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Regulation of a Mitogen-Activated Protein Kinase Kinase Kinase, MLTK by PKN

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PKNα is a fatty acid- and Rho-activated serine/threonine protein kinase having a catalytic domain homologous to members of the protein kinase C family. Recently it was reported that PKNα is involved in the p38 mitogen–activated protein kinase (MAPK) signaling pathway. To date, however, how PKNα regulates the p38γ MAPK signaling pathway is unclear. Here we demonstrate that PKNα efficiently phosphorylates MLTKα (MLK-like mitogen-activated protein triple kinase), which was recently identified as a MAPK kinase kinase (MAPKKK) for the p38 MAPK cascade. Phosphorylation of MLTKα by PKNα enhances its kinase activity *in vitro*. Expression of the kinase-negative mutant of PKNα inhibited the mobility shift of MLTKα caused by osmotic shock in SDS-PAGE. Furthermore, PKNα associates with each member of the p38γ MAPK signaling pathway (p38γ, MKK6, and MLTKα). These results suggest that PKNα functions as not only an upstream activator of MLTKα but also a putative scaffold protein for the p38γ MAPK signaling pathway.

Key words: osmotic shock, PKN, p38γMAPK, MLTK, scaffold protein.

Abbreviations: GST, glutathione S-transferase; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MAPKKKK, MAPKKK kinase; MLTK, MLK-like mitogen-activated triple kinase; PKC, protein kinase C.

PKN is a serine/threonine protein kinase with a catalytic domain highly homologous to PKC in the carboxyl-terminal region and a unique regulatory domain in the amino-terminal region (*1–3*). This protein kinase makes a family that comprises at least three gene products, including PKNα/PRK1, PKNβ, and PRK2 (*4*, *5*). The amino-terminal region of PKNα contains three repeats of a leucine zipper–like motif and its kinase activity is stimulated by fatty acids such as arachidonic acid (*3*). The amino-terminal region of PKNα also functions as a binding interface with various proteins. Indeed, we have isolated several binding proteins such as small GTP-binding protein Rho (6–8), intermediate filament proteins (*9*, *10*), actin cross-linking protein α-actinin (*11*), phospholipase D1 (*12*), the potential transcription factor PCD-17 (*13*), the basic Helix-Loop-Helix transcription factor NDRF/NeuroD2 (*14*), and centrosome and the Golgi-localized giant anchoring protein CG-NAP (*15*). We reported that PKN translocates from the cytosol to the nucleus upon exposure to stresses such as heat shock and serum starvation (*16*) and that PKN is cleaved during apoptosis, presumably by caspase-3, generating a constitutively active kinase fragment (*17*). Furthermore, we have found that PKN delays mitotic timing by inhibiting the mitotic regulatory phosphatase, Cdc25C (*18*). Thus, we speculate that PKN is a multifunctional protein kinase involved in both cytoskeletal reorganization and nuclear events. Recently, a novel biochemical route linking Rho to the nucleus was found (*19*). This signaling pathway involves PKN and a recently identified member of the p38 MAPK superfamily, p38γ (ERK6) (*20*, *21*). It is unclear, however, how PKN regulates the p38γ MAPK signaling pathway. In the present study, therefore, we have tried to elucidate the functional linkage between PKN and the p38γ MAPK cascade.

The MAPK pathways function in a variety of physiological aspects in cells from yeast to human (*22–25*). In mammals, at least three MAPK cascades have been identified. The MAPKs in each pathway are extracellular signal-regulated kinases (ERK-1, 2), c-JUN amino-terminal kinases/stress-activated protein kinases (JNK/SAPK-1, 2, 3), and p38 MAPKs (p38α, β, γ, δ). The ERK cascade is mostly responsive to mitogenic and differentiation stimuli, whereas the JNK and p38 MAPK pathways are primary signaling pathways that are activated by proinflammatory cytokines and extracellular stresses such as UV irradiation and osmotic shock (*26–28*). The central core of each MAPK pathway is a conserved cascade of three protein kinases, which are commonly referred to as MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK) (*29*). An activated MAPKKK phosphorylates and activates a specific MAPKK, which then activates a specific MAPK.

Here we analyze the activation mechanism of the p38γ MAPK signaling pathway by PKNα. We demonstrate that PKNα phosphorylates and activates MLTKα (MLK-like mitogen-activated protein triple kinase) (*30*), which was recently identified as a MAPKKK for the p38 MAPK

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cascade. Furthermore we show that PKNα associates with each member of the p38γ MAPK signaling pathway (p38γ, MKK6, and MLTKα). These results suggest that PKNα acts as not only a MAPKKK kinase (MAPKKKK) but also a putative scaffold protein for the p38γ MAPK signaling pathway.

EXPERIMENTAL PROCEDURES

Antibodies—Mouse monoclonal anti-FLAG (M2) and rat monoclonal anti-HA (3F10) were purchased from Sigma and Roche Molecular Biochemicals, respectively. Agarose-conjugated mouse monoclonal anti-HA (F-7), goat polyclonal anti-ERK6 (p38γ), and goat polyclonal anti-MEK6 (MKK6) were obtained from Santa Cruz Biotechnology, Inc. The phosphorylated peptide-specific antibodies (anti-phospho-ATF2, anti-phospho-p38 MAPK, and anti-phospho-MKK3/MKK6) and rabbit polyclonal anti-ATF2 were purchased from Cell Signaling Technology.

Plasmids—The open reading frames (ORFs) of human p38α, p38γ, MKK3, and MKK6 were amplified by PCR. The products were treated with restriction endonucleases, and inserted into expression plasmids. For the expression of HA- and FLAG-tagged proteins, pTB701HA and pTB701FLAG were used, respectively (*9*, *11*). For the expression of GST- and His-tagged proteins, pGEX4T (Amersham Bioscience) and pRSET (Invitrogen) were used, respectively. HA-tagged wild type and kinase negative MLTKα expression plasmids were kindly provided by E. Nishida (*30*). The expression plasmids for PKNα of FLAG-tagged full length (pRc/CMV/PKN-FL), the constitutive active form (pRc/CMV/PKN/AF3-FL), and the kinase-negative mutant (pRc/CMV/PKN/AF3-T774A-FL) were described previously (*17*, *31*).

Recombinant Proteins—GST-fused and His-tagged proteins were expressed in *E. coli* and purified on glutathione Sepharose 4B (Amersham Bioscience) and nickel-NTA agarose (Qiagen), respectively, according to the manufacturers' protocols. The GST-fused-constitutively active form of human PKNα was expressed in Sf9 cells and purified on glutathione Sepharose 4B as described previously (*31*).

Cell Culture—HEK293T and COS-7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Transfection of the expression plasmids to HEK293T cells was performed using LipofectAMINE PLUS (Invitrogen) according to the manufacturer's protocol. Transfection to COS-7 cells was performed by electroporation.

In Vitro Treatment of p38γ by PKNα—Two micrograms of the purified GST-p38γ MAPK was treated with the constitutively active form of PKNα at 30°C for 15 min in a kinase buffer (25 mM Tris-HCl at pH 7.5, 2 mM DTT, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and10 mM $MgCl_2$) containing 200 μM ATP. After incubation, the reactions were stopped by the addition of SDS sample buffer. Samples were subjected to SDS-PAGE and analyzed by immunoblotting using an anti–phospho-p38 antibody. Immunoreactive bands were visualized with ECL Western Blotting Detection Reagents (Amersham Bioscience).

Immunoprecipitation and Kinase Assays—After transfection, cells were cultured for 24 h and incubated overnight in serum-free medium. Cells were lysed at 4°C in lysis buffer (20 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin). The cleared lysates containing HA-tagged kinases were immunoprecipitated at 4°C for 1 h with an agarose-conjugated mouse monoclonal anti-HA antibody. The immunocomplexes were washed twice with lysis buffer and twice with kinase buffer, and the resultant immunoprecipitates were then used for *in vitro* kinase assay or analyzed by immunoblotting using the phospho-specific antibody. In the case of the kinase assay for p38, immunoprecipitates were resuspended in 50 μl of kinase buffer containing 200 μM ATP and 1 μg of GST–ATF2 (Cell Signaling Technology) as a substrate. The mixtures were incubated at 30°C for 15 min and the reactions were stopped by adding SDS sample buffer. Samples were subjected to SDS–PAGE and analyzed by immunoblotting using an anti-phospho-ATF2 antibody. In the case of the kinase assay of MLTKα, first, immunoprecipitates containing MLTKα were treated with or without the constitutively active form of PKNα in 50 μl of kinase buffer containing 200 μM ATP at 30°C for 5 min. Then 2 μg of His-tagged MKK6 was added as a substrate for MLTKα and the mixtures were incubated at 25°C for 5 min. After incubation, the reactions were stopped by the addition of SDS sample buffer. Samples were subjected to SDS–PAGE and analyzed by immunoblotting using an anti-phospho-MKK3/MKK6 antibody. For *in vitro* phosphorylation of MLTKα by PKNα, immunoprecipitates containing MLTKα were phosphorylated by the constitutively active form of PKNα in 50 μl of kinase buffer containing 100 μM ATP and 37 kBq [γ-^{32}P]ATP under the conditions described in the figure legends. After incubation, the reactions were stopped by the addition of SDS sample buffer. Samples were subjected to SDS–PAGE and the gel was dried under vacuum. Phosphorylation was visualized by a BAS2000 imaging analyzer (Fuji Film).

Co-Immunoprecipitation Assays—COS-7 cells were transfected with an equal amount of the expression plasmids for HA- and FLAG-tagged proteins. After 2 days, the cells were lysed in a lysis buffer. The cleared lysates were incubated with an anti-FLAG antibody at 4°C for 2 h, and then protein G-Sepharose (Amersham Bioscience) was added and the reaction was continued for 1 h. After washing the resin with the same buffer, the bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting with an anti-HA antibody.

RESULTS AND DISCUSSION

PKNα Activates the p38γ MAPK Signaling Pathway—To test whether PKNα affects the p38γ MAPK pathway, we transiently co-expressed HA-tagged p38γ MAPK with the FLAG-tagged constitutively active form of PKNα in HEK293T cells. HA-tagged p38γ MAPK was immunoprecipitated and tested for kinase activity *in vitro* using GST fused-ATF2 protein as a specific substrate. As shown in Fig. 1A, consistent with the report by Marinissen *et al.* (*19*), expression of the active form of PKNα resulted in a

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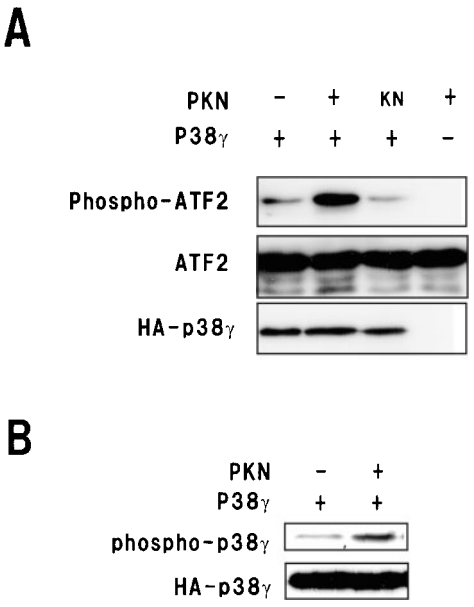

Fig. 1. **PKNα activates the p38γ MAPK signaling pathway.** A: HEK293T cells were transfected with expression plasmids encoding HA-tagged p38γ MAPK together with or without the FLAG-tagged constitutively active form of PKNα. After transfection, cells were cultured for 24 h and incubated in serum-free medium overnight. HA-p38γ MAPK was immunoprecipitated and then subjected to *in vitro* kinase assays using GST fused-ATF2 protein as an exogenous substrate. Phosphorylation of ATF2 protein was analyzed by immunoblotting using an anti-phospho-ATF2 antibody (upper). Input ATF2 protein was monitored by re-probing with an anti-ATF2 antibody (middle). The expression of HA-p38γ MAPK in total cell lysates was monitored by an anti-HA antibody (lower). As a negative control, the effect of the kinase-negative mutant of PKNα on p38γ activity was also examined (indicated as KN). B: Immunoprecipitates containing HA-p38γ MAPK as in *A* were analyzed by immunoblotting using an anti–phospho-p38 antibody (upper). The expression level of HA-p38γ MAPK in total cell lysates was monitored by an anti-HA antibody (lower).

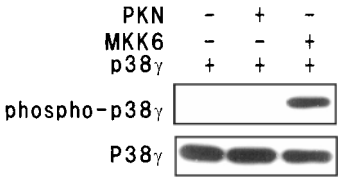

Fig. 2. **PKNα does not phosphorylate the p38γ MAPK *in vitro*.** GST-p38γ MAPK was treated with the constitutively active form of PKNα *in vitro*. After incubation at 30°C for 15 min, samples were analyzed by immunoblotting using an anti-phospho-p38 antibody (upper). The input GST-p38γ MAPK protein was monitored by an anti-ERK6 (p38γ) antibody (lower). As a positive control, GST-MKK6 instead of PKNα was added (lane 3).

potent elevation of the enzymatic activity of p38γ in HEK293T cells. We also analyzed the effect of PKNα on the phosphorylation of the activating phosphorylation sites of the p38γ MAPK by immunoblotting using an anti–phospho-p38 antibody that recognizes the dual phosphorylation site TGY on its regulatory domain. Figure 1B shows PKNα enhances the specific phosphorylation of the activating phosphorylation sites of the p38γ MAPK. These results indicate that PKNα can activate the p38γ MAPK signaling pathway.

PKNα Phosphorylates and Activates a MAPKKK, MLTKα—We addressed the question of how PKNα activates the p38γ MAPK signaling pathway. One of the most likely mechanisms is that PKNα directly phosphorylates and activates the protein kinase(s) participating in the p38γ MAPK cascade.

First, we examined whether PKNα can directly phosphorylate and activate the p38γ MAPK. As shown in Fig. 2, PKNα did not phosphorylate the activating phosphorylation site of GST-fused p38γ MAPK in an *in vitro* phosphorylation reaction. Furthermore, PKNα did not change the activity of the p38γ MAPK *in vitro* (data not shown). We then examined the phosphorylation of the activating phosphorylation sites of MKK6 and MKK3, which are known as MAPKKs for the p38 MAPK family, using an anti–phospho-MKK3/MKK6 antibody. Again, however, PKNα did not show any specific phosphorylation of MKK6 and MKK3 (Fig. 4A, lane 3; and data not shown). These results indicate that PKN directly regulates neither p38γ MAPK nor MKK3/6. Thus we tried to test whether PKNα regulates MAPKKK.

In this paper we have focused on MLTKα (also known as MLK-related kinase α, MRKα) as a MAPKKK, because it has been reported to preferentially activate the p38γ pathway (*30*, *32*). To investigate whether PKNα can phosphorylate MLTKα *in vitro*, we prepared wild-type, a kinase-negative mutant, and a deletion mutant of the HA-tagged MLTKα protein expressed in HEK293T cells by immunoprecipitation and used them as the substrates for PKNα. As shown in Fig. 3A, PKNα efficiently phosphorylated the kinase-negative mutant of MLTKα, with the phosphorylation by PKNα found in the carboxyl-terminal region of MLTKα (Fig. 3C). The level of phosphorylation of the wild type of MLTKα was apparently enhanced by incubation with PKNα (Fig. 3, A and B). The phosphorylation by PKNα caused a mobility shift of MLTKα in SDS-PAGE (Fig. 3, A and C, lower). Gotoh *et al*. reported that the mobility shift correlates with the kinase activity of MLTKα (*30*). Thus we examined whether PKNα activates MLTKα activity by direct phosphorylation. The wild type HA-tagged MLTKα protein expressed in HEK293T cells was purified by immunoprecipitation, treated with or without PKNα, and subjected to an *in vitro* kinase assay using His-MKK6 as an exogenous substrate. MLTKα activity was evaluated by analyzing the phosphorylation level of the activation phosphorylation site of MKK6 by immunoblotting using an anti–phospho-MKK3/MKK6 antibody. As shown in Fig. 4A, when MLTKα was treated with PKNα, the kinase activity of MLTKα for the phosphorylation of the activating phosphorylation site of MKK6 was clearly enhanced. The phosphorylation could not be seen in the absence of MLTKα (Fig. 4A, lane 3), and the kinase-negative mutant of PKNα did not enhance the phosphorylation level of MKK6 by MLTKα (data not show). Furthermore, to confirm that PKNα is involved in the regulation of MLTKα *in vivo*, we first analyzed the effect of co-expression of the constitutively active form of PKNα on MLTKα activity. As shown in Fig. 4B, phosphorylation of MKK6 by MLTKα was apparently enhanced when the

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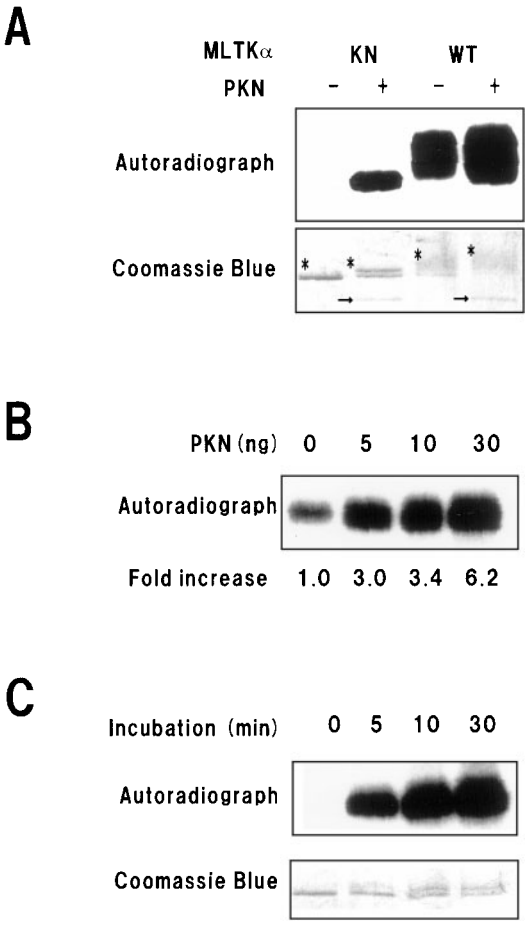

Fig. 3. **PKNα phosphorylates MLTKα *in vitro*.** A: The wild-type (WT) and the kinase-negative mutant (KN) of the HA-tagged MLTKα protein expressed in HEK293T cells were purified by immunoprecipitation and used as substrates for PKNα. Reactions were performed at 30°C for 15 min in the presence of the constitutively active form of PKNα. Phosphorylated proteins were detected by autoradiography following SDS-PAGE (upper). The lower panels indicate Coomassie Blue staining of the gel. Asterisks and arrows indicate the protein bands of HA-tagged MKTKα and the GST-fused constitutively active form of PKNα, respectively. B: Phosphorylation of the wild type MLTKα was enhanced by incubation with the constitutively active form of PKNα in a dose-dependent manner. HA-tagged MLTKα expressed in HEK293T cells was purified by immunoprecipitation and used as a substrate for PKNα. Reactions were performed at 25°C for 3 min in the presence of the indicated amounts of the constitutively active form of PKNα. Phosphorylated proteins were detected by autoradiography following SDS-PAGE. Fold increases in phosphate incorporation into MLTKα are shown at the bottom of each lane. C: The HA-tagged carboxyl-terminal region of MLTKα (amino acids, 284–802) expressed in HEK293T cells was purified by immunoprecipitation and used as a substrate for PKNα. Reactions were performed at 30°C for the indicated time in the presence of the constitutively active form of PKNα. Phosphorylated proteins were detected by autoradiography following SDS-PAGE (upper). The lower panel indicates Coomassie Blue staining of the gel.

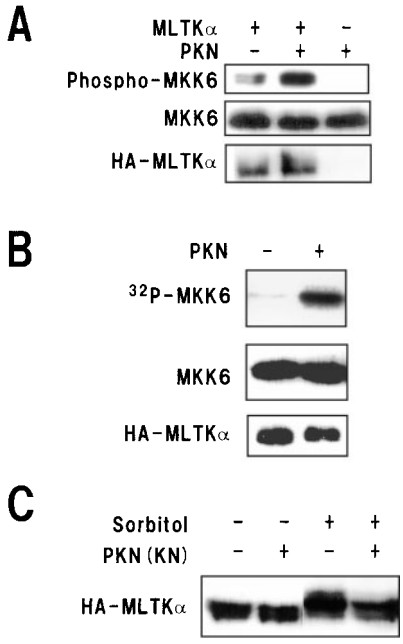

Fig. 4. **PKNα regulates MLTKα.** A: Activation of MLTKα by PKN *in vitro*. HA-tagged MLTKα expressed in HEK293T cells was purified by immunoprecipitation, treated with or without the constitutively active form of PKNα, and subjected to *in vitro* kinase assay using His-tagged MKK6 as an exogenous substrate. MKK6 phosphorylation was performed at 25°C for 5 min. MLTKα activity was analyzed by immunoblotting using a phospho-MKK3/MKK6 antibody (upper). The input His-tagged MKK6 protein was monitored by re-probing with an anti-MKK6 antibody (middle). HA-MLTKα was also monitored by an anti-HA antibody (lower). B: Effect of co-expression of the constitutively active form of PKNα on MLTKα activity. HEK293T cells were tranfected with expression plasmids encoding HA-tagged MLTKα together with or without the constitutively active form of PKNα. HA-tagged MLTKα was purified by immunoprecipitation, and subjected to the *in vitro* kinase assay using His-tagged MKK6 as an exogenous substrate. MKK6 phosphorylation was performed at 25°C for 5 min. The results of autoradiography are shown (upper). The input His-tagged MKK6 protein was monitored by probing with an anti-MKK6 antibody (middle). HA-MLTKα was also monitored by an anti-HA antibody (lower). C: Effect of co-expression of the kinase negative mutant of PKNα on the mobility shift of MLTKα in response to osmotic shock. HEK293T cells were tranfected with expression plasmids encoding HA-tagged MLTKα together with or without the kinase-negative mutant of PKNα. The cells were then left either untreated or stimulated with 0.5 M sorbitol for 20 min. HA-tagged MLTKα was detected by immunoblotting the cell lysates with an anti-HA antibody.

constitutively active form of PKNα was co-transfected. Next, we co-expressed HA-tagged MLTKα together with or without the kinase-negative mutant of PKNα in HEK293T cells. The cells were then left either untreated or stimulated with sorbitol. It is known that osmotic shock, such as treatment with sorbitol, causes a mobility shift of MLTKα in SDS-PAGE (*30*). Indeed, a clear mobility shift of MLTKα was seen in response to osmotic shock (Fig. 4C, lane 3). However, when the cells were transfected with MLTKα together with the kinase-negative mutant of PKNα, the mobility shift of MLTKα in response to osmotic shock was apparently reduced (Fig. 4C, lane 4). These results suggest that PKNα functions as an upstream activator of MLTKα and may lie downstream of an osmosensor system.

Despite the growing knowledge of the signaling elements involved in each MAPK cascade, little is known about the regulation of MAPKKK. In *Saccharomyces cer-*

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evisiae, Ste20 functions as a MAPKKKK in the mating pheromones pathway (*33*). Ste20 binds to and phosphorylates the MAPKKK Ste11. The amino-terminal regulatory half of Ste11 binds to its catalytic domain and blocks its activity. Phosphorylation of the amino-terminus by Ste20 prevents this intramolecular association, leading to the activation of Ste11 (*33*). In mammalian cells, a group of kinases called the p21-activated kinases, or PAKs are known to be homologues of Ste20 (*34*, *35*). Since PAKs can mediate JNK activation by the small GTP-binding protein Rac and Cdc42, PAKs may connect Rac/Cdc42 to the MEKK1-SEK-JNK module (*35*, *36*). The p38γ signaling pathway involving PKNα may be regulated by a similar mechanism to that described above. The carboxyl-terminal region of MLTKα has a α-motif just after the leucine zipper motif, and is supposed to be a regulatory region (*30*). Phosphorylation experiments using deletion mutants of MLTKα have revealed that PKNα phosphorylates mainly the carboxyl-terminal region of MLTKα and that phosphorylation causes an apparent mobility shift of the protein band in SDS-PAGE (Fig. 3C). It is suggested, that therefore, phosphorylation by PKNα on the carboxyl-terminal region of MLTKα causes a conformational change in MLTKα resulting in the activation of this kinase.

PKNα Associates with Each Member of the p38 MAPK Signaling Pathway—It is known that the specificity and efficiency of MAPK cascades are maintained by scaffold proteins and/or physical interactions of each member of the MAPK cascades (*37*). Thus we studied the interaction of PKNα with the p38γ MAPK, MKK6, and MLTKα by co-immunoprecipitation experiments. First, the interaction between PKNα and the p38γ MAPK was examined. The association of PKNα with the p38α MAPK was also analyzed to clarify the binding specificity of PKNα for the p38 MAPK family. We transiently expressed FLAG-tagged full-length PKNα with HA-tagged p38γ MAPK or HA-tagged p38α MAPK in COS-7 cells. FLAG-tagged full-length PKNα was recovered from the cell extracts by immunoprecipitation with an anti-FLAG antibody, and the immunoprecipitates were then probed with an anti-HA antibody. The p38γ MAPK clearly interacted with PKNα, while weak binding of the p38α MAPK to PKNα was observed (Fig. 5A). When HA-tagged p38α MAPK was co-expressed with the constitutively active form of PKNα in HEK293T cells and the p38 activity for ATF2 phosphorylation was assayed using the immunoprecipitates of HA-tagged p38α MAPK, no apparent enhancement of the enzymatic activity of p38α could be observed (data not shown). These results suggest that among p38 MARK family members, PKNα preferentially regulates the p38γ MAPK signaling pathway. To define the region of PKNα responsible for its interaction with the p38γ MAPK, we used deletion mutants of PKNα. Fig. 5B shows that the carboxyl-terminal region, corresponding to the constitutively active form of PKNα, is sufficient for its interaction with the p38γ MAPK. Next, we examined the interaction of PKNα with MKK3 and MKK6. As shown in Fig. 5C, both MAPKKs clearly associated with the carboxyl-terminal region of PKNα. Finally, we tested the association between PKNα and MLTKα. Again, PKNα interacted with MLTKα, and the carboxyl-non-catalytic region of MLTKα preferentially bound to PKNα (Fig. 5D).

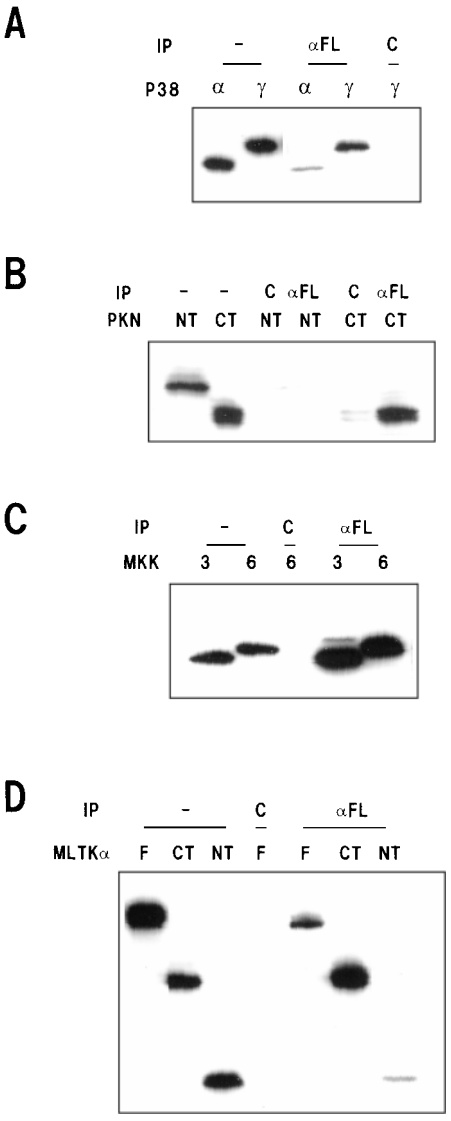

Fig. 5. **PKNα associates with an each member of the p38 MAPK signaling pathway.** A: COS-7 cells were transfected with expression plasmids encoding FLAG-tagged full-length PKNα with an HA-tagged p38γ MAPK or p38α MAPK. The FLAG-tagged protein was recovered from the cell extracts by immunoprecipitation with an anti-FLAG antibody (α-FL), and the immunoprecipitates were then probed with an anti-HA antibody (α-HA). Immunoprecipitation with normal mouse IgG was done as a control (indicated as C). B: COS-7 cells were transfected with expression plasmids encoding FLAG-tagged p38γ MAPK and HA-tagged amino-terminal region (NT) (*10*) or carboxyl-terminal region of PKNα (CT) (*10*). Association was analyzed as in A. C: COS-7 cells were transfected with expression plasmids encoding the FLAG-tagged carboxyl-terminal region of PKNα and HA-tagged MKK3 or MKK6. Association was analyzed as in A. D: COS-7 cells were transfected with expression plasmids encoding the FLAG-tagged carboxyl-terminal region of PKNα and HA-tagged full length (F), amino-terminal region (NT) (amino acids, 1–291), or carboxyl-terminal region (CT) (amino acids, 284–802) of MLTKα. Association was analyzed as in A. Equal amounts of FLAG-tagged proteins were expressed in each experiment.

MAPKs regulate a wide array of biological functions; therefore, mechanisms must exist to achieve signaling

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specificity and ensure the correct biological response to extracellular stimulation. One mechanism that can achieve signaling specificity is the presence of scaffold proteins (*37*). Scaffold proteins assemble components of signaling cascades into modules, and this may lead to the efficient activation of certain pathways within a restricted region of cells in response to a particular stimulus. We demonstrate here that PKNα associates with MLTKα, MKK6, and the p38γ MAPK (Fig. 5). At present, it is unclear whether these signaling molecules form a physiologically functional complex in cells and how each association is regulated. However, there is a possibility that PKNα serves as a scaffold protein for the p38γ MAPK–specific signaling pathway. The physiological significance of this association should be examined in future studies.

In summary, we suggest that PKNα acts as not only a MAPKKKK but also a putative scaffold protein for the p38γ MAPK signaling pathway. Further work will be necessary to investigate the physiological role of this novel signaling pathway.

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