis program (PSORT, GenomeNet WWW server, Japan) predicted that the amino-terminal region of MIPP65 contained a mitochondrial targeting signal. Mitochondrial targeting signal sequences do not show extensive amino acid sequence homology but are rich in basic, hydroxylated, and hydrophobic amino acids [22]. This characteristic feature was observed in the amino-terminal region of MIPP65. Outside the homologous regions (amino acid residues 64–411), the MIPP65 sequence showed no significant similarity to other known proteins. Interestingly, the nonsimilar region contained a serine cluster (residues 141–154) found in several proteins such as Nopp140, a shuttle protein between the nucleus and the cytoplasm [23], or phosvitin, a constituent protein of oocytes of several species [24], although the function of the cluster is still unclear. The predicted molecular weight of the MIPP65 protein encoded by the cDNA was 49,299, which is considerably less than that of the MIPP65 protein partially purified from rat liver. To confirm whether the cDNA contained the entire coding region for the MIPP65 protein, COS7 cells were transfected with an MIPP65 expression vector. As expected, immunoblotting analysis with polyclonal antiserum raised against the bacterially expressed amino-terminal region of MIPP65 showed that the recombinant protein migrated as a 65-kDa band on SDS–PAGE, indicating that the cloned cDNA encoded the full-length MIPP65 protein (Fig. 6B, lane 6). The difference between calculated and observed molecular masses of MIPP65 might be due to

FIG. 5. Northern blot analysis of MIPP65 RNA in rat tissues. β-Actin expression is shown at the bottom as the internal control.

FIG. 6. Immunoblot analysis of MIPP65 in various rat tissues and cultured cells. For each lane, 10 μg of protein of crude lysate was loaded and the proteins were resolved by 10% SDS–PAGE. The position of MIPP65 is indicated by a white arrowhead. Molecular mass markers are indicated at left. (A) Immunoblot analysis of various rat tissues. Lanes: 1, brain; 2, heart; 3, kidney; 4, liver; 5, lung; 6, spleen; 7, testis; 8, thymus. (B) Immunoblot analysis of various cultured cells. Lanes: 1, rat C6 glioma cells; 2, NG108-15 hybridoma cells; 3, rat pheochromocytoma PC12 cells; 4, Rat1 rat embryo fibroblast cells; 5, monkey kidney COS7 cells; 6, COS7 cells transfected with pTB-MIPP.

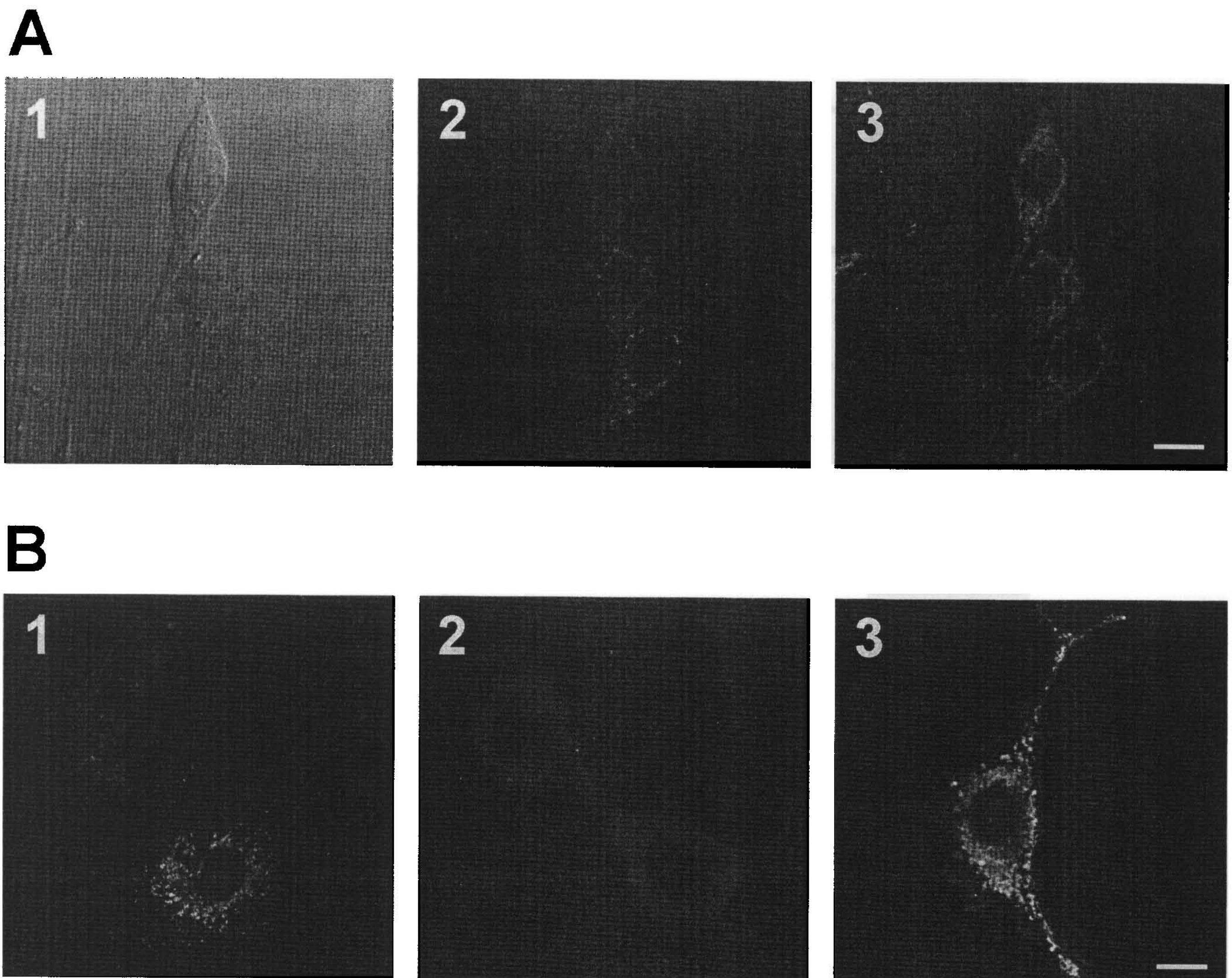

FIG. 7. Immunofluorescence localization of MIPP65 in cultured cells. Bars, 10 μm. (A) Double-labeling with the antiserum against MIPP65 and a mitochondrial fluorescent dye in C6 cells. C6 cells incubated with MitoTracker dye were fixed and incubated sequentially with the antiserum and fluorescein-conjugated goat anti-rabbit IgG and then viewed by phase-contrast microscopy (1) or by fluorescence microscopy with FITC (2), or a rhodamine B isothiocyanate (3) filter. (B) Immunofluorescence localization of various recombinant MIPP65 in COS7 cells. COS7 cells were transiently transfected with pTB-MIPP (1), pTB-MIPP-ΔN (2), or pTB-MIPP-ΔC (3) plasmid DNA, incubated with antiserum against MIPP65, and then viewed by fluorescence microscopy with an FITC filter.

posttranslational modification. Northern blotting analysis using an ~800-bp cDNA probe corresponding to the unique midportion of MIPP65 revealed that ~1.5-kb MIPP65 mRNA was expressed ubiquitously in various rat tissues (Fig. 5); it was abundant in liver and kidney and was expressed at moderate levels in other tissues.

Immunoblotting Analysis of MIPP65

Antisera raised against recombinant amino-terminal or carboxyl-terminal regions of MIPP65 reacted consistently with a major 65-kDa protein in various rat tissues (Fig. 6A). MIPP65 was relatively abundant in brain, liver, and thymus. The antisera also reacted with faint minor bands that varied between tissues and that were possibly due to the nonspecific cross-reaction. Moreover, C6, NG108-15, PC12, and Rat1 cells, which are all derived from rat tissues, showed MIPP65 immunoreactivity as a single band of 65 kDa, suggesting that MIPP65 is ubiquitously expressed in glial, neuronal, and fibroblastic cells (Fig. 6B). COS7 cells, derived from monkey tissues, showed no detectable levels of MIPP65 immunoreactivity, which suggests that the antiserum

scarcely cross-reacts with the monkey homolog of MIPP65 or that the native expression of monkey homolog of MIPP65 is low in COS7 cells.

Immunolocalization of MIPP65 in Mammalian Cells

We examined the subcellular localization of the endogenous MIPP65 in C6 cells by using antisera against MIPP65. No detectable immunofluorescence was obtained using nonimmune serum or immune serum preabsorbed with the antigen (data not shown). As shown in Fig. 7A-2, most of the MIPP65 immunofluorescence was associated with structures that appeared to be mitochondria on the basis of their cytoplasmic localization and shape. Double-labeling with antiserum against MIPP65 and a rhodamine-like dye which is specifically imported into mitochondria (MitoTracker) confirmed that a large portion of MIPP65 was associated with mitochondria (Fig. 7A-3). Biochemical subcellular fractionation of rat brain revealed that MIPP65 immunoreactivity was abundant in the P2D fraction, supporting the mitochondrial localization of MIPP65 (data not shown). Moreover, the fluorescence pattern of COS7 cells transfected with the expression vector encoding the full length of MIPP65 (Fig. 7B-1) was similar to that seen in C6 cells. To determine the region of MIPP65 responsible for mitochondrial localization, deletion mutants of the protein were constructed and expressed in COS7 cells. Cells transfected with an expression vector encoding a mutant MIPP65 lacking part of the putative mitochondrial targeting signal (amino acid residues 1–20) showed diffuse staining in the cytosol (Fig. 7B-2). On the other hand, deletion of the carboxyl-terminal region of MIPP65 (lacking amino acid residues 123–458) resulted in a staining pattern similar to that seen with the full-length MIPP65 (Fig. 7B-3). These results suggest that the amino-terminal region of MIPP65 functions as a mitochondrial targeting signal.

Phosphorylation of MIPP65 in Vivo

Phosphorylation of MIPP65 *in vivo* was investigated using C6 cells incubated in [^{32}P]orthophosphate. Labeled cells were lysed and the endogenous MIPP65 was immunoprecipitated from the supernatant with the antiserum. Then, the immunoprecipitant was subjected to SDS–PAGE and autoradiography. As shown in Fig. 8, the major band of radioactivity occurred at a position corresponding to approximately 65 kDa. Some weak bands of radioactivity were present at a high-molecular-weight position and were possibly due to contamination. Further evidence to support the endogenous presence of the MIPP65 as a phosphoprotein was obtained by analysis of the electrophoretic mobility of MIPP65. Dephosphorylation of the partially purified MIPP65 from rat liver resulted in an apparent increase

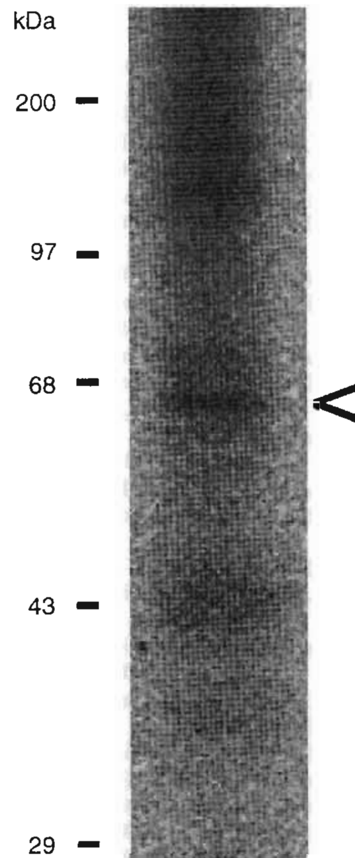

FIG. 8. SDS–PAGE and autoradiography of MIPP65 phosphorylated *in vivo*. C6 cells were incubated for 2 h in [^{32}P]orthophosphate. Proteins were extracted and MIPP65 was immunoprecipitated with antiserum against MIPP65. Molecular mass markers are indicated to the left. The arrowhead indicates the band of radioactivity at a position corresponding to 65 kDa.

in mobility (~1 kDa) on SDS–PAGE (data not shown). These results suggest that the protein was metabolically phosphorylated as a posttranslational modification in the cells and that some protein kinase was involved in this phosphorylation of MIPP65 *in vivo*. From the results of *in vitro* phosphorylation experiments, PKN is a likely candidate for the protein kinase responsible. However, further investigations at the cellular level are required to confirm the role of MIPP65 in the PKN signaling pathway.

The function of MIPP65 is not known at present. Since the N-terminal and the C-terminal regions of MIPP65 were highly similar to the 9-kDa subunit of Complex I, MIPP65 may be a rat isoform of the 9-kDa subunit separated by insertion of an ~340-amino-acid unique sequence. Masui *et al.* indicated by structural analysis that the 9-kDa subunit is a constituent of only the mammalian Complex I [25], the major entry point for electrons into the respiratory chain. However, this

9-kDa subunit does not contain FMN or iron–sulfur centers which are involved in electron transfer between NADH and ferricyanide, and thus the function of this subunit is not yet clear. Consensus phosphorylation sites for casein kinase and PKC were found in the primary sequence of the 9-kDa subunit. However, phosphorylation of this subunit has not yet been reported. In our *in vitro* phosphorylation experiments using various deletion mutants of MIPP65 as substrates, PKN efficiently phosphorylated the unique midportion of MIPP65 (data not shown). MIPP65 thus might be a new subunit of oxidoreductase that was acquired during evolution and that confers regulation via phosphorylation.

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