Front. Biol. 2010, 5(3): 263–271
DOI 10.1007/s11515-010-0050-5

RESEARCH ARTICLE

RNAi screen to identify protein phosphatases that regulate the NF-kappaB signaling

Guoxin WANG, Suping LI, Feifei WANG, Shufang HUANG, Xian LI, Wei XIONG, Biliang ZHANG (✉)

Laboratory of RNA Chemical Biology, Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China

Abstract NF-kappaB plays a critical role in cell survival, apoptosis, and inflammatory responses. Serine/threonine-specific phosphatases (PPs) represent the second major class of enzymes that catalyze the dephosphorylation of proteins. The roles of PPs regulating NF-kappaB activities are poorly understood. Here we describe an RNAi-based screen to identify the PPs that involve in regulating NF-kappaB signaling. Thirty-four candidate PPs siRNAs were synthesized and primarily screened by NF-kappaB reporter gene assay in HeLa cells. PHLPP, one of the protein phosphatase type 2C family members (PP2C), was identified as a positive regulator of NF-kappaB signaling. Knock-down of PHLPP dramatically attenuated TNFα-stimulated NF-kappaB transcriptional activation. Knock-down of PHLPP led to enhancement of NF-kappaB/p65 nuclear import and retention, but decreased TNFα-induced phosphorylation at Ser276 on p65. This critical phosphorylation was also drastically reduced by knock-down of PKCalpha and Akt1, two important serine/threonine kinases dephosphorylated by PHLPP. The results together suggest that PHLPP-Akt-PKC may represent an important signaling loop that activates NF-kappaB/p65 signaling through critical serine phosphorylation.

Keywords nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB), protein serine/threonine phosphatases, PH domain leucine-rich repeat protein phosphatase (PHLPP), RNA interference

1 Introduction

Nuclear factor-kappaB (NF-κB) represents a family of transcription factors that play crucial roles in cell survival, apoptosis, and inflammatory responses (Hayden and Ghosh, 2004; 2008). The NF-κB family consists of five members, p50, p52, p65 (RelA), c-Rel, and RelB, which share an N-terminal Rel homology domain (RHD) responsible for DNA binding, dimerization and interaction with inhibitors of NF-κB (IκB). Binding to IκB maintains NF-κB dimmers, mainly the p50/p65 heterodimer, in an inactive state in the cytoplasm. In response to extracellular stimuli, such as tumor necrosis factor alpha (TNFα), the signal pathway proceeds via activation of the IκB kinase (IKK) complex, degradation of IκB proteins, release and nuclear translocation of NF-κB dimmers. NF-κB signaling pathway consists of various signaling molecules, including TNF receptor associated factor (TRAF) family, IKK complex, IκB proteins, and NF-κB dimmers (Li and Lin, 2008). The activity of NF-κB is regulated by phosphorylation of the positive and negative signaling molecules, which is controlled by the opposing actions of protein kinases and phosphatases. Dozens of kinases have been identified to be involved in the phosphorylation of the components in the pathway (Viatour et al., 2005). However, the roles of phosphatases in NF-κB signaling remain to be further defined.

Protein serine/threonine phosphatases (PPs) are classified into the PP1, PP2A, PP2B, and PP2C/PPM families based on their biochemical properties. These family members have shown to be involved in many signaling pathways and cellular functions. Recent evidence indicates that PP1 regulates Akt1 signal transduction pathway to control the expression of downstream genes, promoting cell survival and modulating differentiation (Xiao et al., 2010). Distinct PP2A phosphatases are identified by RNA interference (RNAi) screen to associate with and dephosphorylate the IKK, NF-κB, and TRAF2 complexes leading to inhibition of NF-κB transcriptional activity in mouse astrocytes (Li et al., 2006). PPM1A/PP2Cα acts as a Smad phosphatase to abolish TGF-β-induced anti-proliferative and transcriptional responses (Lin et al., 2006). PPM1A and PPM1B dephosphorylate IKKβ at Ser177 and

Received March 31, 2010; accepted April 21, 2010

E-mail: zhang_biliang@gibh.ac.cn

Ser181, resulting in termination of TNFα-mediated NF-κB activation (Sun et al., 2009). PP2Ceta-2 suppresses interleukin-1 (IL-1) induced NF-κB activation by selectively dephosphorylating IKKβ (Henmi et al., 2009). PPs may represent a potential therapeutic target in the treatment of cancer, as well as of other related human diseases (Lu and Wang, 2008).

In this report, we describe an RNAi-based method to screen out the potential additional PPs involved in regulating NF-κB transactivation activity. Several PPs small interfering RNAs (siRNAs) were found to enhance the activities of NF-κB reporter gene induced by tumor necrosis factor-α (TNFα), whereas two, including PH domain leucine-rich repeat protein phosphatase (PHLPP) siRNA, impaired NF-κB activities. The cycling NF-κB/p65 shuttle between the nucleus and the cytoplasm was moderately inhibited by PHLPP siRNA. Besides, both the cellular IκBα content and TNFα-induced phosphorylation at serine 276 (Ser276) on p65 were weakened by PHLPP siRNA. Among several reported serine/threonine kinases involved in NF-κB signaling, Akt1/PKB and PKCα were considered as the potential targets of PHLPP by RNAi screening, according to the above mentioned cellular events. These results help to gain new insights in understanding the molecular mechanism by which protein kinases and phosphatases accurately regulate the NF-κB transcriptional activity.

2 Materials and methods

2.1 siRNA design and synthesis

Thirty-four PPs were chosen from the GenBank. The siRNAs (three target sequences per gene) were designed by siCatch™ siRNA Design (http://www.sirna.cn/). The scoring sequences were blasted in GenBank to ensure more than 15 nt mismatch with other human genes, and synthesized with 3′ dTdT overhangs (Guangzhou RiboBio, China). The efficiency of siRNAs was assessed by real time RT-PCR. Only the ones with an efficiency higher than 75% were used in the subsequent screening experiments. The efficiency of siRNA treatment has also been validated by Western blots (Supplementary Fig. S1).

2.2 Cell culture, transfection, and treatment

The HeLa and A549 cells (ATCC) were maintained in the recommended medium supplemented with 10% fetal bovine serum (FBS; HyClone). Cells were grown at 37°C in a humidified 5% CO_2 air incubator and subcultured twice a week. Transfections were performed routinely using Lipofectamine 2000 (Invitrogen). Forty eight hours post-transfection, the cells were starved overnight, followed by exposed to or not to 10 ng/mL TNFα (Sigma) for an indicated time before harvesting.

2.3 Luciferase reporter gene assay

The luciferase reporter gene assay was performed using a dual luciferase reporter assay system. Briefly, cells grown in 96-well plate were co-transfected with control siRNA or PPs siRNAs at a final concentration of 50 nmol/L, and 0.2 μg NF-κB reporter gene (pNF-κB-Luc) as well as 0.02 μg *Renilla* luciferase expression plasmid pRL-TK (control for transfection efficiency) in each well. Before harvesting, cells were exposed to TNFα (10 ng/mL) for 4 h, and subjected to luciferase activity assay according to the manufacturer's instruction (Promega). Luciferase activity was normalized to *Renilla* luciferase activity.

2.4 NF-κB/p65 nuclear translocation assay

Cells were grown in 96-well plate and transfected with the control siRNA or the candidate PPs siRNAs. The cells were exposed to or not to TNFα (10 ng/mL) for an indicated time prior to harvesting, and then fixed in 4% (w/v) paraformaldehyde in Phosphate Buffered Saline (PBS) for 30 min at room temperature, followed by penetration with 0.5% Triton X-100 in PBS for 10 min. The nuclear-cytoplasmic distribution of NF-κB/p65 was probed with mouse anti-p65 antibody (Santa Cruz) and FITC-labeled goat anti-mouse IgG antibody, respectively diluted 1∶100 in 1% bovine serum albumin (BSA) for 2 h-incubation at room temperature. The nuclei were visualized by Hoechst 33258 staining. The image was captured and analyzed by BD pathway system (BD Pathway Bioimager 855). The p65 nuclear translocation application and data management were described in Fig. S2 in the supplemental data.

2.5 Western blot

Cells grown in 60 mm dishes were transfected with the control siRNA, the candidate PPs siRNAs, or the candidate kinase siRNAs, followed by treatment with TNFα (10 ng/mL) for an indicated time. The cells were washed with cold PBS and harvested by RIPA lysis buffer (Beyotime Institute of Biotechnology, China). Otherwise, the nuclear and cytoplasmic protein was respectively extracted by a nuclear-cytosol extraction kit according to the manufacturer's instruction (Beyotime Institute of Biotechnology, China). Briefly, 0.2 mL cytosol extraction buffer (containing protease inhibitors) was added to the harvested cells followed by vortexing vigorously on the highest setting for 15 s and incubation on ice for 10 min. The supernatant after centrifugation was the cytoplasmic extract. Then 0.1 mL nuclear extraction buffer was used to resuspend and lyse the pellet (containing nuclei). The protein concentrations of lysates were determined using a BCA kit and equal amounts (100 μg) of protein were separated by 12% SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes (PVDF; BIO-RAD) and

blocked 1 h at room temperature with 8% BSA in TBS. Thereafter, the antibodies for p65 (Santa Cruz), phospho-Ser276 p65 (Cell signal), IκBα (Cell signal), phospho-IκBα (Cell signal), and β-actin (Santa Cruz) diluted in 1% BSA were added, followed by a 2-h incubation at room temperature. Then horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse IgG antibodies (Santa Cruz) were added and incubated for a further 2 h at room temperature. The target protein bands were visualized using an enhanced chemiluminescence detection system (Pierce). Relative band densities were quantified by BD pathway software.

2.6 Quantitative RT-PCR

Cells grown in 60 mm dishes were transfected with the control siRNA, the RelA/p65 siRNA, or the candidate PPs siRNAs, and then incubated with TNFα (10 ng/mL) for 24 h. An amount of 2 μg total RNAs extracted by Trizol reagent was reverse transcribed using M-MLV reverse transcriptase (Promega). Aliquots (10%) of the oligo dT-primed cDNAs, a volume of 8 μL of 2.5 × RealMasterMix/SYBR solution (Beijing TianGen Biotech, China), and 1 μL of 10 μmol/L forward and reverse primers, were mixed and subjected to qPCR amplification for 40 cycles of incubation at 95°C for 10 s, 60°C for 20 s, and 70°C for 10 s. The mRNA levels of NF-κB target gene cyclooxygenase-2 (COX-2) in the samples were normalized against β-actin. The primers were designed as follows: COX-2: 5′-CAGGGTTGCTGGTGGTAGGA-3′ and 5′-GCATAAAGCGTTTGCGGTAC-3′; β-actin: 5′-CGTACCACTGGCATCGTGAT-3′ and 5′-GTGTTGGCGTACAGGTCTTTG-3′.

2.7 Data analysis

All experiments were conducted at least three times. Groups were compared by analysis of variance, and statistical difference between two separate groups was determined by Student's *t*-test (unpaired, two-tailed) as calculated using SigmaPlot for Windows software. A value of $P < 0.05$ was considered statistically significant.

3 Results

3.1 PHLPP activates the NF-κB transcriptional activity

We used the NF-κB luciferase reporter gene, located in the plasmid pNF-κB-luc in which the luciferase transcription is under the control of NF-κB activation, to identify the candidate PPs that involved in regulation of NF-κB signaling in HeLa cells. Thirty-four PPs, including 16 PP2C family members, were chosen in primary RNAi screen. The siRNAs resulting in two standard deviations (SD) away from the mean increase or reduction in NF-κB activity compared with the control siRNA (Notarget) were considered as potential hits (Fig. 1A). Two SD approximately equaled a 70% change in the reporter gene activity. According to the criteria, five candidate phosphatase genes were identified (Fig. 1A). These siRNAs were further tested by NF-κB reporter assay in A549 cells. As shown in Fig. 1B, knock-down of the five PPs led to similar results. We therefore identified two NF-κB-activating phosphatases and three NF-κB-suppressing phosphatases (Table 1).

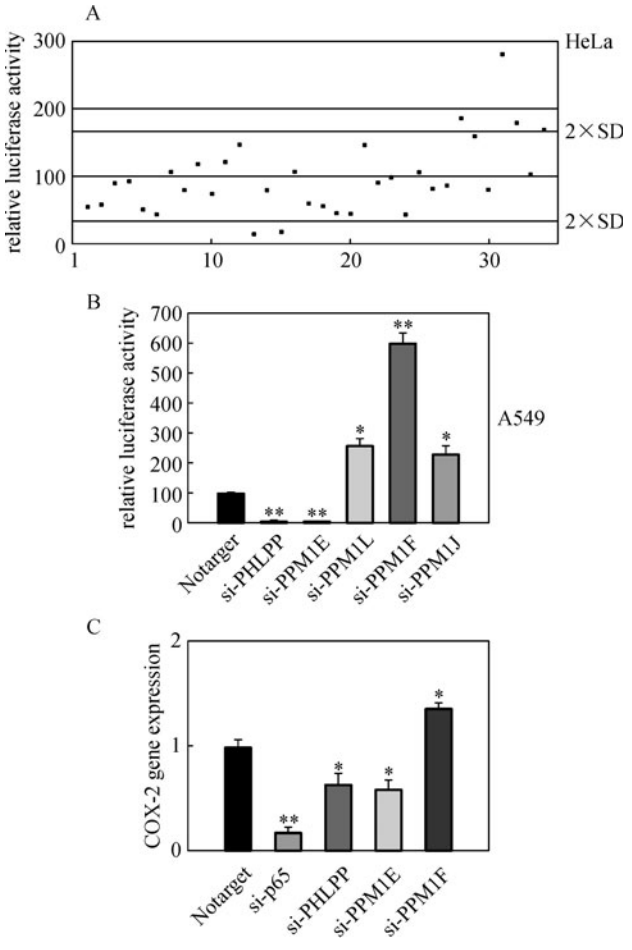

Fig. 1 Effects of protein serine/threonine phosphatases (partial) siRNAs on NF-κB transcriptional activities. A: The NF-κB transcriptional activity in HeLa cells following TNFα stimulation was determined by the luciferase reporter gene assay as described in **Materials and methods** section. The data were normalized to the percentage of the no targeting siRNA control (Notarget), and the means of three independent experiments were presented. B: The NF-κB reporter assay in A549 cells transfected with positive candidates in primary screen was measured. A value of $P < 0.05$, compared with Notarget, was considered significant. C: The transcriptional activity of NF-κB was determined by qRT-PCR detection of the expression of a well-known NF-κB target gene COX-2 in HeLa cells stimulated by TNFα. The NF-κB/p65 siRNA was used as positive control. And the efficiency of corresponding siRNAs was shown in Fig. S1 in the supplemental data. The results were normalized against the control, and presented as mean±SE of three experiments. **: $P < 0.001$; *: $P < 0.05$.

Table 1 Identification of phosphatase genes regulating NF-κB activity in HeLa cells

number of the corresponding siRNAs	GenBank accession number	symbol	name	RNAi reporter activity (% control)
NF-κB-activating phosphatases				
13	NM_194449	PHLPP	PH domain leucine-rich repeat protein phosphatase	7.2
15	NM_014906	PPM1E	protein phosphatase 1E, POPX1	9.1
NF-κB-suppressing phosphatases				
28	NM_139245	PPM1L	protein phosphatase 2C epsilon	193.3
31	NM_014634	PPM1F	protein phosphatase 1F, POPX2	291.8
32	NM_005167	PPM1J	protein phosphatase 2C zeta	189.9

Among these five phosphatase genes, PPM1L and PPM1J (also named as PP2Cζ) have been previously identified to suppress NF-κB transcriptional activity in mouse astrocytes, which is consistent with our results (Li et al., 2006). And the others were novel PPs identified to modulate NF-κB activity. To confirm the results, we performed real-time quantitative RT-PCR (qRT-PCR) to detect the expression of an NF-κB targeted gene COX-2 in HeLa cells following stimulation by TNFα. It shows that the change of COX-2 expression by PHLPP, PPM1E and PPM1F siRNAs was similar to that of the reporter luciferase expression (Fig. 1C). PHLPP was chosen for further investigation in the present report because it was lately indicated as a potential tumor suppressor (Liu et al., 2009).

3.2 PHLPP inhibits the TNFα-induced p65 nuclear translocation

The activity of the transcription factor NF-κB is regulated mainly through translocation of NF-κB/p65 to the nucleus. We next investigated the nuclear translocation of p65 in HeLa cells upon TNFα stimulation in the presence of the control siRNA or the p65-targted siRNA. Translocation of NF-κB/p65 was measured by quantification of the nuclear-cytoplasmic distribution of NF-κB/p65 in each cell using a BD Pathway Bioimager. The BD Pathway Bioimager defines the nucleus of each cell based on Hoechst 33258 staining, and then measures the amount of fluorescent signal (FITC) reflecting NF-κB/p65 level in nuclear and cytoplasmic areas. This measurement allows the calculation of the nuclear fraction of the labeled p65 in each cell (see Fig. S2 in the supplemental data).

Figure 2A shows that NF-κB/p65 translocation into nucleus occurred 10 min after TNFα stimulation, peaking at 30 min, and then gradually decreased. Unexpectedly, the nuclear translocation of p65 was significantly ($P < 0.05$) enhanced by PHLPP siRNA treatment, even at the time point of TNFα treatment (Fig. 2A). Western blot analysis of the p65 levels in the cytoplasmic and nuclear extracts further demonstrated that PHLPP siRNA-treatment enhanced the NF-κB/p65 translocation, which might not depend on TNFα-stimulation (Fig. 2B); the enhancement at 0.5 and 2 h of the TNFα stimulation, respectively, was 136.71% ($P < 0.05$) and 149.86% ($P < 0.05$), compared with that of the control siRNA treatment.

These results strongly suggest that PHLPP does not activate NF-κB/p65 transcription through the canonical NF-κB signaling pathway. Instead, it plays a negative role in regulating the nuclear localization of NF-κB/p65.

3.3 IκBα degradation may be uncoupled from its phosphorylation when PHLPP expression is silenced

The intracellular localization of NF-κB is controlled by the family of IκB proteins. Upon activation, IκBα is phosphorylated by the IKK complex, leading to its rapid degradation. To further clarify the negative regulation of the nuclear localization of NF-κB/p65 by PHLPP, we assessed the total and phosphorylated level of IκBα protein upon TNF-α-stimulation using specific antibodies. Consistent with the active role of IκBα in regulating NF-κB/p65 localization (Fig. 2A), Western blot analysis of different forms of IκBα protein revealed a dramatic increase of the phosphorylated IκBα protein 10 min after TNFα-stimulation. Concomitantly, a drastic decrease of the total IκBα level was observed (Fig. 3). PHLPP siRNA treatment did not alter either the phosphorylated or the total level of IκBα protein at this point (Fig. 3). A prolonged stimulation (30 min) continuously elevated the phosphorylated IκBα level, while the total level of IκB started to return, probably due to an attenuation of the polyubiquitination of the phosphorylated IκB (Hayden and Ghosh, 2008). Interestingly, PHLPP siRNA treatment significantly reduced the total IκBα level, indicating that PHLPP might involve in the attenuation of IκB polyubiquitination (Fig. 3). The low level of total IκB in cells treated by PHLPP siRNA is consistent with the increased nuclear localization of NF-κB/p65 shown in Fig. 2. However, the level of the phosphorylated IκBα at this time point was decreased by PHLPP siRNA treatment as well, indicating that PHLPP could positively regulate IκBα phosphorylation through a yet unidentified target (Fig. 3). Nevertheless, the positive regulation of both the total and phosphorylated forms of IκBα protein level by PHLPP indicates that IκBα degradation might be uncoupled from its phosphorylation when PHLPP level is reduced.

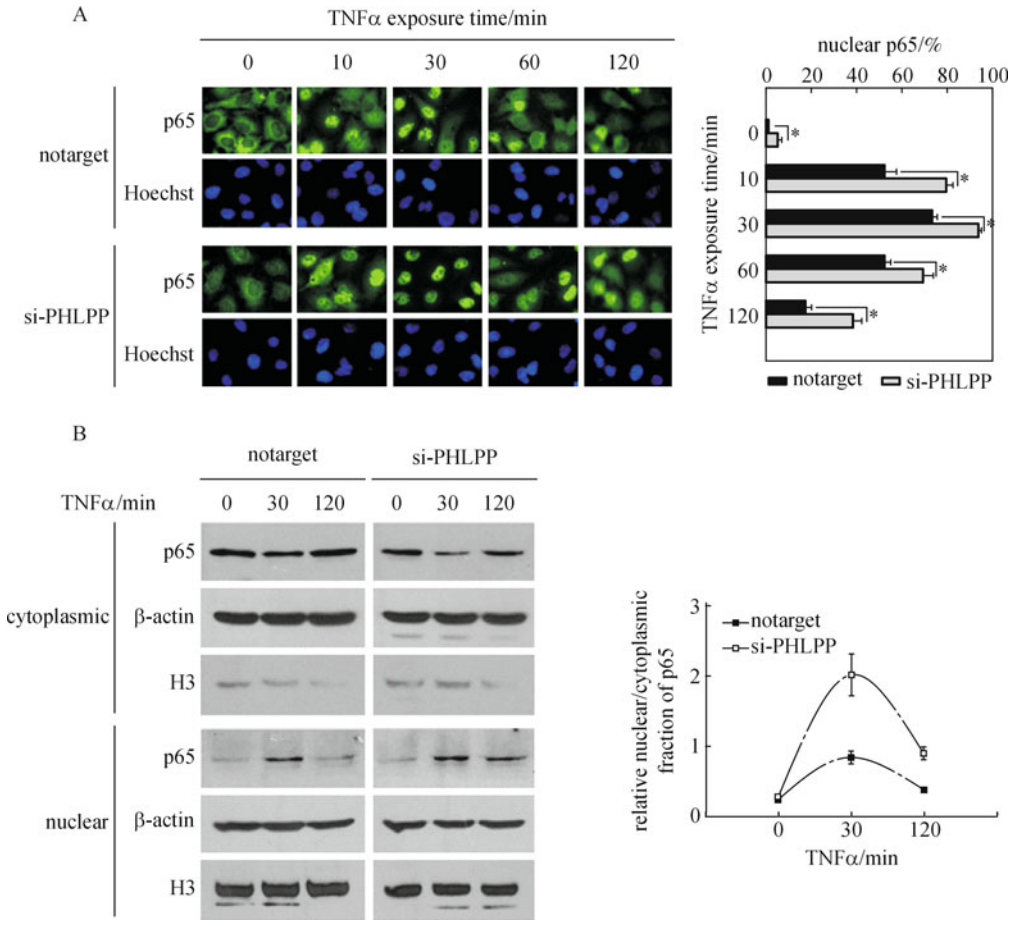

Fig. 2 Effects of PHLPP siRNA on nuclear translocation of p65. The distribution of p65 in HeLa cells transfected with control siRNA or candidate siRNAs for 48 h, followed by treatment with or without TNFα for an indicated time, was determined by p65 nuclear translocation assay (A) and by Western blot analysis of nuclear-cytoplasmic fractions of p65 (B) as described in **Materials and methods** section. Histone H3 (H3) was used as a marker of the nuclear extract. The data were shown as mean±SE of three experiments. *: $P < 0.05$.

3.4 PHLPP may target phosphatidylinositol 3-kinase/Akt and PKC signaling pathways to activate NF-κB/P65 transcription

In addition to nuclear translocation, post-translational modifications, particularly phosphorylation of NF-κB proteins at Ser276, Ser311 or Ser536 residue, are critical for the optimal activation of NF-κB target genes (Viatour et al., 2005; Hayden and Ghosh, 2008). A number of signaling pathways can respond to the cell stimulation by TNFα, a pro-inflammatory cytokine, and activate NF-κB transcriptional activity. The canonical IKK-IκB-NF-κB pathway involves the phosphorylation and degradation of IκB, and therefore the shift of the dynamic balance of NF-κB localization between cytosol and nucleus. The noncanonical pathways, conversely, mainly regulate NF-κB activity by a cascade of phosphorylation events, which eventually phosphorylates NF-κB proteins (Viatour et al., 2005).

We therefore asked whether PHLPP could control NF-kB/p65 transcriptional activity through regulating the

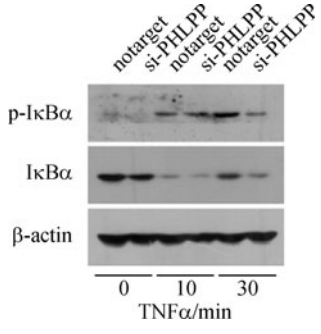

Fig. 3 Effects of PHLPP siRNA on cellular IκBα content and phosphorylation of IκBα. HeLa cells were transfected with the control siRNA and PHLPP siRNA for 48 h, followed by treatment with or without TNFα for an indicated time. Phosphorylation of IκBα and total IκBα were respectively detected by Western blot as described in **Materials and methods** section.

phosphorylation of the critical serine residue 276. Strikingly, we found that TNFα-induced phosphorylation of NF-kB/p65 at Ser276 was significantly ($P < 0.05$) blocked

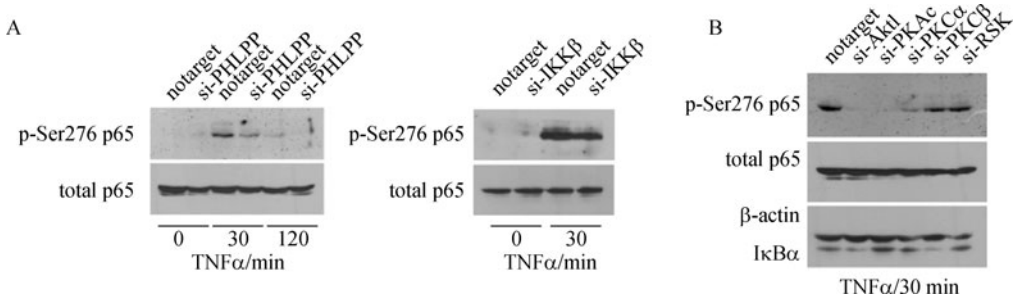

Fig. 4 Effects of PHLPP and candidate kinases siRNAs on p65 phosphorylation and cellular IκBα. A: Effects of PHLPP and IKKβ on TNFα-induced phosphorylation of p65 Ser276. The efficiency of IKKβ siRNA was shown in Fig. S1 in the supplemental data. B: Effects of candidate kinases siRNAs on phosphorylation of p65 Ser276 and cellular IκBα exposed to TNFα.

by PHLPP siRNA treatment, indicating that PHLPP positively, yet indirectly, regulates the NF-kB/p65 phosphorylation (Fig. 4A). IKKβ has never been shown to phosphorylate Ser276 of NF-κB/p65, although it phosphorylates Ser536 (Sakurai et al., 1999). Consistently, we found that siRNA silencing of IKKβ expression (Supplementary Fig. S1) did not alter the phosphorylation of NF-κB/p65 at Ser276 (Fig. 4A), confirming that the decreased phosphorylation of Ser276 by PHLPP siRNA treatment is gene-specific. Positive regulation of NF-kB/p65 phosphorylation by PHLPP might provide a mechanism for its activation of NF-kB/p65 transcriptional activity.

Protein kinase A (PKAc) phosphorylates NF-κB/p65 at Ser276, which likely promotes the p65 transcriptional activity by enhancing its DNA binding activity and provides an additional interaction site for the transcriptional coactivator CBP/p300 (Zhong et al., 1998; 2002). TNFα also triggers mitogen- and stress-activated protein kinase-1 (MSK1)-mediated phosphorylation of p65 at Ser276 in the nucleus via an ERK-dependent pathway (Vermeulen et al., 2003). In response to DNA damaging stimuli, p53 induces NF-κB activation by a mechanism involving phosphorylation of p65 at Ser536 by ribosomal s6 kinase (RSK) (Bohuslav et al., 2004). Akt1, a serine-threonine protein kinase mediating the apoptosis suppression by survival factors, has been shown to be a direct target of PHLPP (Gao et al., 2005; Brognard et al., 2007). TNFα treatment activates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, which phosphorylates p65 through a p38- and IKK-dependent mechanism (Viatour et al., 2005). Akt1 phosphorylates Ser536 and stimulates the transactivation potential of p65 (Madrid et al., 2000; 2001).

We then asked whether PHLPP could positively regulate NF-κB/p65 phosphorylation of Ser276 through Akt1 upon TNFα-stimulation. In this assay, PKAc and RSK were used as the positive and negative controls, respectively. Two members in protein kinase C family were also included, because PHLPP has been recently shown to control the amplitude and duration of both Akt and PKC signaling by its phosphatase activity (Brognard and Newton, 2008). Expression of each of these kinases was specifically silenced by the corresponding siRNA. As shown in Fig. 4B, phosphorylation of Ser276 was significantly blocked ($P < 0.05$) by knock-down of Akt1, PKAc and PKCα, suggesting that both Akt1 and PKCα are required for phosphorylating NF-κB/p65 Ser276. Taken together, our results indicate that PHLPP may activate the TNFα-stimulated NF-κB/p65 transcriptional activity by noncanonical pathways involving Akt and PKC signaling pathways. The detailed mechanisms remain to be further studied.

4 Discussion

The activation of NF-κB is strongly associated with tumorigenesis, progression, and resistance to therapy (Lee et al., 2007). Inhibition of NF-κB signaling thus offers a potential cancer therapy strategy. IKK inhibitors have been shown to exert significant anti-tumor effects in a broad spectrum of cancer cells (Lee et al., 2007). The use of an IκB super-repressor molecule, which blocks IκB degradation and thus inhibits NF-κB nuclear translocation, reduces the resistance of tumors to chemotherapy in a mouse model (Sun and Andersson, 2002). The present preclinical and clinical applications, however, have limitations due to lack of specificity and inhibition of other signaling systems (Yamamoto and Gaynor, 2001; Lee et al., 2007). The RNAi-based gene therapy is assumed to provide a more effective and specific strategy for inhibition of NF-κB signaling.

The siRNA-based screen in this study has revealed that two out of 34 candidate PPs siRNAs block TNFα-induced NF-κB activities in HeLa cells. Knock-down of PHLPP led to enhancement of NF-κB/p65 nuclear import and retention and lowered cellular IκBα content, indicating that PHLPP could not activate TNFα-induced NF-κB activity through the canonical signaling pathway. Importantly, this report shows that silencing PHLPP expression reduces TNFα-induced phosphorylation of Ser276 on p65. These results are consistent with the previous report, which demonstrates that reduction of phosphorylation at Ser276

by mutation leads to nuclear retention of NF-κB/p65 (Hochrainer et al., 2007).

Ser276 within the RHD domain (DNA binding and dimerization domain) of NF-κB/p65 subunit is a major phosphorylation site, and its phosphorylation is essential for p65-dependent NF-κB activity (Okazaki et al., 2003). It has been demonstrated that Ser276 is subjected to phosphorylation by PKAc (Zhong et al., 1998), as well as by MSK1 (Vermeulen et al., 2003). This report shows that TNFα-induced phosphorylation of Ser276 is also impaired by knock-down of PHLPP, Akt1 and PKCα, suggesting that PHLPP-regulated Akt1 and PKC signaling may play critical roles in regulating phosphorylation of this site, correlating with the distinct role of PHLPP in activating TNFα-induced NF-κB/p65 activity.

The serine/threonine kinase PKB/Akt has recently gained special attention. It acts as a mediator of many functions including nutrient metabolism, cell survival and proliferation initiated by PI3K in response to external signals (Song et al., 2005). For its inactivation, several PPs are identified to be associated with Akt. PHLPP specifically dephosphorylates Akt1 at Ser473 (Gao et al., 2005), PP2A dephosphorylates it at Thr308 (Kuo et al., 2008), and PP1 is a major phosphatase that directly dephosphorylates Akt at Thr450 to modulate its activation (Xiao et al., 2010). Akt has long been shown to activate the transcription factor NF-κB to provide cell survival functions, but the mechanism whereby Akt activates NF-κB remains controversial. Some evidences support induction of nuclear translocation of NF-κB via activation of IκB kinase activity, while others suggest the stimulation of the transcriptional function of NF-κB rather than inducing NF-κB nuclear translocation via IκB degradation (Madrid et al., 2000, 2001). Data presented in this report reveal that Akt1 may activate NF-κB/p65 transcription through enforcing TNFα-induced phosphorylation of Ser276 on p65 in HeLa cells.

PHLPP belongs to the PP2C/PPM family, which is distinct from other PP families including PP1, PP2A and PP2B because it acts as a monomer and requires Mg^{2+} or Mn^{2+} for full activity (Mumby and Walter, 1993). PHLPP is a potential tumor suppressor in colon cancer (Liu et al., 2009), and has recently been demonstrated to terminate Akt signaling by dephosphorylating the hydrophobic motif on Akt (Gao et al., 2005; Brognard et al., 2007). PHLPP has also been found to control the cellular levels of PKC by specifically dephosphorylating the hydrophobic motif on the enzyme (Gao et al., 2008). As both Akt and PKC are strongly implicated in tumorigenesis, PHLPP is believed to play key roles in this process (Brognard and Newton, 2008).

Based on the finding that sustained activation of PKC represses NF-κB signaling (Park et al., 2007) and the concordant regulation of the Ser276 phosphorylation on NF-κB/p65 subunit by PHLPP in this report, we propose a model in which PHLPP dephosphorylation of Akt1 and PKCα allows these kinases to promote NF-κB signaling through phosphorylation of NF-κB/p65 at Ser276. More experimental efforts are called to test this model and purse the mechanism and biological function of the engagement of PHLPP-PKC-Akt loop in NF-κB/p65 signaling.

Acknowledgements This research was supported by the National High Technology Research and Development Program of China (863 Program) (No.2006AA02Z191), the Bureau of Science and Technology of Guangzhou, China (No. 2007Z1-E4041) and Guangzhou Economic & Technological Development District (GETDD S & T Project) (2007G-P029).

References

Bohuslav J, Chen L F, Kwon H, Mu Y, Greene W C (2004). p53 induces NF-kappaB activation by an IkappaB kinase-independent mechanism involving phosphorylation of p65 by ribosomal S6 kinase 1. J Biol Chem, 279(25): 26115–26125

Brognard J, Newton A C (2008). PHLiPPing the switch on Akt and protein kinase C signaling. Trends Endocrinol Metab, 19(6): 223–230

Brognard J, Sierecki E, Gao T, Newton A C (2007). PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. Mol Cell, 25(6): 917–931

Gao T, Furnari F, Newton A C (2005). PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol Cell, 18(1): 13–24

Gao T, Brognard J, Newton A C (2008). The phosphatase PHLPP controls the cellular levels of protein kinase C. J Biol Chem, 283(10): 6300–6311

Hayden M S, Ghosh S (2004). Signaling to NF-kappaB. Genes Dev, 18 (18): 2195–2224

Hayden M S, Ghosh S (2008). Shared principles in NF-kappaB signaling. Cell, 132(3): 344–362

Henmi T, Amano K, Nagaura Y, Matsumoto K, Echigo S, Tamura S, Kobayashi T (2009). A mechanism for the suppression of interleukin-1-induced nuclear factor kappaB activation by protein phosphatase 2Ceta-2. Biochem J, 423(1): 71–78

Hochrainer K, Racchumi G, Anrather J (2007). Hypo-phosphorylation leads to nuclear retention of NF-kappaB p65 due to impaired IkappaBalpha gene synthesis. FEBS Lett, 581(28): 5493–5499

Kuo Y C, Huang K Y, Yang C H, Yang Y S, Lee W Y, Chiang C W (2008). Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. J Biol Chem, 283(4): 1882–1892

Lee C H, Jeon Y T, Kim S H, Song Y S (2007). NF-kappaB as a potential molecular target for cancer therapy. Biofactors, 29(1): 19–35

Li H, Lin X (2008). Positive and negative signaling components involved in TNFalpha-induced NF-kappaB activation. Cytokine, 41 (1): 1–8

Li S, Wang L, Berman M A, Zhang Y, Dorf M E (2006). RNAi screen in mouse astrocytes identifies phosphatases that regulate NF-kappaB signaling. Mol Cell, 24(4): 497–509

Lin X, Duan X, Liang Y Y, Su Y, Wrighton K H, Long J, Hu M, Davis C

M, Wang J, Brunicardi F C, Shi Y, Chen Y G, Meng A, Feng X H (2006). PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. Cell, 125(5): 915–928

Liu J, Weiss H L, Rychahou P, Jackson L N, Evers B M, Gao T (2009). Loss of PHLPP expression in colon cancer: role in proliferation and tumorigenesis. Oncogene, 28(7): 994–1004

Lu G, Wang Y (2008). Functional diversity of mammalian type 2C protein phosphatase isoforms: new tales from an old family. Clin Exp Pharmacol Physiol, 35(2): 107–112

Madrid L V, Wang C Y, Guttridge D C, Schottelius A J, Baldwin A S Jr, Mayo M W (2000). Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. Mol Cell Biol, 20(5): 1626–1638

Madrid L V, Mayo M W, Reuther J Y, Baldwin A S Jr (2001). Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J Biol Chem, 276(22): 18934–18940

Mumby M C, Walter G (1993). Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. Physiol Rev, 73 (4): 673–699

Okazaki T, Sakon S, Sasazuki T, Sakurai H, Doi T, Yagita H, Okumura K, Nakano H (2003). Phosphorylation of serine 276 is essential for p65 NF-kappaB subunit-dependent cellular responses. Biochem Biophys Res Commun, 300(4): 807–812

Park K A, Byun H S, Won M, Yang K J, Shin S, Piao L, Kim J M, Yoon W H, Junn E, Park J, Seok J H, Hur G M (2007). Sustained activation of protein kinase C downregulates nuclear factor-kappaB signaling by dissociation of IKK-gamma and Hsp90 complex in human colonic epithelial cells. Carcinogenesis, 28(1): 71–80

Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W (1999). IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. J Biol Chem, 274(43): 30353–30356

Song G, Ouyang G, Bao S (2005). The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med, 9(1): 59–71

Sun Z, Andersson R (2002). NF-kappaB activation and inhibition: a review. Shock, 18(2): 99–106

Sun W, Yu Y, Dotti G, Shen T, Tan X, Savoldo B, Pass A K, Chu M, Zhang D, Lu X, Fu S, Lin X, Yang J (2009). PPM1A and PPM1B act as IKKbeta phosphatases to terminate TNFalpha-induced IKKbeta-NF-kappaB activation. Cell Signal, 21(1): 95–102

Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, Haegeman G (2003). Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). EMBO J, 22(6): 1313–1324

Viatour P, Merville M P, Bours V, Chariot A (2005). Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem Sci, 30(1): 43–52

Xiao L, Gong L L, Yuan D, Deng M, Zeng X M, Chen L L, Zhang L, Yan Q, Liu J P, Hu X H, Sun S M, Liu J, Ma H L, Zheng C B, Fu H, Chen P C, Zhao J Q, Xie S S, Zou L J, Xiao Y M, Liu W B, Zhang J, Liu Y, Li D W (2010). Protein phosphatase-1 regulates Akt1 signal transduction pathway to control gene expression, cell survival and differentiation. [Epub ahead of print] Cell Death Differ. DOI?

Yamamoto Y, Gaynor R B (2001). Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest, 107(2): 135–142

Zhong H, Voll R E, Ghosh S (1998). Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell, 1(5): 661–671

Zhong H, May M J, Jimi E, Ghosh S (2002). The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol Cell, 9(3): 625–636

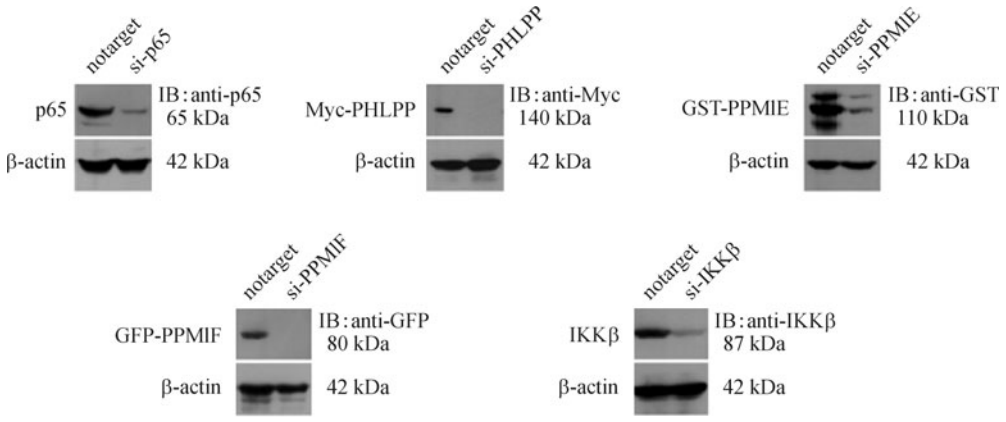

Fig. S1 The efficiency of siRNAs used in corresponding experiments. The efficiency of si-p65 and si-IKKβ were assessed by Western blot detection of the endogenous expressions of p65 and IKKβ in HeLa cells, respectively. And the efficiency of PHLPP, PPM1E and PPM1F siRNAs were determined by knock-down of the expressions of exogenous plasmids pcDNA-Myc-PHLPP, pReceiver-GST-PPM1E (Guangzhou FulenGen, China) and pcr-GFP-PPM1F, respectively, in HeLa cells. The antibody used and the approximate molecular weight of target protein are shown.

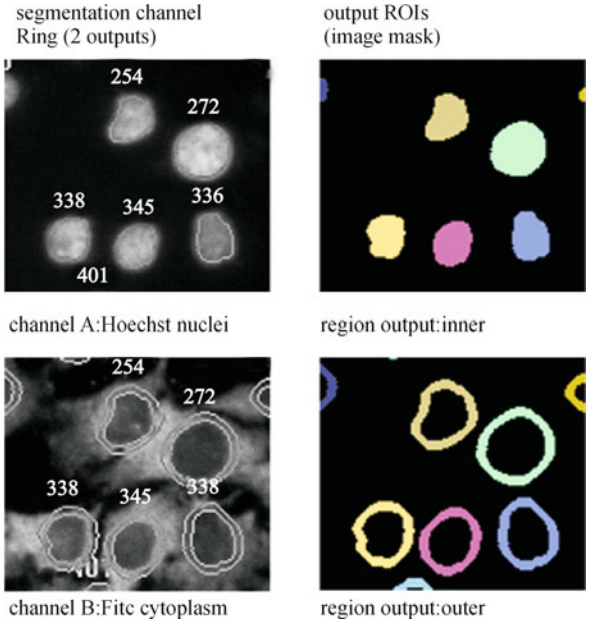

Fig. S2 Illustration of p65 nuclear translocation assay. As described in BD pathway system user's manual, the nuclear translocation assay identifies the translocation of p65 protein from the cytoplasm into the nucleus. It defines the nucleus and cytoplasm, measures the amount of fluorescent signal (FITC) in each, and then quantifies the proportion of labeled p65 in each cell compartment as a measure of cellular response. Image mask represents the measured region of the nucleus and the cytoplasm, respectively. The ratio of nuclear/cytoplasmic intensity of fluorescent signal $>$ a cutoff value (two SD above mean of no TNFα treated cells) was categorized as positive nuclear translocation. The percentage of positive nuclear translocation cells accounting for the total cells in each well was determined by BD image data explorer (BD Biosciences, Version 2.2.15).