Pim-1 controls NF- κ B signalling by stabilizing RelA/p65

K Nihira¹, Y Ando¹, T Yamaguchi¹, Y Kagami¹, Y Miki^{*1} and K Yoshida^{*1}

Post-translational modification and degradation of proteins by the ubiquitin–proteasome system are key regulatory mechanisms in cellular responses to various stimuli. The NF- κ B signaling pathway is controlled by the ubiquitin-mediated proteolysis. RelA/p65, which is a main subunit of NF- κ B, is ubiquitinated for degradation by SOCS-1, but the functional mechanism of its ubiquitination remains poorly understood. In this study we show that phosphorylation of RelA/p65 at Ser276 prevents its degradation by ubiquitin-mediated proteolysis. In contrast, impairment of Ser276 phosphorylation affects constitutive degradation of RelA/p65. Importantly, we identify Pim-1 as a further kinase responsible for the phosphorylation of RelA/p65 at Ser276. Depletion of Pim-1 hinders not only Ser276 phosphorylation but also transactivation of RelA/p65 target genes. We also show that Pim-1 contributes to recruitment of RelA/p65 to κ B-elements to activate NF- κ B signalling after TNF- α stimulation. In concert with these results, the knockdown of Pim-1 impairs IL-6 production and augments apoptosis by interfering RelA/p65 activation. These findings provide a model in which Pim-1 phosphorylation of RelA/p65 at Ser276 allows defense against ubiquitin-mediated degradation and whereby exerts activation of NF- κ B signalling.

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NF- κ B is an inducible transcription factor that controls the expression of a number of proteins involved in the regulation of cell survival and immune response.¹ It is a dimer formed from a multisubset family consisting of RelA/p65, RelB, c-Rel, p105/p50 (NF- κ B1), and p100/p52 (NF- κ B2). It is activated by a bewildering array of stimuli and its activation is regulated by multiple distinct signalling cascades, including inhibitors of the NF- κ B (I κ B) kinase (IKK) signalosome. IKK phosphorylates I κ B- α at Ser32 and Ser36 in response to a variety of stimuli, resulting in its ubiquitination and subsequent proteasomal degradation. The released NF- κ B targets to the nucleus and thereby induces the expression of specific target genes. In addition to nuclear translocation of the NF- κ B complex, previous studies have shown that a subunit of NF- κ B, RelA/p65, is post-translationally modified, such as phosphorylation, ubiquitination, or acetylation, and these changes influence its transcriptional activity. In particular, phosphorylation of RelA/p65 at Ser276, Ser529, or Ser536 could be indispensable for its ability to function as an activator of gene expression.² However, recent findings showing a role for Ser529/536 phosphorylation on RelA/p65 activation in response to TNF- α arise conflicting evidences.^{3–5} In contrast, accumulating evidences have revealed that Ser276 phosphorylation is critical for transactivation of RelA/p65 at least in response to TNF- α . Moreover, Ser276 is the major phosphorylation site of RelA/p65 induced by TNF- α . Phospho-RelA/p65 at Ser276 forms a stable complex with co-activator CBP/p300, a modification apparently required for assembly of a functional

enhanceosome on the responsive promoters.^{6,7} As nuclear translocation or DNA-binding activity of the mutant, which was substituted by Ala for Ser276, was not impaired, it is most likely that the phosphorylation of Ser276 regulates the transcriptional activity of RelA/p65.^{3,6} In this context, Ser276 kinases should be of importance in the regulation of RelA/p65. Previous report showed that protein kinase A catalytic subunit (PKAc) is involved in this phosphorylation.⁸ However, other studies showed the inhibitory actions of PKA on the NF- κ B-dependent gene expression by stabilizing I κ B.^{9–11} Besides PKA, another report showed the identification of mitogen- and stress-activated protein kinase-1 (MSK1) as a nuclear kinase for RelA/p65 at Ser276.¹² However, recent study has shown that MSK1 is unlikely to phosphorylate Ser276 in response to TNF- α .⁶ Taken together with these conflicting evidences, it would be still plausible that there could be other Ser276 kinases, whereas requisition of Ser276 phosphorylation on RelA/p65 transactivation has been thoroughly uncovered. In this context, mechanisms for RelA/p65 transactivation by Ser276 phosphorylation after nuclear translocation remain to be elucidated.

In this study we show that phosphorylation of Ser276 prevents degradation of RelA/p65 from the ubiquitin–proteasome machinery. We also show that Pim-1 kinase is a novel kinase responsible for phosphorylation of RelA/p65 at Ser276 upon exposure to TNF- α . Importantly, Pim-1 phosphorylation of Ser276 activates RelA/p65.

¹Department of Molecular Genetics, Medical Research Institute, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8510, Japan

*Corresponding authors: K Yoshida or Y Miki, Department of Molecular Genetics, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8510, Japan. Tel: +81 3 5803 5826; Fax: +81 3 5803 0242; E-mail: yos.mgen@mri.tmd.ac.jp

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Abbreviations: NF- κ B, nuclear Factor- κ B; SOCS-1, suppressor of cytokine signaling 1; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; PKAc, protein kinase A catalytic subunit; MSK1, mitogen- and stress-activated protein kinase-1; RT-PCR, reverse transcription-PCR; ELISA, enzyme-linked immunosorbent assay; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; TUNEL, terminal dUTP nick-end labeling

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Results

Phosphorylation of Ser276 is crucial for constitutive expression of RelA/p65. To determine the biological significance of RelA/p65 phosphorylation at Ser276, we established HeLa cells stably expressed with Flag-vector (HeLa/vector), Flag-tagged RelA/p65 wild-type (HeLa/RelA-WT), or the S276A mutant (HeLa/RelA-SA) in which Ser276 was mutated to Ala. Toward this end, we have obtained each of several clones; however, expression levels of the SA mutants were markedly lower than those of the WT (Figure 1a). Importantly, each of mRNA levels was comparable (Figure 1a), indicating the possibility that the S276A mutants are, at least in part, constitutively degraded by the post-translational modification. To exclude the possibility that endogenous RelA/p65 affects expression levels of stably introducing exogenous RelA/p65, we used an RelA(5'UTR) siRNA that recognizes 5'-untranslated region of RelA/p65 mRNA and thereby depletes endogenous RelA/p65 without any effect upon exogenous RelA/p65. The results showed that depletion of endogenous RelA/p65 had little, if any, effect on the expression levels of stably overexpressing RelA/p65 (Supplementary Figure 1). Previous studies have shown that RelA/p65 is degraded by the ubiquitin-proteasome system.¹³ To determine whether the SA mutants are constitutively ubiquitinated, cells were left untreated or treated with proteasome inhibitor MG-132. There was little, if any, ubiquitinated form of RelA/p65 in HeLa/RelA-WT cells (Figure 1b). In sharp contrast, substantial poly-ubiquitination was observed in MG-132-treated HeLa/RelA-SA cells (Figure 1b). In concert with this finding, expression levels of RelA/p65 SA were recovered to levels equivalent to those of WT after treatment with MG-132 (Figure 1b). As shown previously, mRNA expression remained unchanged and comparable between WT and SA, thus indicating that the SA mutants are constitutively degraded by the ubiquitination-proteasome machinery. Other studies have shown that a ubiquitin ligase, SOCS-1, mediates ubiquitination of RelA/p65 between residues 220 and 335, and subsequent degradation by proteasome.¹⁴ To explore the involvement of SOCS-1 on RelA/p65 expression, SOCS-1 was depleted in cells by transfection with SOCS-1 siRNA. There was no significant effect on expression of Flag-RelA/p65 WT by knocking down SOCS-1 (Figure 1c). In contrast, attenuation of Flag-RelA/p65 SA expression was partially cancelled in cells silenced for SOCS-1 (Figure 1c). Consistent with previous results, mRNA expression remained unchanged and comparable between WT and SA regardless of SOCS-1 silencing (Figure 1c). Taken together, these results indicate that Ser276 phosphorylation prevents RelA/p65 from SOCS-1-dependent ubiquitination and subsequent degradation. Accumulating studies have shown that phosphorylation of RelA/p65 at Ser276 is indispensable for its transactivation.^{3,6} In this regard, to further define whether Ser276 phosphorylation is necessary for RelA/p65 activation in response to TNF- α , we performed chromatin immunoprecipitation (ChIP) assays on the I κ B- α promoter. HeLa/RelA-WT or HeLa/RelA-SA cells were left untreated or treated with TNF- α in the presence of MG-132. Chromatin was isolated from cells and immunoprecipitated with an anti-

Flag antibody. Immunoprecipitated DNA was analyzed using real-time PCR with primers amplifying the I κ B- α promoter region encompassing κ B sequences. In HeLa/RelA-WT cells, DNA fragments containing κ B sequences were specifically immunoprecipitated with anti-RelA/p65, but not IgG, suggesting that RelA/p65 actually binds to κ B sequences on the I κ B- α promoter *in vivo* (Figure 1d). Moreover, occupancy of RelA/p65 to I κ B- α promoter was substantially increased after TNF- α stimulation. In concert with these results, Ser276 phosphorylation was enhanced upon exposure to TNF- α (Figure 1d and Supplementary Figure 2). In contrast, there was profoundly less occupancy of RelA/p65 to the promoter in HeLa/RelA-SA cells (Figure 1d). These results thus suggest that phosphorylation of RelA/p65 at Ser276 is required for its transactivation in response to TNF- α . In this context, Ser276 kinase(s) contributes to RelA/p65 stabilization and activation, and thus identification of such kinase(s) is of importance in understanding the precise mechanisms of NF- κ B signalling pathways.

Identification of Ser276 kinases by expression cloning. To identify kinases that phosphorylate RelA/p65 at Ser276, we performed expression-cloning analysis by using anti-phospho-RelA/p65(Ser276) antibody (S276-Ab) (Figure 2a).¹⁵ The LE392 strain of *E. coli* transformed with GST-RelA/p65(221–319) WT or the GST-RelA/p65(221–319) S276A mutant, in which Ser276 is substituted with Ala, was infected with phage expression libraries prepared from the human fetal brain. After IPTG induction and transfer of plaques to the nitrocellulose membrane, phosphorylation of GST-RelA/p65 at Ser276 was detected using immunoblot analysis with S276-Ab. A three-stage screening process was carried out to identify positive plaques (Figure 2b). As a control, there was no detectable signal in *E. coli* transformed with the GST-RelA/p65(221–319) S276A mutant, suggesting that S276-Ab specifically reacts with phospho-Ser276 (Figure 2b). As a result of the screening performed using the human cDNA library, we isolated three positive clones in total. To identify the cDNA present in the positive phages, the phage DNA was excised into pBluescript (Stratagene, La Jolla, CA, USA) and then subjected to sequencing. We found that these clones encoded Pim-1, suggesting that Pim-1 kinase is capable of phosphorylating GST-RelA/p65(221–319) at Ser276 in *E. coli*. To determine whether Pim-1 recombinant kinase phosphorylates purified RelA/p65, GST-RelA/p65(221–319) WT or the GST-RelA/p65(221–319) S276A mutant were incubated with ATP in the presence or absence of the recombinant kinase. The finding that purified Pim-1 phosphorylated GST-RelA/p65(221–319) shows that Pim-1 is a Ser276 kinase *in vitro* (Figure 2c). Notably, co-incubation of Pim-1 with the GST-RelA/p65(221–319) S276A mutant completely abrogated reaction with S276-Ab (Figure 2c), indicating the specificity of this antibody against phospho-Ser276. To extend these findings to cellular kinases, COS-7 cells were co-transfected with Flag-RelA/p65 WT or SA and, GFP vector, GFP-Pim-1 WT, or the catalytically inactive GFP-Pim-1 K67R mutant. Cell lysates were immunoprecipitated with anti-Flag followed by immunoblot analysis with S276-Ab. Expression of WT Pim-1 was associated with substantial phosphorylation of RelA/p65

Figure 1 The RelA/p65 S276A mutant is degraded by ubiquitin-proteasome machinery. **(a)** HeLa cells stably expressed with Flag-vector (HeLa/vector, one clone), Flag-tagged RelA/p65 wild-type (HeLa/RelA-WT, two clones), or the S276A mutant (HeLa/RelA-SA, two clones), in which Ser276 was mutated to Ala, were lysed and analyzed using immunoblotting (IB) with anti-Flag or anti-tubulin. Total RNA samples were analyzed by RT-PCR using Flag-RelA/p65-specific or GAPDH-specific primers. **(b)** HeLa/vector, HeLa/RelA-WT, or HeLa/RelA-SA cells were left untreated or treated with MG-132. Cell lysates were subjected to immunoprecipitation (IP) with anti-Flag followed by immunoblotting with anti-ubiquitin or anti-Flag. Cell lysates were also analyzed using immunoblotting with anti-Flag or anti-tubulin. Total RNA samples were analyzed by RT-PCR using Flag-RelA/p65-specific or GAPDH-specific primers. **(c)** HeLa/vector, HeLa/RelA-WT, or HeLa/RelA-SA cells were transfected with scramble siRNA or SOCS-1 siRNA. Cell lysates were immunoblotted with anti-Flag, anti-SOCS-1, or anti-tubulin. Total RNA samples were analyzed by RT-PCR using Flag-RelA/p65-specific or GAPDH-specific primers. **(d)** HeLa/RelA-WT or HeLa/RelA-SA cells were pre-treated with MG-132 for 18 h followed by treatment with 20 ng/ml TNF- α for 1 h. ChIP assays were performed using primer sequences containing the κ B-elements in the $I\kappa$ B- α promoter. Real-time PCR was performed with immunoprecipitated chromatin fragments using anti-Flag or IgG and normalized to input DNA. The input DNA indicates PCR amplification of total chromatin before immunoprecipitation. The results are represented as mean $\pm$ S.D. obtained from at least two independent experiments, each performed in triplicate. * P < 0.05. Cell lysates were subjected to immunoprecipitation with anti-Flag followed by immunoblot analysis with anti-phospho-RelA/p65(Ser276) or anti-Flag. Lysates were also immunoblotted with anti-tubulin. A ratio of phosphorylated RelA/p65 was determined by the densitometric analysis using 'ImageJ' (NIH, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>) program. The phosphorylation level in untreated HeLa/RelA-WT cells was defined as 1.00

Figure 2 Identification of Ser276 kinase. **(a)** Schematic diagram of the Ser276 kinase screening (see Materials and Methods section for detail). **(b)** In the first screening step, bacteria expressing GST-RelA/p65(221–319) wild-type (WT) were infected by phage cDNAexpression libraries from human fetal brain. Phosphorylated RelA/p65 was detected by anti-phospho-RelA/p65(Ser276) (upper left panel). Positive phages were replated for the second screening (upper right panel). After the second screening, selected positive clones were infected with a bacterium that either expressed GST-RelA/p65(221–319) WT or S276A (lower panels). The arrow indicates a positive plaque. **(c)** Recombinant GST-Pim-1 was incubated with purified GST-RelA/p65(221–319) WT or S276A and ATP. Reaction products were analyzed using immunoblotting with anti-phospho-RelA(Ser276) or anti-GST. **(d)** COS-7 cells were co-transfected with Flag-RelA/p65 WT or Flag-RelA/p65 S276A and GFP vector, GFP-Pim1 WT, or GFP-Pim1 K67R, which is characterized as the dominant-negative kinase mutant. Cell lysates were subjected to immunoprecipitation with anti-Flag followed by immunoblotting with anti-phospho-RelA/p65(Ser276) or anti-Flag. Cell lysates were also analyzed using immunoblotting with anti-GFP or anti-tubulin

at Ser276 (Figure 2d). In contrast, Ser276 phosphorylation was completely abrogated by expression of the dominant-negative kinase mutant (Figure 2d). As expected, no phosphorylation was observed in cells ectopically expressed with the Flag-RelA/p65 SA (Figure 2d). These findings collectively support a novel role for Pim-1 as a Ser276 kinase *in vitro*.

Pim-1 kinase is capable of phosphorylating Ser276 in the cellular response to TNF- α . To determine whether Pim-1 physiologically phosphorylates RelA/p65 at Ser276, HeLa cells were transfected with scramble siRNA or Pim-1 siRNA followed by TNF- α stimulation. Immunoblot analysis with S276-Ab revealed that, after exposure to TNF- α , Ser276 phosphorylation was increased and was maximal at 1 h (Figure 3). In contrast, Ser276 phosphorylation was completely abrogated in cells silenced for Pim-1, suggesting that Pim-1 functions as a Ser276 kinase in response to TNF- α

(Figure 3). Similar results were obtained in U2OS cells (Supplementary Figure 3). Comparable findings were obtained with another stimulus, interleukin-1 β (Supplementary Figure 4). Moreover, inhibition of Ser276 phosphorylation by Pim-1 siRNA was associated with a slight but substantial attenuation of RelA/p65 expression in HeLa cells (Figure 3). Intriguingly, more profound attenuation was observed in U2OS cells (Supplementary Figure 3). In concert with these results, *de novo* transcription of I κ B- α was substantially attenuated in cells depleted for Pim-1 (Figure 3 and Supplementary Figure 3). Inhibitor of apoptosis protein 1 (c-IAP1) is also induced by TNF- α , which depends on the NF- κ B signalling.¹⁶ As shown for I κ B- α , mRNA expression of c-IAP1 increased after TNF- α stimulation in HeLa cells (Figure 3). In contrast, knockdown of Pim-1 abrogated TNF- α -induced upregulation of c-IAP1 (Figure 3). Similar findings were obtained in U2OS cells (Supplementary Figure 3). These results imply the possibility that depletion of

Figure 3 Pim-1 phosphorylates RelA/p65 at Ser276. HeLa cells were transfected with scramble siRNA or Pim-1 siRNA. At 48 h after transfection, cells were treated with 20 ng/ml TNF- α for indicated times. Cell lysates were subjected to immunoblot analysis with anti-phospho-RelA/p65(Ser276), anti-RelA/p65, anti-I κ B- α , or anti-tubulin. Nuclear lysates were also subjected to immunoblot analysis with anti-RelA/p65 or anti-PCNA. A ratio of RelA/p65 expression in whole cell lysates was determined by the densitometric analysis using 'ImageJ' program. The expression level in untreated cells transfected with scramble siRNA was defined as 1.00. Total RNA samples were analyzed by RT-PCR using RelA/p65-specific, I κ B- α -specific, Pim-1-specific, c-IAP1-specific, or GAPDH-specific primers

Pim-1 impairs TNF- α -induced NF- κ B signalling to disrupt regulation of apoptosis, mainly because of the lack of Ser276 phosphorylation. Given the finding that there was considerable attenuation of RelA/p65 expression with knocking

Figure 4 Silencing Pim-1 elevates poly-ubiquitination of RelA/p65. HeLa cells transfected with scramble siRNA or Pim-1 siRNA were treated with MG-132 for 5 h. Cell lysates were immunoprecipitated with IgG or anti-RelA/p65 followed by immunoblot analysis with anti-ubiquitin or anti-RelA/p65. IgH indicates immunoglobulin heavy chain. Lysates were also analyzed using immunoblotting with anti-Pim-1 or anti-tubulin

down Pim-1, depletion of Pim-1 affected little, if any, nuclear translocation of RelA/p65 in response to TNF- α (Figure 3). It is noteworthy that Pim-1 expression was increased gradually after TNF- α exposure, conceivably because of its partial abrogation from proteasome-mediated degradation.¹⁷ Previous study showed that Pim-2 activates NF- κ B-dependent gene expressions through phosphorylation of the oncogenic serine/threonine kinase Cot.¹⁸ In this context, to examine the possibility that Pim-2 directly affects NF- κ B activation by phosphorylating RelA/p65 at Ser276, HeLa cells were transfected with scramble siRNA or Pim-2 siRNA followed by treatment with TNF- α . In contrast to depletion of Pim-1, TNF- α -induced Ser276 phosphorylation was substantially detectable in cells silenced for Pim-2, even though its level was to a slightly lesser extent compared when with mocked cells (Supplementary Figure 5). Thus, it could be unlikely that Pim-2 directly affects phosphorylation of RelA/p65 at Ser276, at least upon exposure to TNF- α . To assess that the attenuation of endogenous RelA/p65 is mainly due to the ubiquitination, we carried out the ubiquitination assays. Endogenous RelA/p65 was slightly but pronouncedly poly-ubiquitinated in HeLa cells treated with MG-132 (Figure 4, lane 2). Importantly, the finding that silencing Pim-1 remarkably augmented its poly-ubiquitination (Figure 4, lane 4) further provided the protective effect of Ser276 phosphorylation by Pim-1 on ubiquitination of RelA/p65. Similar results were obtained in U2OS cells (data not shown). To confirm that Pim-1 interacts with RelA/p65, HeLa cells were left untreated or treated with TNF- α . Analysis of anti-Pim-1-immunoprecipitates with anti-RelA/p65 revealed that Pim-1 constitutively associates with RelA/p65 and that this

interaction remained unchanged after TNF- α exposure (Supplementary Figure 6). Similar findings were obtained in U2OS cells (data not shown). These results indicate constitutive binding of Pim-1 to RelA/p65 in cells. To further define whether Pim-1-dependent Ser276 phosphorylation induces transactivation of RelA/p65 target genes, we performed ChIP assays on the κ B- α promoter. Occupancy of RelA/p65 to κ B- α promoter was markedly increased after TNF- α stimulation (Figure 5a). In contrast, there was no occupancy of RelA/p65 to the promoter in cells silenced for Pim-1, regardless of TNF- α exposure. Comparable findings were obtained in U2OS cells (Supplementary Figure 7). To examine whether Pim-1 enhances TNF- α -induced RelA/p65 activation, 293 cells stably transfected with the luciferase-reporter vector containing κ B-elements were treated with TNF- α . Cells were then transfected with scramble siRNA,

Figure 5 Pim-1 enhances RelA/p65 recruitment to the κ B-elements on the promoters in response to TNF- α . (a) HeLa cells were transfected with scramble siRNA or Pim-1 siRNA. Cells were left untreated or treated with 20 ng/ml TNF- α for 1 h. Soluble chromatin was subjected to ChIP analysis using anti-RelA or IgG for κ B- α promoter binding. Total RNA samples were analyzed by RT-PCR using Pim-1-specific or GAPDH-specific primers. (b) 293 cells expressing pNF- κ B-luc were transfected with scramble siRNA, Pim-1 siRNA, or RelA/p65 siRNA. Cells were then treated with TNF- α for 8 h. Luciferase assays were performed and the results are represented as mean $\pm$ S.D. obtained from three independent experiments, each performed in triplicate. *P < 0.05 and **P < 0.01

Pim-1 siRNA, or RelA/p65 siRNA. As expected, silencing of RelA/p65 was associated with a pronounced inhibition of NF- κ B activity in response to TNF- α (Figure 5b). Importantly, depletion of Pim-1 significantly diminished TNF- α -induced NF- κ B activation (Figure 5b). We further examined whether knockdown of PKAc influenced TNF- α -induced NF- κ B activation. The results showed that, in contrast to deficit in Pim-1, silencing PKAc had little, if any, effect on luciferase production driven by NF- κ B through κ B-elements (Supplementary Figure 8). Taken together, these findings indicate that Pim-1-mediated phosphorylation of RelA/p65 at Ser276 is essential for its transactivation in response to TNF- α .

Pim-1 kinase affects cellular function of NF- κ B by controlling RelA/p65 in response to TNF- α . To examine the biological significance of Pim-1-dependent regulation of RelA/p65 transcriptional activity, we analyzed TNF- α -induced interleukin-6 (IL-6) IL-6 production. U2OS cells were left untreated or treated with TNF- α in the presence or absence of Pim-1 siRNA. As expected, TNF- α stimulation substantially imposed IL-6 production (Figure 6). In contrast, inhibition of Pim-1 expression impaired production of IL-6 in response to TNF- α (Figure 6). These data show that Pim-1 induces transcriptional activity of RelA/p65 to produce IL-6 in response to TNF- α .

Depletion of Pim-1 expression with siRNA sensitizes cells to TNF- α -induced cell death. To further access the biological significance of RelA/p65 regulation by Pim-1, we analyzed TNF- α -induced apoptotic cell death. HeLa cells were transfected with scramble siRNA or Pim-1 siRNA followed by treatment with TNF- α . Cell survival was monitored using MTS assays. The results showed that knocking down Pim-1 severely impaired cell survival after TNF- α stimulation (Figure 7a). To define whether this impairment is provoked by apoptosis induction, we evaluated with TUNEL assays in the same experimental settings. Analysis of TUNEL assays revealed that apoptotic cells were slightly increased after TNF- α exposure (Figure 7b). Importantly, TNF- α -induced apoptotic induction was significantly enhanced in cells silenced for Pim-1 (Figure 7b). Similar results were obtained in U2OS cells

Figure 6 Pim-1 induces IL-6 production upon exposure to TNF- α . U2OS cells were transfected with scramble siRNA or Pim-1 siRNA. After 48 h, cells were left untreated or treated with TNF- α for 24 h. The amount of secreted IL-6 production was measured using ELISA. The results are represented as mean $\pm$ S.D. obtained from three independent experiments, each performed in triplicate

Figure 7 Pim-1 inhibits TNF- α -induced apoptosis. (a) HeLa cells were transfected with scramble siRNA or Pim-1 siRNA. At 48 h after transfection, cells were left untreated or treated with TNF- α for 24 h. Surviving cells were measured using MTS assays. The data indicate the mean $\pm$ S.D. obtained from two independent experiments, each performed in triplicate. (b) HeLa cells were transfected with scramble siRNA or Pim-1 siRNA. At 48 h after transfection, cells were left untreated or treated with 100 ng/ml TNF- α for 24 h. Apoptotic cells were quantitated using TUNEL assays. The results are represented as the percentage of TUNEL-positive cells. The data indicate the mean $\pm$ S.D. obtained from two independent experiments each performed in triplicate. ** $P < 0.01$. (c) HeLa/RelA-WT or HeLa/RelA-SA cells were transfected with scramble siRNA or RelA/p65(5'UTR) siRNA. Total RNA samples were analyzed by RT-PCR using endogenous RelA/p65-specific, Flag-RelA/p65-specific, or GAPDH-specific primers. (d) HeLa/RelA-WT or HeLa/RelA-SA cells were transfected with scramble siRNA, RelA/p65(5'UTR) siRNA, or Pim-1 siRNA. At 48 h after transfection, cells were left untreated or treated with 100 ng/ml TNF- α for 24 h. Apoptotic cells were quantitated using TUNEL assays. The results are represented as the percentage of TUNEL-positive cells. The data indicate the mean $\pm$ S.D. obtained from two independent experiments each performed in triplicate. ** $P < 0.01$

silenced for Pim-1 (Supplementary Figure 9). These results suggest that abrogation of Pim-1 expression, at least in part, diminishes TNF- α -induced NF- κ B activation that is essential for defense from apoptotic cell death. As a result, cells undergoing apoptosis were significantly increased. Finally, we have examined whether Pim-1 phosphorylation of RelA/p65 at Ser276 confers resistance to TNF- α -induced apoptosis. To address this issue, we used HeLa cells stably expressed with RelA/p65 WT (HeLa/RelA-WT) or the S276A mutant (HeLa/RelA-SA) (Figure 1a). To deplete endogenous RelA/p65, HeLa/RelA-WT or HeLa/RelA-SA cells were transfected with the RelA(5'UTR) siRNA (Figure 7c). After TNF- α exposure, there was no significant increase in apoptosis in HeLa/RelA-WT cells silenced for endogenous RelA/p65 (Figure 7d). In contrast, co-depletion of Pim-1 increased TNF- α -induced cell death. More importantly, upon exposure to TNF- α , apoptotic induction was markedly enhanced in HeLa/RelA-SA cells silenced for endogenous RelA/p65 (Figure 7d), indicating that absence of Ser276 phosphorylation is substantially sensitized to TNF- α -induced apoptosis. These results indicate that Pim-1

phosphorylation of RelA/p65 at Ser276 is required for the protective effect from TNF- α -induced apoptosis.

Taken together, these findings thus support a model in which, upon exposure to TNF- α , Pim-1 phosphorylates RelA/p65 at Ser276 to activate transcription function on the promoters (Figure 8). Pim-1 has a crucial role in NF- κ B activation by controlling RelA/p65 to regulate cellular function and fate in response to TNF- α (Figure 8).

Discussion

The serine/threonine kinase *pim* gene is a proto-oncogene.¹⁹ The Pim kinase has three family gene products, which are Pim-1, Pim-2, and Pim-3.²⁰ Pim kinases contribute to cell-cycle progression and cell growth. Moreover, Pim kinases have been implicated in the control of tumorigenesis. Through the analysis of *pim-1* or *pim-2* transgenic mice, Pim kinase has been shown to enhance development of lymphoma and leukemia.^{21,22} Indeed, the expression levels of Pim kinases are frequently elevated in patients with lymphoma, leukemia,

Figure 8 A hypothetical model for the NF- κ B activation pathway. In response to TNF- α stimulation, Pim-1 phosphorylates rapidly RelA/p65 at Ser276. Pim-1-mediated phosphorylation affects RelA/p65 functions such as promoter binding and transactivation of target genes. In addition, phosphorylation of Ser276 protects RelA/p65 from SOCS-1-mediated ubiquitination and subsequent proteolysis

and prostate cancer.^{21,23} In particular, the expression levels of Pim-1 have been associated with the clinical outcome in prostate cancer.²⁴ Furthermore, accumulating studies have revealed that *pim-1* gene is a target of aberrant somatic hypermutation in non-Hodgkin's lymphoma and B-cell lymphoma,^{25,26} and some of the mutations markedly increase an enzymatic activity of Pim-1.²⁷ Although the ability of Pim kinase to stimulate cell growth and inhibit apoptosis may contribute to the promotion of tumorigenesis,²⁰ aberrant Pim kinase activation may induce tumorigenesis. Thus, both NF- κ B and Pim kinase are implicated in tumorigenesis. In this context, a recent study showed that protective effect of Pim-1 depends, at least in part, upon NF- κ B activity; however, there was no evidence that Pim-1 directly affects NF- κ B.²⁸ This study is the first to show that Pim-1 directly activates NF- κ B signalling by phosphorylating RelA/p65 at Ser276.

Intriguingly, reconstitution of RelA/p65^{-/-} MEFs with various serine to alanine substituted mutants of p65 recently was found to completely rescue TNF- α -induced IL-6 production with S529A, S536A, and S529A/S536A mutants, whereas S276A was completely unresponsive to TNF- α to induce IL-6 expression, indicating that this phosphorylation is critical for RelA/p65 activity.³ In this regard, Pim-1 specifically phosphorylates RelA/p65 at Ser276, thus leading to its targeting to the κ B-elements containing promoter sections and selective stimulation of particular NF- κ B-driven genes, such as I κ B- α . Interestingly, Pim-1 is normally regulated by the JAK/STAT pathway, especially involved in STAT3 and STAT5.²⁹ On the basis of this evidence and given that treatment of cells with TNF- α activates STAT5, it is conceivable that TNF- α stimulation also contributes to activation of Pim-1 through STAT5. In this context, even although there would be residual constitutive activity of Pim-1 without stimulation, TNF- α could rapidly elevate Pim-1 activity to a greater extent. As a result, phosphorylation of Ser276 was transiently upregulated in response to TNF- α

(Figure 3 and Supplementary Figure 3). Previous studies have shown that after RelA/p65 phosphorylation, a conformational switch allows engagement of the CREB binding protein to create a transcriptionally competent enhanceosome. Although deacetylase inhibitors have been shown to prolong phosphorylation of RelA/p65,³⁰ it remains uncertain how Pim-1 phosphorylation of RelA/p65 is linked further to acetylation.

Our findings also suggest that the RelA/p65 S276A mutant converted to an extremely unstable protein, mainly due to rapid protein degradation. Significantly, either inhibition of phospho-Ser276 RelA/p65 formation or mutation of Ser276 hinders the ability of RelA/p65 to transactivate target genes. In this context, Ser276 phosphorylation could be necessary for inhibitory effect of ubiquitin-mediated degradation of RelA/p65 in TNF- α signalling. Consistent with this notion, abrogation of Ser276 phosphorylation by knockdown of the Ser276 kinase, Pim-1, substantially attenuated expression levels of RelA/p65 (Figure 3 and Supplementary Figure 3). Previously, a ubiquitin ligase SOCS-1 was supposed to ubiquitinate RelA/p65, inducing subsequent degradation by proteasome.¹⁴ However, it remains unclear whether SOCS-1 ubiquitinates RelA/p65 in the physiological condition or in response to any stimuli. Nevertheless, we showed that Ser276 is involved in the target of SOCS-1-mediated ubiquitination, resulting in the down-regulation of NF- κ B activity, and that phosphorylation on Ser276 protects RelA/p65 from degradation. Given the previous findings that the *socs-1* gene is silenced in many human malignancies,³¹ RelA/p65 would be highly stable intrinsically, thereby constitutively active in cancer cells. Interestingly, previous studies have shown that Pim-1 stabilizes SOCS-1 by phosphorylation.³² Meanwhile, this study showed that Pim-1 also phosphorylates RelA/p65 at Ser276 to prevent from SOCS-1-mediated ubiquitination. We do not have any rationale to connect these distinct roles of Pim-1. However, as Pim-1 is expressed both at the nucleus and the cytoplasm whereas SOCS-1 is localized exclusively in the cytoplasm, it is thus conceivable that TNF- α -induced nuclear targeting of RelA/p65 can hardly enable SOCS-1 to its ubiquitination and subsequent degradation.

Accumulating studies suggest that activation of NF- κ B can lead to tumor cell proliferation, invasion, angiogenesis, and metastasis. Thus, suppression of NF- κ B in cancer cells may provide an additional target for prevention of cancer. NF- κ B blockers can also be considered for the therapy of cancer, perhaps in combination with chemotherapeutic agents or γ -irradiation. In this regard, the key activator of NF- κ B, Pim-1 kinase, would be one of the appropriate targets for the development of kinase inhibitors. Indeed, small molecule inhibitors of the Pim-1 kinase have been recently described *in vitro* and in cell-based systems.^{33–35} Targeting the Pim-1 kinase may be a beneficial addition to a traditional cancer chemotherapy regimen.

In summary, we show that Pim-1-mediated phosphorylation of RelA/p65 is a novel mechanism that is critical for modulating NF- κ B function, including its stability and activity. Deregulation of these pathways can thus have a crucial role in constitutive activation of NF- κ B in some human diseases, such as cancer. Moreover, in these human diseases, Pim-1 kinase might offer promising new targets for controlling the aberrant activation of NF- κ B signalling.

Materials and Methods

Screening of the kinases that phosphorylate RelA/p65 at Ser276. Identification of Ser276 kinases using phospho-specific antibodies was performed as described previously,^{15,36} with minor modification. In brief, LE392 strain of *E. coli* were transfected with GST-RelA/p65(221–319) WT or S276A by electroporation. The bacteria were infected with cDNA library phages from human fetal brain (Stratagene, La Jolla, CA, USA), plated onto LB agar, and incubated for 3 h at 42°C. GST-RelA/p65(221–319) and library-originated protein were expressed in the bacteria by overlaying the nitrocellulose membranes containing IPTG for 4 h at 37°C, and transferred onto the membranes. The membranes were immunostained with anti-phospho-RelA/p65(Ser276) (Rockland Immunochemicals, Gilbertsville, PA, USA). Proteins were visualized using Western Lighting (PerkinElmer, Wellesly, MA, USA). Positive clones were selected and isolated. To analyze cDNA inserted into phages, the phage DNA was excised into pBluescript according to the standard protocol.

In vitro kinase assays. *In vitro* kinase assays were performed as described.^{37,38} In brief, recombinant GST-RelA/p65(221–319) proteins corresponding to WT and S276A purified from plasmid-transduced *E. coli* were incubated in kinase buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0, 10 mM MgCl₂, 0.1 mM Na₃VO₄, and 2 mM dithiothreitol (DTT)) with recombinant GST-Pim-1 protein (Cell Signaling Technology, Danvers, MA, USA) and ATP for 20 min at 30°C. The reaction products were boiled for 5 min and subjected to immunoblot analysis.

Cell culture. COS-7, HeLa, U2OS, and 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37°C in 5% CO₂. Cells were treated with 1.25 µM MG-132 (Merck KGaA, Darmstadt, Germany), recombinant human TNF-α (PeproTech, Rocky Hill, NJ, USA), or purified human interleukin-1-β (PeproTech).

Plasmid construction. RelA/p65 and Pim-1 cDNAs were isolated and amplified by reverse transcription PCR (RT-PCR). Various mutations were introduced by site-directed mutagenesis and all mutations were confirmed using sequencing. RelA/p65 cDNA was cloned into pcDNA3-Flag vector.³⁹ RelA(221–319) fragment was cloned into pGEX4T-1 vector. Pim-1 cDNA was cloned into pE GFP-C1 vector.

Cell transfections. Cells were transiently transfected using FuGENE 6 (Roche, Basel, Switzerland) according to the instructions of the manufacturer. HeLa cells were stably transfected with pcDNA3-Flag, pcDNA3-Flag-RelA/p65 WT, or pcDNA3-Flag-RelA/p65(S276A) by using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer and selected in the presence of G418. Transfections of siRNA were performed using LipofectAMINE RNAi MAX (Invitrogen) according to the instructions of the manufacturer. The sequences of siRNAs are 5'-AUUGUCGGCUGCCACCUG GUUGUGU-3' for SOCS-1, 5'-AGAACAUUCGCAUCCAUUGGAUGGU-3' for Pim-1, 5'-UCCUCUGGUAGAUGGCAUCCAGUC-3' for PKAc, 5'-UACAUCCUCGGCU GGUGUUUGCAUC-3' for Pim-2, 5'-GCCCUAUCUUUACGUCATT-3' for RelA/p65, and 5'-GUGCACUACAGACGAGCCATT-3' for RelA(5'UTR).

Immunoblot and immunoprecipitation analysis. Immunoprecipitation and immunoblot analyses are previously described,⁴⁰ with minor modification. In brief, cells were harvested, washed in cold PBS, and re-suspended in the lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 1 mM DTT, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1% NP-40). After centrifugation, the supernatants were isolated and used as whole cell lysates. Cell lysates were incubated with anti-Flag agarose (Sigma-Aldrich, St Louis, MO, USA) for 2 h at 4°C. The beads were washed thrice in lysis buffer, and eluted with Flag-peptide (Sigma-Aldrich). Cell lysates and immunoprecipitated proteins were boiled for 5 min, separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with anti-Flag (Sigma-Aldrich), anti-tubulin (Sigma-Aldrich), anti-ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-SOCS-1 (Millipore, Billerica, MA, USA), anti-phospho-RelA/p65(Ser276) (Thermo Fisher Scientific Anatomical Pathology, Waltham, MA, USA), anti-GST (Nacalai Tesque, Kyoto, Japan), anti-GFP (Nacalai Tesque), anti-Pim-1 (Santa Cruz Biotechnology), anti-Pim-2 (Cell Signaling Technology), anti-PKAc (Santa Cruz Biotechnology), or anti-IκB-α (Santa Cruz Biotechnology). Immune complexes were visualized using Western Lighting (PerkinElmer).

RT-PCR analysis. Total RNA from cells was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) according to the instructions of the manufacturer. A total of 900 ng RNA was amplified using Super Script III One Step RT-PCR System with Platinum Taq Kit (Invitrogen). For *flag-rela/p65* gene expression, the nucleotide sequence of 5'-ATGGACTACAAGGACGATGACGATAAG-3' was used as the sense primer, and 5'-TGGCGTGACTGATAGCCTGCTCCAGGT-3' was used as the antisense primer. For *endogenous rela/p65* gene expression, the nucleotide sequence of 5'-CGAATGGCTCGTCTGTAGTGCA-3' was used as the sense primer, and 5'-TGGCGTGACTGATAGCCTGCTCCAGGT-3' was used as the antisense primer. For *ikb-α* gene expression, the nucleotide sequence of 5'-GAAAAGGCATGACCATGGAAGTGATC-3' was used as the sense primer, and 5'-ACACCAGGTCAGGATTTTGCAGGTCCA-3' was used as the antisense primer. For *pim-1* gene expression, the nucleotide sequence of 5'-AT GCTCTTGTCAAAATCAACTCGCTTGCC-3' was used as the sense primer, and 5'-TGATGAAGTCGAAGAGATCTTGACCCGGCT-3' was used as the antisense primer. For *c-iap1* gene expression, the nucleotide sequence of 5'-GACATCTCTTCATCGAGGACTAACCCCTAC-3' was used as the sense primer, and 5'-CCTTTGGTTCCAGTACTAGCTTCCAC-3' was used as the antisense primer. For *gapdh* gene expression, the nucleotide sequence of 5'-AAGGCTGTGGGCAAGGTCATCCCT-3' was used as the sense primer, and 5'-TTACTCCTTGGAGGCCATGTGGGC-3' was used as the antisense primer. The reaction products were separated on 2% agarose gels.

Subcellular fractionation. Subcellular fractionation was performed as described previously.^{41,42} Purity of the fractions was monitored using immunoblot analysis.

Chromatin immunoprecipitation (ChIP) assay. Cells were harvested and washed with chilled PBS once followed by incubation in 1% formaldehyde for 15 min at room temperature for chromatin cross-linking. The cells were then collected and washed with chilled PBS again. After centrifugation, the cell pellets were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM PMSF, 1 mM Na₃VO₄, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A), and the lysates were sonicated to obtain DNA fragments for 200–500 base pairs in length. After centrifugation, 50 µl of the supernatant was used as an input, and the remainder was diluted 2- to 2.5-fold in washing buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% NP-40, and protease inhibitors as described above). This diluted fraction was subjected to immunoprecipitation with 2 µg of indicated antibodies for 2 h to overnight at 4°C with rotation. The immunocomplexes were collected with 30 µl protein A-Sepharose beads (Santa Cruz Biotechnology) for 1–2 h at 4°C with rotation. The beads were then pelleted by centrifugation and washed sequentially with 300 µl of the following buffers: wash buffer I (500 mM NaCl, 0.1% SDS, 2 mM EDTA, and 20 mM Tris-HCl, pH 8.0), wash buffer II (250 mM LiCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 1% deoxycholate) and then twice with TE buffer. Precipitated chromatin complexes were removed from the beads by shaking with 150 µl elution buffer (1% SDS and 0.1 M NaHCO₃) for 15 min, and this step was repeated. All the eluate was collected and then the cross-linking was reversed by adding NaCl to a final concentration of 200 mM for overnight at 65°C. The remaining proteins were digested with the extraction buffer (50 mM Tris-HCl, pH 6.8, 10 mM EDTA, and 40 µg/ml proteinase K) for 1 h at 45°C. DNA was recovered by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. For analysis of the binding to the IκB-α promoter, the nucleotide sequence of 5'-GACGACCCCAATCAAATCG-3' was used as the sense primer, and 5'-TCAGGCTCGGGGAATTTCCC-3' was used as the antisense primer. The PCR reaction was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) or KODFX (TOYOBO, Osaka, Japan) according to the instruction manual of the manufacturer. The results of quantitative real-time PCR analysis were normalized for the level of input control.

Reporter gene assays. 293 cells stably transfected with pNF-κB-luc and pTK-hyg (Panomics, Fremont, CA, USA) were treated with TNF-α. The luciferase activity was determined using Bright-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The relative fold increase in activity compared with untreated cells was determined.⁴³ The data represent mean ± S.D. from at least 3–4 independent experiments, each performed in triplicate.

IL-6 production. Cells were plated onto 24-well plates and stimulated with TNF- α . After 24 h, the culture supernatants were assayed for IL-6 production using OptEIA human IL-6 ELISA kit II (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction.

Measurement of cell viability. To measure cell viability, MTS assays were performed by adding 20 μ l CellTiter 96 Aqueous One Solution Reagent (Promega) directly into 100 μ l culture media in 96-well plates. After incubation for 3 h at 37°C in a humidified 5% CO₂ atmosphere, the absorbance was measured at 490 nm using a multilabel counter (PerkinElmer).

Apoptosis assays. Cells cultured in poly-D-lysine-coated four-well chamber slides were transfected with siRNAs, and then treated with TNF- α for 24 h. At 24 h after treatment, apoptotic cells were detected by TUNEL assays using DeadEnd Fluorometric TUNEL System (Promega).

Conflict of interest

The authors declare no conflict of interest.

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