Negative regulation of constitutive NF-κB and JNK signaling by PKN1-mediated phosphorylation of TRAF1

Tomohisa Kato Jr[1,a,*], Yusuke Gotoh[1], Alexander Hoffmann[3] and Yoshitaka Ono[1,2]

[1]*Biosignal Research Center, and*
[2]*Graduate School of Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan*
[3]*Signaling Systems Laboratory, Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0375, USA*

Inhibitor of NF-κB (IκB) kinase (IKK) and c-Jun NH_2-terminal kinase (JNK) are stress inducible kinases that critically regulate numerous physiological and pathological processes. Transient activation of the downstream transcription factors NF-κB and AP-1, allows for stress inducible, inflammatory and innate immune gene expression programs. However, elevated chronic activity is associated with cancer and chronic inflammatory disease. Despite its relevance to human health, little is known about the molecular mechanisms that control constitutive activity of IKK and JNK. Here, we demonstrate that the serine/threonine kinase PKN1 plays a critical role in regulating constitutive IKK/JNK activity in unstimulated cells and report on the molecular mechanism. We identify TRAF1 as a substrate of PKN1 kinase activity *in vitro* and *in vivo*, and show that this phosphorylation event is required for attenuating downstream kinase activities. Furthermore, this silencing was dependent on TNFR2. Mutagenesis of the phospho-acceptor residue in TRAF1 abrogated PKN1-dependent recruitment to TNFR2. Our results suggest a model by which the stoichiometric ratio of TRAF1 and TRAF2 heteromeric complexes associated with TNFR2 control the tonic activity of JNK and IKK. TRAF1 phosphorylation by the ubiquitously expressed kinase PKN1 thereby plays a critical role in the negative regulation of tonic activity of the two central inflammatory signaling pathways.

Introduction

Cellular stress and inflammatory responses are critically mediated by two signaling pathways that are co-ordinately regulated in response to cellular exposure to cytokines, pathogens and environmental noxious stimuli. The c-Jun NH_2-terminal kinase (JNK) and the inhibitor of NF-κB (IκB) kinase (IKK) control the activities of the transcription factors AP-1 and NF-κB, respectively; together these are responsible for the gene expression programs comprising hundreds of genes that are activated in response to cellular stimulation.

The dimeric transcription factor NF-κB plays a role in myriad physiological functions (Ghosh & Karin 2002; Hayden & Ghosh 2004). In unstimulated cells, NF-κB is held in the cytoplasm in an inactive state with one of the three inhibitory proteins, IκB (Hoffmann *et al.* 2002). Activation involves IKK-mediated phosphorylation of IκB protein at two serines in the regulatory domain, which targets the IκB for proteolysis through the ubiquitin-proteasome system (Karin & Ben-Neriah 2000). Degradation of inhibitory IκB liberates NF-κB to translocate into the nucleus.

JNK is a member of an evolutionarily conserved family of a mitogen-activated protein (MAP) kinase superfamily, which integrates signals from a diverse range of extracellular stimuli (Davis 2000; Kyriakis & Avruch 2001). One of the major functions of JNK is to phosphorylate the leucine zipper protein c-Jun, thereby potentiating its activation potential when bound to promoters as a dimer with other leucine zipper family members (Hibi *et al.* 1993; Derijard *et al.* 1994).

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*Correspondence: Email: tkato@kuhp.kyoto-u.ac.jp
[a]*Present address*: Department of Radiation Oncology and Image-applied Therapy, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

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The IKK/NFκB signaling and JNK group of MAP kinases represent intensively studied model systems for understanding how extracellular stimuli activate latent transcription factors. As stimulus-responsive signal transduction pathways, these proteins transmit information to the nucleus by intricate control of their activity. Recent studies have indeed revealed that the dynamic control of these kinases plays a role in determining cellular responses (Werner *et al.* 2005; Ventura *et al.* 2006). Both signaling pathways have evolved numerous mechanisms to attenuate activity subsequent to activation; these often involve negative feedback. Indeed, elevated constitutive activity of JNK and IKK is associated with numerous human diseases, including chronic inflammatory disease and cancer (Antonyak *et al.* 2002; Li & Verma 2002; Manning & Davis 2003; Bacher & Schmitz 2004; Lu & Stark 2004; Karin & Gallagher 2005; Courtois & Gilmore 2006; Karin 2006). While some basal activity may be required for normal cellular functions and proliferation (Sabapathy *et al.* 2004; Ventura *et al.* 2004; Gerondakis *et al.* 2006), the regulatory mechanisms that control it remains enigmatic.

Inflammatory signaling is triggered by the cytokine-responsive tumor necrosis factor receptor (TNFR) (Locksley *et al.* 2001) and the pathogen-sensing toll-like receptor (TLR) superfamilies (Dunne & O'Neill 2003). Both transduce the signal by recruiting members of the TNF receptor-associated factor (TRAF) family (Rothe *et al.* 1994) to the receptor. To date, seven TRAF proteins have been reported for mammals that share structural and functional similarities (Arch *et al.* 1998; Inoue *et al.* 2000; Bradley & Pober 2001; Wajant *et al.* 2001; Bouwmeester *et al.* 2004; Xu *et al.* 2004). Recent studies have focused on characterizing interaction domains between TRAFs and the receptors to understand their coordinated functions during signaling. Among the TRAF family, TRAF1 is unique as it lacks N-terminal RING finger domain present in all other TRAFs. The RING finger domain is known as an effector domain crucial for activating NF-κB and JNK, and RING finger domain-deficient mutants have been shown to act as dominant negatives. Given that TRAF1 lacks a RING domain, a proposed negative regulatory role of TRAF1 found confirmation in the observation that TRAF1-deficient mice show enhanced TNF signaling (Tsitsikov *et al.* 2001).

In the present study, we addressed the regulation of constitutive inflammatory signaling. By using RNA interference (RNAi), we identified a member of the PKC superfamily, the kinase PKN (also called PRK) as a negative regulator of constitutive JNK and NF-κB activity. To date, three members of the PKN sub-family have been identified in mammals and its biochemical activity as a serine–theronine kinase have been characterized in detail (Mukai 2003). However, its physiological regulation and function have not been addressed. In the work reported here, we identified TRAF1 as the key molecule that links PKN1 kinase activity and the silencing of constitutive activity of IKK/NF-κB and JNK signaling. We found that TRAF1 physically interacts with and is phosphorylated by PKN1 *in vivo*. We demonstrate that phosphorylation of TRAF1 by PKN1 is required for the suppressive function of TRAF1 in IKK/NF-κB and JNK signaling. We also found this suppressive function depends on TNFR2, and moreover, we demonstrated that phosphorylation of TRAF1 enhances its recruitment to TNFR2, which resulted in increase of stoichiometric ratio of TRAF1 toward TRAF2 in the TNFR2 complex. Our results suggest that phosphorylation of TRAF1 by PKN1 regulates the ratio of TRAF1 and TRAF2 in receptor–proximal heteromeric complexes, thereby determining the level of constitutive NF-κB and JNK signaling.

Results

Knockdown of PKN1 results in enhancement of IKK/NF-κB and JNK basal activity

As PKN1 has been implicated in stress signaling (Mukai 2003) and, in the previous study, we identified PKN1 as a candidate regulator of inflammatory signaling (Gotoh *et al.* 2004). To assess the role of PKN1 in these signaling in more detail, we employed RNAi technique to knockdown the expression of PKN1. We constructed RNAi vector, harboring two copies of short hairpin RNA (shRNA) targeted against PKN1 mRNA, pcPUR(U6)-2x(shRNA^{PKN1}). Cells were transfected with pcPUR(U6)-2x(shRNA^{PKN1}) followed by selection with puromycin. This procedure efficiently reduced the level of PKN1 in all cell lines tested (HeLa, U2OS, HEK293, 293T cells; data not shown) more than 90% when compared to the cells transfected with control shRNA, which differs two nucleotides from shRNA^{PKN1}. HeLa cells were transfected with control vector or pcPUR(U6)-2x(shRNA^{PKN1}), selected with puromycin and treated with tumor necrosis factor (TNF) α. As shown in Fig. 1A (upper panel), knockdown of PKN1 resulted in enhancement of NF-κB activity in unstimulated cells (indicated by arrow) as evidenced by an electrophoretic mobility shift assay (EMSA). In stimulated cells, there was only a slight increase of the activity.

As NF-κB activity is dependent on IKK (Rothwarf & Karin 1999; Hayden & Ghosh 2004), we measured the IKK activity in PKN1 knocked-down (KD) cells. As

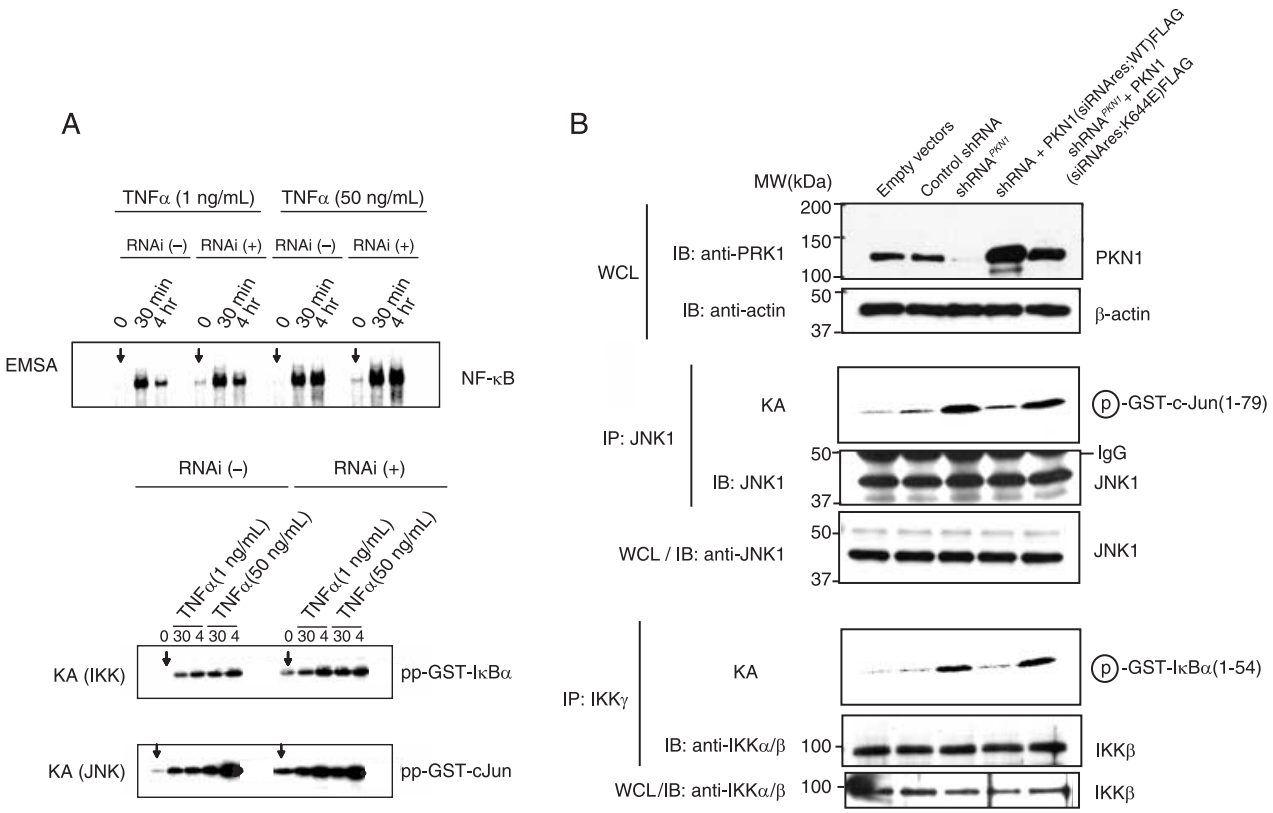


Figure 1 PKN1 is required for silencing of constitutive NF-κB, IKK and JNK activities. (A) (Upper panel) HeLa cells were transfected with either empty vector, pcPUR(U6)-*icassette*, or that harboring two copies of shRNA against PKN1, pcPUR(U6)-2x(shRNA^{PKN1}), and selected with puromycin. Before harvesting, the cells were left unstimulated or stimulated with TNFα at a final concentration of 1 or 50 ng/mL and incubated for times indicated. Nuclear extracts (NE) were prepared and EMSA was carried out. RNAi(–) indicates samples from cells transfected with empty vector and RNAi(+) indicates those transfected with pcPUR(U6)-2x(shRNA^{PKN1}). (Lower panel) Cytoplasmic extracts (CEs) prepared in upper panel were used for assessing the kinase activity of IKK and JNK by immunocomplex kinase assay (KA). (B) HeLa cells were co-transfected with empty vectors, control shRNA plus empty vector, siRNA vector harboring shRNA against PKN1 (shRNA^{PKN1}) plus empty vector, shRNA^{PKN1} plus expression vector for wild-type PKN1 which is resistant to siRNA (PKN1(WT; siRNAres)) or shRNA^{PKN1} plus expression vector for kinase-dead PKN1 which is resistant to siRNA (PKN1(K644E; siRNAres)). After selecting with puromycin, cells were harvested and whole cell lysates (WCLs) were prepared. WCLs were subjected to immunocomplex kinase assay to assess the activities of JNK and IKK using GST-cJun(1-79) and GST-IκBα(1-54) as a substrate, respectively.

shown in Fig. 1A (lower panel), IKK basal activity was significantly up-regulated in PKN1 KD cells (indicated by arrow). In cells treated with TNFα, IKK activity was slightly higher than that of control cells. This result is consistent with NF-κB activity, and indicates that a decrease in the PKN1 level by RNAi results in enhancement of basal activity of the IKK–NF-κB signaling axis. Similarly, we examined the JNK activity in PKN1 KD cells, as well. As shown in Fig. 1A (lower panel), the basal level of JNK kinase activity was significantly increased in PKN1 KD cells.

To confirm the effect of PKN1 knockdown, that is, enhancement of the basal level of IKK/NF-κB and JNK signaling, and to control for potential "off-target" effects, we constructed cDNAs of PKN1 wild-type and kinase-dead mutant in which the target sequence for shRNA is changed but still encode for the same amino acids. Resultant cDNAs encoding either the wild-type or kinase-dead mutant PKN1 (K644E), which are resistant to shRNA designed to knockdown endogenous PKN1, were co-transfected with shRNA against PKN1 into the cells followed by selecting with puromycin. This resulted in knockdown of endogenous PKN1 and expression of either exogenous wild-type or kinase-dead mutant of PKN1 (Fig. 1B). We assessed the cells for IKK and JNK kinase activity in this context. As shown in Fig. 1B,

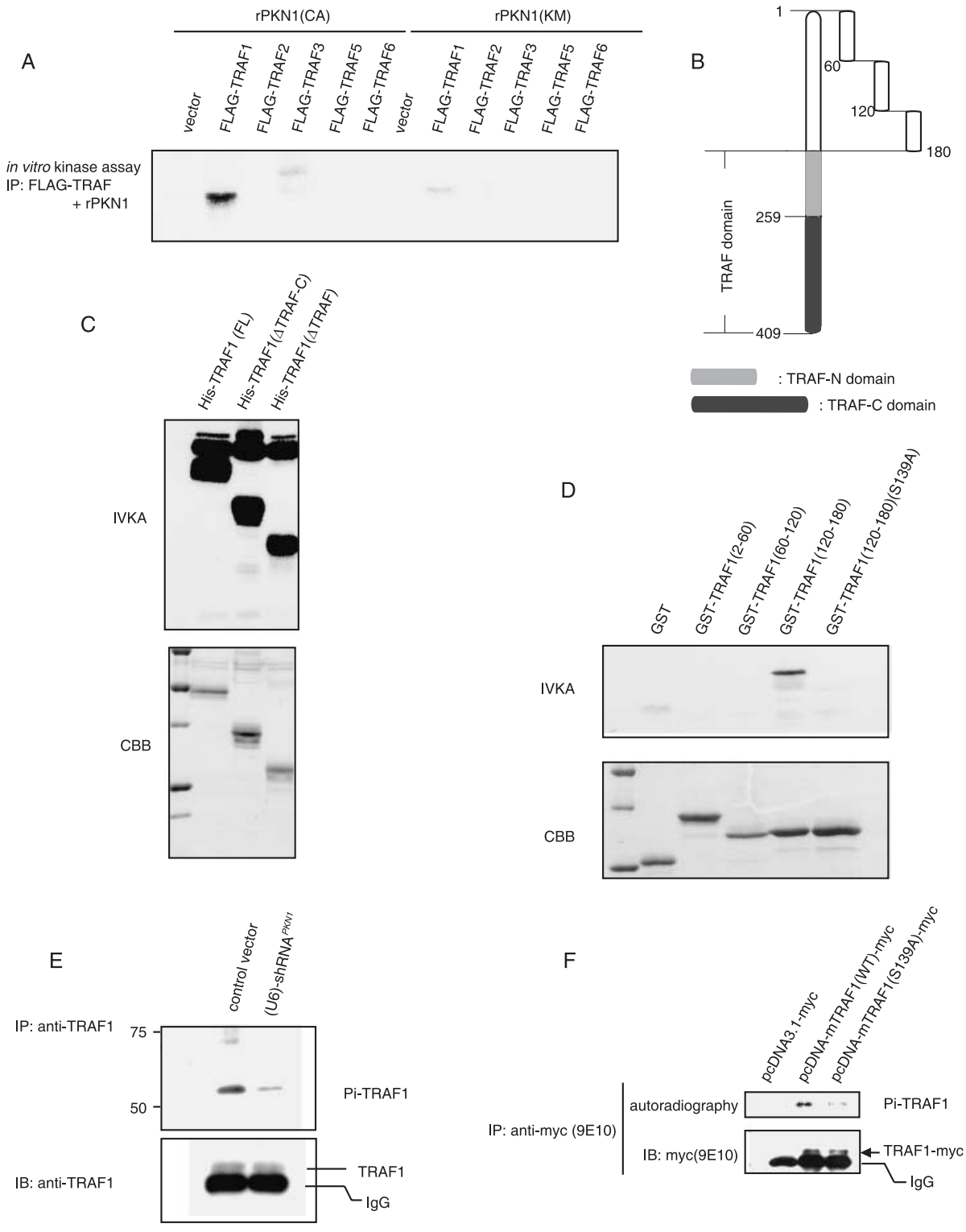
when knockdown of PKN1 was complemented with wild-type (kinase-intact) PKN1, the basal activity of both IKK and JNK were silenced as in control cells, that is, cells transfected with control shRNA against PKN1. In contrast, complementing with a kinase-dead mutant of PKN1 resulted in even higher basal activities for IKK and JNK. These results strongly suggest that PKN1 suppresses basal activity of IKK and JNK in a kinase activity-dependent manner.

PKN1 phosphorylates TRAF1 both *in vitro* and *in vivo*

In related work, we identified that PKN1 interacts with proteins of the TNF receptor-associated factor (TRAF) family member; TRAF1, 2, 3, 5 and 6 (TRAF4 was not tested; T. Kato Jr and Y. Gotoh, unpublished results). Those are known to play important roles in TNFR and TLR superfamily-dependent NF-κB and JNK activation (Arch *et al.* 1998; Inoue *et al.* 2000; Bradley & Pober 2001; Wajant *et al.* 2001). As PKN1 is a protein kinase and TRAFs interact with it, we asked whether TRAFs could be substrates of PKN1. We immunopurified each TRAF member from cells transfected with FLAG-tagged plasmid vector and kinase assay was carried out with recombinant PKN1 produced in insect cells. Intriguingly, as shown in Fig. 2A, only TRAF1 was found to be phosphorylated by constitutively active (CA) PKN1, but not by kinase dead (KM) PKN1. TRAF1 is unique among the TRAF family members as it lacks the characteristic N-terminal RING finger domain. Interestingly, T cells derived from *Traf1* knockout mouse display enhanced activity of IKK and JNK under both unstimulated and TNFα-stimulated conditions (Tsitsikov *et al.* 2001). We therefore set out to examine the role of PKN1-mediated phosphorylation of TRAF1 by identifying the exact phosphorylation site(s) in TRAF1. To this end, we produced $(His)_6$-tagged full length, TRAF-C domain deleted (ΔTRAF-C) and both TRAF-N and C deleted (ΔTRAF) version of murine TRAF1 proteins (Fig. 2B) in bacteria, and performed an *in vitro* kinase assay using purified recombinant active PKN1 produced in insect cells. As shown in Fig. 2C, all the proteins were phosphorylated equally, indicating that the phospho-acceptor(s) resides in the N-terminal region of the protein (amino acid residue 1–180).

Next, we divided the N-terminal region into three parts (Fig. 2B), produced each in bacteria as glutathione-S-transferase (GST)-fusion proteins, and tested each for phosphorylation by purified recombinant PKN1 (Fig. 2D). Our results indicated that the phospho-acceptor(s) resides between amino acid residue 120 and 180 in TRAF1. There are seven serine–threonine residues in this portion of murine TRAF1 and, among them, serine 139 is embedded within a sequence that, matched with the PKC consensus K/R-X-X-S/T. As PKN1 is a PKC superfamily member, we constructed a mutant in which serine 139 was replaced with alanine (S139A), produced a GST-fusion protein of this mutant and examined for phosphorylation by PKN1. As shown in Fig. 2D, replacement of serine 139 with alanine abolished the phosphorylation by PKN1. These results indicate that PKN1 phosphorylates TRAF1 on serine 139 *in vitro*. Alignment of human and mouse TRAF1 (data not shown) reveals that murine serine 139 corresponds to human

Figure 2 PKN1 phosphorylates TRAF1 both *in vitro* and *in vivo*. (A) *In vitro* phosphorylation of TRAF proteins (i.e. TRAF1, 2, 3, 5 and 6) by PKN1. Immunopurified FLAG-tagged TRAF protein was subjected to *in vitro* kinase assay with either recombinant constitutive active (CA) or recombinant kinase-dead mutant (KM) PKN1 protein produced in insect cells. Kinase reaction was terminated by adding SDS-sample buffer (final 1× conc.), separated on SDS-PAGE and subjected to autoradiography. (B) Schematic illustration of TRAF1 proteins used in this study. (C) PKN1 phophorylates the N-terminal half of TRAF1 *in vitro*: Wild type and truncated version of His_6-tagged TRAF1 proteins were produced in *E. coli* (BL21) and purified with Ni-NTA beads. TRAF1(FL), TRAF1(ΔTRAF-C) and TRAF1(ΔTRAF) indicate mouse full length TRAF1, mouse TRAF1 deleted with TRAF-C domain and mouse TRAF1 deleted with whole TRAF domain, respectively. Proteins were used as a substrate in *in vitro* kinase assay with recombinant constitutive active PKN1. (D) Phosphorylation of TRAF1 on serine 139 by PKN1 *in vitro*: Truncated TRAF1 spanning the amino acid number as indicated was fused with GST and produced in *E. coli* (BL21). Proteins were purified with GSH-beads and used as a substrate in *in vitro* kinase assay as described above. (E) TRAF1 is a phospho-protein *in vivo* which depends on PKN1 activity: HeLa cells were transfected with either pcPUR(U6)-2x(shRNA^{PKN1}) to knockdown PKN1 or empty vector to serve as control, metabolically labeled with [^{32}P]-orthophosphate and harvested. TRAF1 was immnunopreicipitated, separated by SDS-PAGE and transferred to PVDF membrane. Membrane was subjected to autoradiography and later probed with anti-TRAF1 antibody to confirm the equal loading. (F) Serine 139 of TRAF1 is phosphorylated *in vivo*: HeLa cells were transiently transfected with empty vector, myc-tagged wild-type TRAF1 or myc-tagged mutant TRAF1 in which serine 139 was replaced with alanine. Then cells were metabolically labeled with [^{32}P]-orthophosphate. TRAF1 was immunoprecipitated with anti-myc antibody, separated by SDS-PAGE and transferred to PVDF membrane. Membrane was subjected to autoradiography and later probed with anti-myc antibody to confirm the equal loading.



serine 146, which indicates that this phospho-acceptor residue is conserved between human and mouse.

To confirm TRAF1 is phosphorylated *in vivo* at the residue identified above, we conducted *in vivo* labeling experiments. HeLa cells were metabolically labeled with ^{32}P-orthophosphate and TRAF1 was isolated by immunoprecipitation. As shown in Fig. 2E, TRAF1 was already phosphorylated in unstimulated cells. Moreover, knockdown of PKN1 was associated with a significant decrease in TRAF1 phosphorylation (Fig. 2E).

We next examined the *in vivo* phosphorylation of exogenously introduced wild-type and (S139A) mutant TRAF1. As shown in Fig. 2F, replacement of serine 139 with alanine decreased the phosphorylation of TRAF1 isolated by immnunoprecipitation. Collectively, these results demonstrate that TRAF1 is a phosphoprotein whose status of phosphorylation depends on PKN1 and the phospho-acceptor is serine 139 residue.

Phosphorylation of TRAF1 is required for silencing of IKK and JNK

The results presented above linked PKN1 and TRAF1 *in vivo*, however, the functional relevance of the phosphorylation event remained enigmatic. To unravel this issue, we knocked down TRAF1 in HeLa cells and complemented with either wild type or (S139A) mutant. Knockdown of TRAF1 was carried out by transfecting a siRNA vector harboring shRNA targeted against TRAF1 mRNA followed by selection with puromycin. As shown in Fig. 3, knockdown of TRAF1 resulted in enhancement of both IKK and JNK basal activity, which is consistent with what was demonstrated with Traf1 knockout T-cells (Tsitsikov *et al.* 2001). Significantly, restoration of the expression of wild-type but not (S139A) mutant of TRAF1 reduced the basal kinase activity of both IKK and JNK back to the level of control cells. These results together with those presented above strongly suggest that phosphorylation of TRAF1 with PKN1 on serine 139 is required for control of constitutive activity of IKK and JNK.

PKN1-mediated control of IKK and JNK depends on TNFR2

Tsitsikov and coworkers have shown that TRAF1 negatively regulates proliferative signals delivered via TNFR2 (Tsitsikov *et al.* 2001). To examine whether the inhibition of constitutive activity of IKK and JNK by PKN1 may also involve TNFR2, we constructed a siRNA vector harboring shRNA targeting TNFR2 (TNFRSF1B) mRNA. We found that elevation of

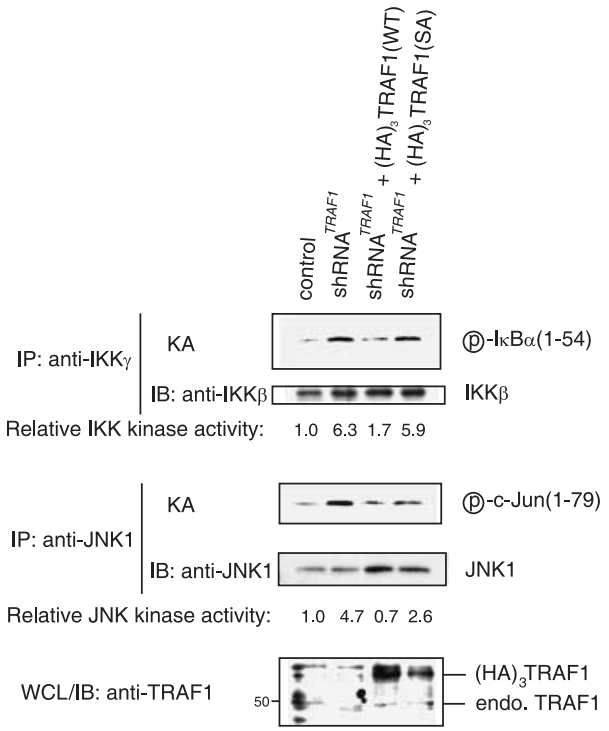


Figure 3 Phosphorylation of TRAF1 is required for silencing of IKK and JNK signalings. HeLa cells were co-transfected with empty vectors (control), siRNA vector harboring shRNA against TRAF1 (shRNA^{TRAF1}) plus empty vector, shRNA^{TRAF1} plus expression vector for HA-tagged wild-type TRAF1 or shRNA^{TRAF1} plus expression vector for HA-tagged mutant TRAF1 in which serine 139 was replaced with alanine. After selecting with drug, cells were collected and assessed for IKK and JNK kinase activities. Cell lysates were assessed for endogenous and exogenously expressed TRAF1 by immunoblotting.

constitutive JNK and IKK activity caused by PKN1 knockdown was indeed reduced by simultaneous knockdown of TNFR2 (Fig. 4A). This result suggests that the control of constitutive activities of IKK and JNK by PKN1 involves its association with TNFR2. To further support the relationship between PKN1 and TNFR2, we examined whether PKN1 and TNFR2 physically interact. Endogenous TNFR2 was immunoprecipitated from cell lysate and the resulted protein complex was analyzed by immunoblotting with the antibody against PKN1. As shown in lanes 1 and 2 of Fig. 4C, PKN1 and TNFR2 physically interact in unstimulated cells. Moreover, as shown in Fig. 4B, results of *in vitro* binding assay using the intracellular domain (icd) of TNFR2 fused to GST (produced in bacteria) and recombinant full length PKN1 (purified from insect cells) indicates that this interaction between PKN1 and TNFR2 is direct.

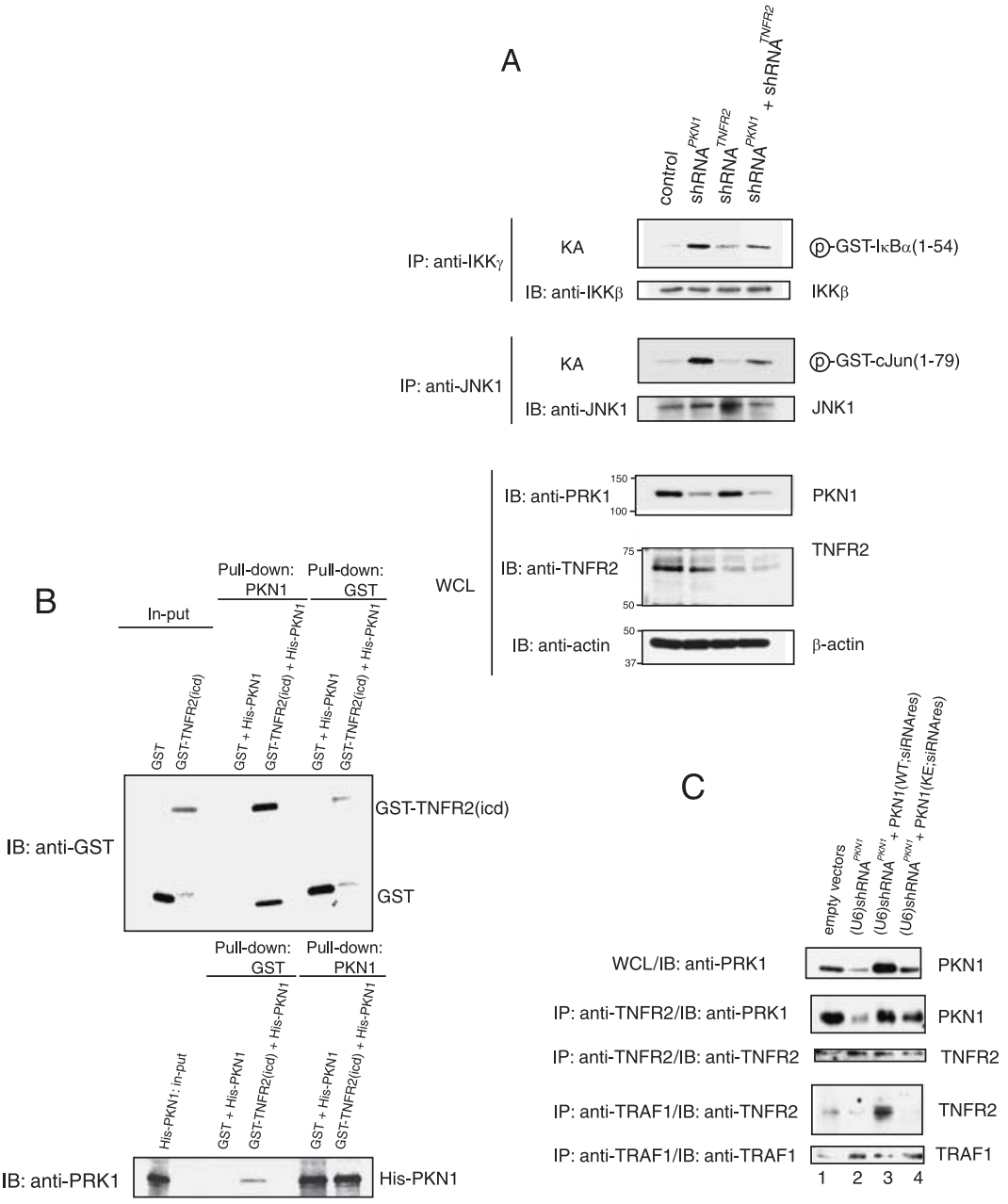


Figure 4 Silencing of IKK and JNK by PKN1 depends on TNFR2. (A) HeLa cells were co-transfected with empty vector (control), siRNA vector harboring shRNA against PKN1 (shRNA^{PKN1}) plus empty vector, siRNA vector harboring shRNA against TNFR2 (shRNA^{TNFR2}) plus empty vector or shRNA^{PKN1} plus shRNA^{TNFR2}. After selecting with puromycin, cells were harvested and assessed for IKK and JNK kinase activities. Cell lysates were assessed for PKN1, TNFR2 and actin by immunoblotting using antibodies as indicated. (B) Association of PKN1 and TNFR2 was assessed by *in vitro* pull-down assay. His-tagged PKN1 produced in insect cells and bacterially produced intracellular domain (icd) of TNFR2 fused with GST were mixed and pulled-down with either anti-PRK1 antibody/protein G and A mixed-beads or GSH-beads. Precipitates were separated on SDS-PAGE and subjected to immunoblot with either anti-GST or anti-PRK1 antibody. (C) HeLa cells were transfected with plasmids as indicated to knockdowon PKN1 or compliment with either wild-type or kinase-dead mutant type of PKN1. Lane 1 served as control. After selection with puromycin, cells were harvested, WCLs were prepared and immunecomplex was assessed for the protein indicated.

Phosphorylation of TRAF1 by PKN1 enhances recruitment of TRAF1 to TNFR2

Our results presented here revealed that PKN1 regulates the constitutive activity of IKK and JNK through phosphorylation of TRAF1 in a TNFR2-dependent manner. Furthermore, when PKN1 was knocked-down, the interaction of endogenous TRAF1 with endogenous TNFR2 was reduced (Fig. 4C; lane 2). And when PKN KD cells were complemented the interaction could be restored when wild-type PKN1 was expressed, but not when the kinase-dead (K644E) mutant was used (Fig. 4C; lanes 3 and 4). To gain a better understanding of the mechanism of TRAF1 phosphorylation-dependent regulation of constitutive signaling, we analyzed the protein complexes associated with the intracellular signaling domain of TNFR2.

Either wild-type or (S139A) mutant TRAF1 tagged with myc-epitope at the C-terminus (TRAF1(WT)-myc or TRAF1(SA)-myc)) was co-transfected with HA-tagged PKN1 (HA-PKN1) and FLAG-tagged TNFR2 (FLAG-TNFR2(WT)) in 293T cells. Cell lysates were prepared and subjected to immunoprecipitation with antibody against FLAG-epitope. Resulting immune-complexes were examined for TRAF1-myc, PKN1 and endogenous TRAF2 by immunoblotting. As shown in Fig. 5, in the lysate prepared from cells expressing the (S139A) mutant TRAF1, the amount of TRAF1 associated with TNFR2 was lower than in cells expressing wild-type TRAF1. Conversely, the amount of TRAF2 was higher in (S139A) TRAF1 mutant expressing cells than in wild-type expressing cells. This observation suggests that phosphorylation of TRAF1 by PKN1 enhances the recruitment of TRAF1 to TNFR2 complex resulting in an increase in the stoichiometric ratio of TRAF1 over TRAF2 in the receptor complex. Our results suggest that PKN1 phosphorylation of TRAF1 regulates the competitive binding event between TRAF1 and TRAF2 to TNFR2 that controls the constitutive signaling activity of the IKK and JNK pathways.

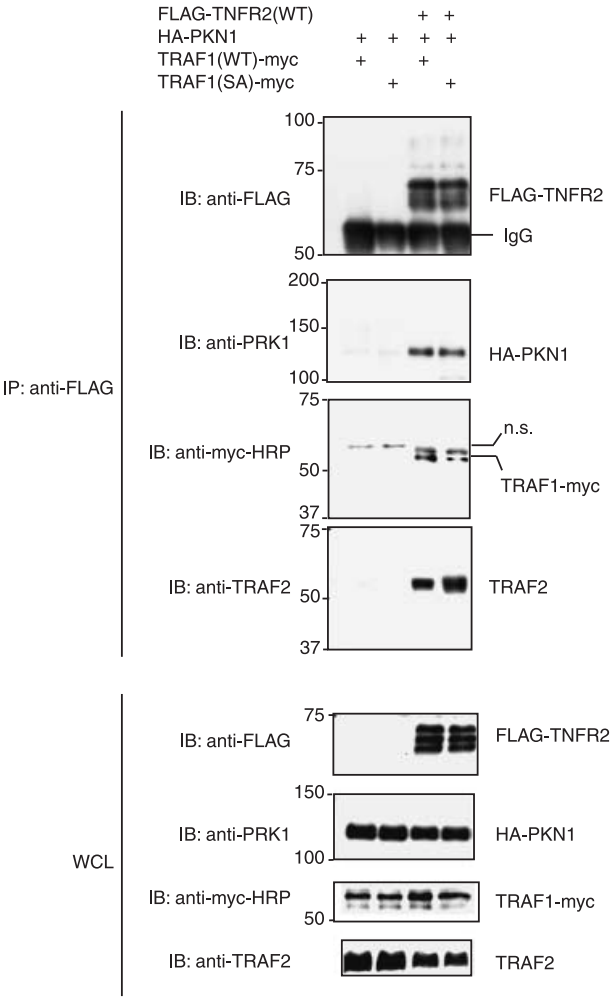


Figure 5 Phosphorylation of TRAF1 is required for increasing the ratio of TNFR2 associated TRAF1 against TNFR2. 293T cells were co-transfected with expression vectors for proteins indicated on top of the panel, harvested and whole cell lysates were prepared. Cell lysates were subjected to immunoprecipitation with anti-FLAG (M-2) antibody and immunecomplex was examined for proteins as indicated by immunoblotting.

Discussion

Signaling pathways of IKK–NF-κB axis and JNK MAP kinase are known to play important roles in numerous pathophysiological processes, and PKN1 was originally identified in the screening for a novel member of PKC family (Mukai & Ono 1994). PKN1 has been suggested to regulate gene expression in response to variety of extracellular stimuli (Mukai 2003). Here we report that PKN1 critically controls the activity of IKK and JNK signaling pathways in the absence of an external inflammatory stimulus by recruiting the signaling attenuator TRAF1 to TNFR2.

Our results presented here have important implications in regards with human health. While chronic activation of IKK and JNK signaling is causally involved in human pathology, such as inflammatory diseases, tumorigenesis and chemoresistance (Antonyak *et al.* 2002; Li & Verma 2002; Manning & Davis 2003; Bacher & Schmitz 2004; Lu & Stark 2004; Karin & Gallagher 2005; Courtois & Gilmore 2006; Karin 2006), tonic NF-κB and JNK activities are also important for cellular homeostasis,

survival and responsiveness. Knockout studies have shown that fibroblasts lacking JNK activity are deficient in cell proliferation (Tournier *et al.* 2000) indicating an important role for constitutive JNK activity. Recently, constitutive RelA/NF-κB was shown to be necessary for PKCδ expression which plays a critical role in UV-induced apoptosis (Liu *et al.* 2006). Furthermore, as the level of constitutive IKK activity controls the responsiveness of the NF-κB signaling module to environmental stress conditions (E. O'Dea and A. H., in preparation), the regulation of constitutive signaling can determine cellular stimulus-responsiveness to noxious stimuli.

Our studies revealed that PKN1-mediated regulation of chronic IKK and JNK activity requires TNFR2, implicating TNFR2 as a source of chronic inflammatory signal in human cells even in the absence of external ligand. However, our studies do not rule out that chronic signaling may also emanate from other receptors. If so, we would expect signaling molecules other than PKN1 to regulate these constitutive signals. The molecule SODD has for example been implicated in down-regulating constitutive signaling downstream of TNFR1 (Jiang *et al.* 1999). However, neither its expression level nor activity has been shown to be regulated.

In the search for the mechanism that mediates PKN1 suppression of IKK/NF-κB and JNK signaling, we identified interactions between PKN1 and the inflammatory signaling adapter proteins TRAFs (TRAF1, 2, 3, 5 and 6; TRAF4 was not tested), and found that only TRAF1, the family member lacking the JNK/IKK signaling effector domain, could be phosphorylated by PKN1 *in vitro*. We further identified serine 139 in mouse and serine 146 in human TRAF1 as the PKN1 phosphorylation site *in vitro* and *in vivo*. Our results indicate that this phosphorylation event regulates recruitment of TRAF1 to TNFR2, which alters the stoichiometry of TRAF1 vs. TRAF2. The stoichiometry of TRAF1–TRAF2 heteromeric complexes ($(TRAF2)_2$-TRAF1 vs. TRAF2-$(TRAF1)_2$) has previously been proposed to determine CD40 signaling without affecting TNF signaling mediated by TNFR1 (Fotin-Mleczek *et al.* 2004). As TNFR2 resembles CD40 (unlike TNFR1) in terms of lacking a 'death domain (DD)' and recruiting TRAF1 with the help of TRAF2 *in vivo* (Rothe *et al.* 1994; Ashkenazi & Dixit 1998; Wallach *et al.* 1999; Locksley *et al.* 2001), it is plausible that increase in the stoichiometric ratio of TRAF1 toward TRAF2 through phosphorylation of TRAF1 by PKN1 tilt the signaling of the receptor complex to be silent.

Intensive research efforts over the past 10 years to understand the molecular mechanism of TRAF-mediated regulation of signaling have revealed ubiquitination as a reversible covalent modification (Sun & Chen 2004; Chen 2005). Interestingly, phosphorylation, a classical covalent modification exerted through phospho-relay, has been reported only for TRAF2 (Chaudhuri *et al.* 1999) but the kinase which phosphorylates it and the physiological relevance of the phosphorylation event remains obscure. Our results demonstrate, for the first time, that a TRAF molecule can be modified by phosphorylation, to regulate the activity of the signaling pathway.

Given that the attenuation activity of TRAF1 is regulated by phosphorylation of a specific residue, our studies have revealed a mechanism that may be subject to signaling crosstalk by which non-inflammatory stimuli regulate chronic inflammatory signaling. In our experimental conditions TRAF1 is a phospho-protein in unstimulated cells implying that PKN1 is at least partially active. As PKN1 interacts with TNFR2 directly, the resulting complex could serve as a scaffold that regulates the recruitment of TRAF1 vs. TRAF2 thereby regulating tonic JNK and IKK kinase activity. It should be noted that PKN1 can be strongly activated *in vitro* by unsaturated fatty acids such as arachidonic and linoleic acids or by a small GTPase Rho (Mukai 2003), all of which reside proximal to the cytoplasm. Thus, recruitment of PKN1 to TNFR2 may sensitize downstream inflammatory pathways to the relative abundance of specific fatty acids. If their distribution in the plasma membrane is not homogenous but is uneven (*c.f.* rafts), PKN1 may provide a sensor for TNFR2 localization within the membrane.

An alternative mechanism to activate PKN1 is proteolytic cleavage catalyzed by caspase-3 or related protease. PKN1 has been shown to be cleaved at specific sites between the N-terminal regulatory domain and the C-terminal catalytic domain, which generates a CA kinase (Takahashi *et al.* 1998). Thus, PKN1 may function as a signaling node integrating apoptotic and inflammatory signals.

During the preparation of this manuscript, Haraga and Miller have reported the isolation of PKN1 as the interacting partner of SspH1 in the yeast two-hybrid screen against human spleen cDNA (Haraga & Miller 2006). SspH1 is an effector of *Salmonella enterica* serovar Typhimurium Type III secretion system (TTSS), and down-modulates production of proinflammatory cytokines by inhibiting NF-κB-dependent gene expression. PKN1 is known to be expressed abundantly in leucocytes (Mukai 2003) that are the mediators of the host defense system. Our results may provide one potential mechanistic explanation in which PKN1 activity is co-opted by a bacterial pathogen to inhibit NF-κB activity during host infection.

Experimental procedures

Cell culture

HeLa cells were grown in DMEM (Sigma, St Louis, MO) supplemented with 5% of fetal bovine serum (FBS; GIBCO, Carlsbad, CA) and penicillin–streptomycin liquid (GIBCO). U2OS, HEK293 and 293T cells were maintained in DMEM supplemented with 10% of FBS and penicillin–streptomycin liquid. Transfection of the cells was carried out using Lipofectamine PLUS reagenats (Invitrogen, Carlsbad, CA) following the manufacture's instruction.

RNA interference (RNAi) experiments

To knockdown PKN1, TRAF1 or TNFR2, 21 nucleoitde target sequences were determined using "siRNA Target Finder and Design Tool" (see Ambion's Web site). "Stem" of hairpin was composed of sense and anti-sense sequence of the target mentioned above and 15 base pair "loop" was design to built shRNA. The nucleotide sequences of shRNA oligonucleotides are as followings: shRNA^{PKN1}(F1); 5′-caccGGAGTTGGAGTTGAGGGAGGGgtgtgctgtccCCCTCCTTCAGCTTCAGCTCCttttt-3′, shRNA^{PKN1}(R1); 5′-gcataaaaaGGAGCTGAAGCTGAAGGAGGGgacagcacacCCCTCCTTCAGCTTCAGCTCC-3′, shRNA^{TRAF1}(F1); 5′-caccGATCTCTAGTCTATAAGTCacgtgtgctgtccgtGGCTTATAGACTGGAGGTCttttt-3′, shRNA^{TRAF1}(R1); 5′-gcataaaaaGCACTCCAGTCTATAAGCCacggacagcacacgtGACTTATAGACTAGAGATC-3′, shRNA^{TNFR2}(F2); 5′-caccATATAGAAATTAGTTGGGCacgtgtgctgtccgtGCCCGGCTAATTTCTGTATttttt-3′, shRNA^{TNFR2}(R2); 5′-gcataaaaaATACAGAAATTAGCCGGGCacggacagcacacgtGCCCAACTAATTTCTATAT-3′. The sequences of mutant oligonucleotides served for control RNAi are as followings: shRNA$^{PKN1(control)}$(F); 5′-caccGGAGTTGGtcTTGAGGGAGGGgtgtgctgtccCCCTCCTTCAGgaTCAGCTCCttttt-3′, shRNA$^{PKN1(control)}$(R); 5′-gcataaaaaGGAGCTGAtcCTGAAGGAGGGgacagcacacCCCTCCTTCAGgaTCAGCTCC-3′.

Complementary oligonucleotides for each shRNA were synthesized (JBios, Saitama, Japan), annealed in buffer containing 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0] and 100 mM NaCl, and 5′-phosphates were added with T4 PNK. Each double stranded shRNA was subcloned into *Bsp*MI site of a siRNA vector, pcPUR(U6)-*icassestte*. To construct pcPUR(U6)-2x(shRNA^{PKN1}), a vector harboring one copy of shRNA against PKN1 was constructed as described above, and resulted siRNA vector was digested with *Bgl* II and *Bam* HI to generate a fragment containing U6 promoter and shRNA. This fragment was purified, subcloned into *Bgl* II-*Bam* HI site of pcPUR(U6)-*icassette*. The copy number of shRNA was assessed by digesting the plasmid with *Bam* HI and *Bgl* II and separating in agarose gel. The resulted shRNAs in the vector were confirmed by sequencing. Each vector was transfected to cells alone or co-transfected with other vector construct(s) as indicated, puromycin was added to the medium a day after transfection at final concentration of 2 μg/mL and the cells were cultured for further 48 h.

Plasmids, recombinant proteins and antibodies

Mammalian expression vectors for PKN1 were described previously (Isagawa *et al.* 2005). cDNA of PKN1, which is resistant to shRNA^{PKN1} was generated using QuikChange Site-Directed Mutagenesis System (Stratagene, La Jolla, CA). The sequence of target site (5′-GGAGCTGAAGCTGAAGG-3′; nucleotides 144–162) was changed to 5′-GGAACTCAAGCTGAAAG-3′. cDNA of mouse TRAF1 was generously provided by Dr Ichijo (The University of Tokyo) and subcloned into appropriate vecteors after PCR amplification in order to generate new restriction enzyme sites. cDNAs of human TRAF1 and human TNFR2 were amplified by PCR using and Human Spleen Marathon-Ready cDNA (Clontech, Palo Alto, CA) as template and subcloned into appropriate vecteors. cDNA of S139A mutant of TRAF1 was generated using QuikChange Site-Directed Mutagenesis System. cDNA of TRAF1(2–60), TARF1(60–120) and TRAF1(120–180) were generated by PCR amplification. All the sequences of PCR amplified cDNAs were confirmed by sequencing.

Production and purification of recombinant proteins in bacteria are described previously (Isagawa *et al.* 2005). Purification of recombinant PKN1 from insect cells is described previously (Isagawa *et al.* 2005).

Anti-IKKα/β, anti-JNK1, anti-TRAF1, anti-TRAF2 and anti-myc antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-TNFRII/TNFRSF1B antibody was from R&D Systems, Inc. (Minneapolis, MN). Anti-IKKγ, anti-JNK1 were from BD Pharmingen (San Diego, CA). Anti-PRK1 antibody was from BD Transduction Labolatories (San Diego, CA). Anti-actin antibody was from Sigma.

Immunoprecipitation and immunoblotting

Cells were lysed in ice-cold lysis buffer (50 mM HEPES-OH [pH7.4], 150 mM NaCl, 1.5 mM $MgCl_2$, 1% NP-40, 20 mM NaF, 1 mM EGTA, 20 mM β-glycerophosphate, 0.5 mM DTT, 0.1 mM Na_3VO_4, 1 mM PMSF, 10 μg/mL leupeptin, 1 μg/mL aprotinin), incubated for 30 min at 4 °C and centrifuged for 20 min. at 10 000 ***g*** to prepare whole cell lysate (WCL). After incubating the WCLs with the indicated antibodies, protein A and protein G Sepharose were added and further incubated for 1 h. Immune-complex was washed twice with lysis buffer and resuspended in Laemmli's sample buffer.

Immunoblotting was performed as previously described (referred as "Western blotting" therein) (Isagawa *et al.* 2005) with the indicated antibodies.

EMSA, kinase assays and *in vivo* metabolic labeling of the cells

NF-κB activity was assessed by electrophoretic mobility shift assay (EMSA) as described (Werner *et al.* 2005).

In vitro kinase assay was carried out by mixing indicated recombinant proteins in kinase reaction buffer (20 mM HEPES-OH [pH 7.4], 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 50 μM Na_3VO_4, 1 mM DTT, 15 μM ATP) and incubating the mixture with γ-[^{32}P]-ATP

at 30 °C for 20 min. For immunecomplex kinase assay, the kinase was immunoprecipitated with the antibody as described, the resulted precipitate was washed once with kinase reaction buffer and incubated in kinase reaction buffer with the substrate as indicated at 30 °C for 20 min. MBP used as a substrate in PKN1 kinase assay was purchased from Sigma.

Metabolic labeling of the cells was carried out with ortho-[^{32}P]-phosphate (PerkinElmer, Boston, MA) as described previously (Kato *et al*. 2003).

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