PKCθ-Mediated PDK1 Phosphorylation Enhances T Cell Activation by Increasing PDK1 Stability

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PDK1 is essential for T cell receptor (TCR)-mediated activation of NF-κB, and PDK1-induced phosphorylation of PKCθ is important for TCR-induced NF-κB activation. However, inverse regulation of PDK1 by PKCθ during T cell activation has not been investigated. In this study, we found that PKCθ is involved in human PDK1 phosphorylation and that its kinase activity is crucial for human PDK1 phosphorylation. Mass spectrometry analysis of wild-type PKCθ or of kinase-inactive form of PKCθ revealed that PKCθ induced phosphorylation of human PDK1 at Ser-64. This PKCθ-induced PDK1 phosphorylation positively regulated T cell activation and TCR-induced NF-κB activation. Moreover, phosphorylation of human PDK1 at Ser-64 increased the stability of human PDK1 protein. These results suggest that Ser-64 is an important phosphorylation site that is part of a positive feedback loop for human PDK1-PKCθ-mediated T cell activation.

Keywords: PDK1, phosphorylation, PKCθ, T cell

INTRODUCTION

Phosphoinositide-dependent protein kinase-1 (PDK1) is a master regulator of the AGC kinase subfamily of protein kinases that includes protein kinase B (PKB), p70 ribosomal S6 kinase (p70S6K), serum and glucocorticoid-inducible kinase (SGK), and protein kinase C (PKC) (Balendran et al., 2000). Activation of AGC kinases by PDK1 regulates cell metabolism, growth, proliferation, survival through substrate phosphorylation(Mora et al., 2004). T cell receptor (TCR)-CD28 stimulation induces activation of PI3K and implements PDK1 recruitment to the plasma membrane (Park et al., 2009). CD28 ligation leads to PDK1 phosphorylation at Thr-513, which is important for binding PDK1 with PKCθ (Kang et al., 2013; Park et al., 2009). PDK1 then induces phosphorylation of PKCθ on Thr-538 and thereby promotes the recruitment of PKCθ to lipid rafts and induces activation of PKCθ (Villalba et al., 2000; 2002; Wang et al., 2012). A previous study provided evidence that PDK1 simultaneously recruits the CARMA1Bcl10-MALT1 (CBM) complex to lipid rafts by binding to CARMA1 and PKCθ, which is important for NF-κB activation (Lee et al., 2005).

PDK1 activity and functions are regulated by phosphorylation. Autophosphorylation of PDK1 at Ser-241 in the activation loop is essential for PDK1 activity (Casamayor et al., 1999; Kenchappa et al., 2004). In addition, phosphorylation of PDK1 at Ser-396 is important for the nuclear shuttling of PDK1 (Scheid et al., 2005), and phosphorylation of mouse PDK1 at Ser-163 is involved in fine-tuning PDK1 activity (Riojas et al., 2006). PDK1 phosphorylation by apoptosis signaling-regulating kinase 1 (ASK1) at Ser-394 and Ser-398 suppresses activity of PDK1 (Seong et al., 2010), and phosphorylation of mouse PDK1 by PKCθ at Ser-504 and Ser-532 negatively regulates PDK1 activity during palmitate-induced

insulin resistance (Wang et al., 2009).

In the present study, we investigated the role of PDK1 phosphorylation at Ser-64, which is mediated by PKCθ during T cell activation. Our data show that PDK1 undergoes phosphorylation by PKCθ in the NF-κB activation pathway. Expression of the phosphomimetic form of PDK1 at Ser-64 increased T cell activation and IL-2 expression, suggesting that PKCθ forms a positive feedback loop with PDK1 that leads to enhanced NF-κB activity during T cell activation.

MATERIALS AND METHODS

Cell culture and antibodies
HEK293 cells were maintained in DMEM supplemented with 5% FBS. Jurkat T cells were maintained in RPMI 1640 supplemented with 5% FBS. Anti-GAPDH antibodies were purchased from Santa Cruz. Anti-Myc antibody was purchased from Cell Signaling. HRP-conjugated anti-mouse IgG and anti-HA antibodies were purchased from Sigma. Anti-human CD3, anti-human CD28, PE-conjugated anti-CD69, and PerCP-conjugated anti-CD25 antibodies were purchased from eBiosciences.

Lentivirus transduction
Recombinant lentiviruses were produced in 293FT cells by transfection of the cells with pLenti vector, pLenti-PDK1-WT, pLenti-PDK1-S64D, or pLenti-PDK1-S64I. Lentiviruses were collected after 48 h, and Jurkat T cells were infected with the lentiviruses by spin inoculation. Cells were pretreated with Polybrene® for 45 min and the medium was removed. Cells were then incubated for 45 min with medium containing recombinant retroviruses and Polybrene® and centrifuged for 90 min at 2,250 rpm. After centrifugation, virus-containing medium was removed and cells were cultured in fresh medium. Blasticidin was used as a selection agent to isolate infected cells.

Flow cytometry
Jurkat cell lines expressing either PDK1 or the PDK1 S64 mutant were activated with anti-human CD3 (5 μg/ml) and anti-human CD28 (5 μg/ml) antibodies for the indicated times, then stained with PE-conjugated anti-CD69 and PerCP-conjugated anti-CD25 antibodies. The stained cells were analyzed on a Guava easyCyte HT (Millipore).

Enzyme-linked immunosorbent assay (ELISA)
Secreted IL-2 was analyzed by IL-2-specific ELISA. Cells were plated at 1×10^5 cells per well in 24-well plates coated with anti-human CD3 (5 μg/ml) and anti-human CD28 (5 μg/ml) and incubated at 37℃ in 5% CO_2. After 24 h, the culture medium was analyzed according to the manufacturer's protocol (eBioscience).

Co-immunoprecipitation
Myc-tagged PDK1 and the PDK1 S64 mutants and HA-tagged PKCθ were used for immunoprecipitation analysis. For the study of molecular interactions, expression vectors were transfected together into HEK293 cells using Lipofectamine 2000 (Invitrogen). After 40 h, target proteins were immunoprecipitated with anti-HA antibody. Samples were separated by SDS-PAGE and analyzed by immunoblotting with anti-Myc or anti-HA antibodies.

NF-κB reporter luciferase assay
HEK293T cells were plated at 5×10^5 cells per well in a 12-well plate and transfected with NF-κB reporter plasmid (pBIIx), pRenilla, and indicated plasmids using Lipofectamine 2000 (Invitrogen). Total DNA was normalized to that of empty pcDNA3 vector. After 48 h, cells were lysed in 1 × passive lysis buffer. Debris was removed by centrifugation at 14,000 rpm for 5 min at 4℃. Firefly and Renilla luciferase activities were measured in 20 μl lysate samples. The relative activity was calculated for each sample by dividing the Firefly luciferase activity in the sample (normalized to Renilla luciferase activity) by the activity of a sample containing only empty expression vector.

Immobilized metal affinity chromatography (IMAC) phosphopeptide enrichment
A fused-silica capillary (250 μm i.d. × 360 μm o.d., Polymicro Technologies) was packed with 3 cm of 5 μm 120 Å ReproSil Pur Aqua C18 (Phenomenex) using a high-pressure bomb for desalting purposes. The tryptic digest samples were loaded onto the column at a flow rate of 2 μl/min. The column was washed with 1 ml of 1% formic acid, and 120 μl of IMAC binding buffer (40% ACN, 0.1% formic acid) was used to elute peptides. PHOS-Select iron affinity gel (15 μl of 50% bead slurry) was incubated with the desalted peptides for 1 h at room temperature. After the beads were washed with 1 ml of IMAC binding buffer, bound phosphopeptides were eluted in 200 μl of IMAC elution buffer (200 mM $NH_4H_2PO_4$) and the resulting phosphopeptide samples were analyzed by micro RPLC-MS/MS. Micro RPLC-MS/MS analysis was carried out for each phosphopeptide-enriched sample to confirm the reproducibility of the label-free quantitative phosphorylation analysis.

Micro RPLC-MS/MS analysis
Micro RPLC-MS/MS analysis was performed using an Agilent 1100 series high performance liquid chromatography (HPLC) pump (Agilent Technologies) coupled to a linear ion trap mass spectrometer (LTQ, Thermo Finnigan, USA) with an in-house manufactured nano-ESI interface. For micro RPLC-MS/MS analysis, samples were injected into a trap column (fused-silica capillary 250 μm i.d. × 360 μm o.d.; packed with 2 cm of Aqua C18) and separated with an analytical column (fused-silica capillary 100 μm i.d. × 360 μm o.d.; packed with 7.5 cm of Aqua C18). Buffer A (0.1% formic acid) and Buffer B (80% aceotonitrile, 0.1% formic acid) were used to elute bound peptides with a split flow system (flow rate: 250 nl/min) for 120 min linear gradient. In positive ion mode, spectra were acquired with cycles of one full MS scan in the LTQ (m/z 400-2,000) followed by ten data-dependent MS/MS scans with normalized collision energy of 35% and dynamic exclusion time of 30 s.

Data analysis
MS/MS spectra were searched against an in-house database containing various PDK1 homologues, GFP, IgG sequences, and bovine alpha casein sequences using SEQUEST algo-

rithm (Bioworks 3.2). Methionine oxidation and phosphorylation of serine, threonine, and tyrosine as variable modifications and carbamidomethylation of cysteine as a fixed modification were applied to the search. DTAselect (v.1.9) was used to filter the search results with the following criteria: fully tryptic digest ends, Xcorr > 1.8 for charge state 1+, Xcorr > 2.5 for charge state 2+, and Xcorr > 3.5 for charge state 3+. Assignments of the phosphopeptide sequences were further confirmed by manual validations on filtered MS/MS spectra. For the quantification of phosphorylation levels, selected ion chromatograms of identified phosphopeptides were constructed using the Xcalibur 2.1.0 SP1 program (Thermo Fisher Scientific), and integrated peak areas were calculated with a built-in feature of the Xcalibur program for comparison purposes. Relative quantification of each phosphopeptide was performed by comparing peak areas of no-activation conditions and those of two kinase-activation samples.

RESULTS

PKCθ kinase activity alters PDK1 mobility through phosphorylation

PI3K and PDK1 are important for TCR-CD28 stimulation-mediated T cell activation (Kang et al., 2013; Park et al., 2009). In our system, both inhibition of PDK1 by OSU03012 (Cho et al., 2010) and inhibition of PI3K by Ly294002 reduced T cell activation, as indicated by reduced surface expression of CD69 and CD25 on activated Jurkat T cells (Fig. 1A). Immunoblot analysis of cells stimulated with TCR-CD28 revealed a shift in the protein band of human PDK1 compared to non-stimulated cells, and our data showed that these mobility changes were mediated by phosphorylation (Fig. 1B). PDK1 plays an important role in PKCθ-mediated phosphorylation of NF-κB. When PDK1 is overexpressed, we found that it binds to PKCθ in HEK293 cells and decreases its mobility in SDS-PAGE (Fig. 1C). This decreased mobility was caused by phosphorylation of PDK1, because the addition of λ protein phosphatase prevented the decrease in mobility (Fig. 1D). Interestingly, the decreased mobility of PKCθ-bound PDK1 was dependent on PKCθ kinase activity, because kinase activity deficient PKCθ(K409W) construct did not induce a decrease in PDK1 mobility (Fig. 1D).

Ser-64, a novel phosphorylation site on PDK1, is phosphorylated by PKCθ

To identify the phosphorylation site on PDK1 that is phosphorylated by PKCθ, we analyzed the phosphorylation status of PKCθ-bound PDK1 by mass spectrometry-based phosphoproteomics techniques. However, if we used wild-type PDK1, we could not obtain enough PKCθ-bound PDK1 for mass spectrometry analysis. Actually, a previous report has
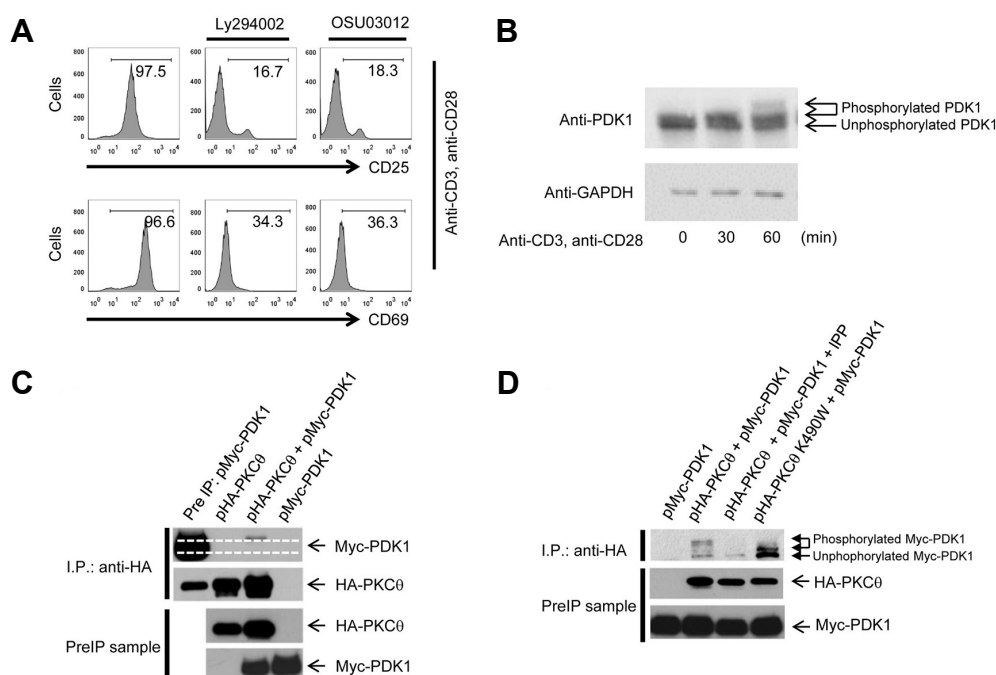

Fig. 1. PKCθ kinase activity alters PDK1 mobility via phosphorylation. (A) Jurkat T cells were treated with Ly294002 or OSU03012 and then stimulated with anti-CD3 and anti-CD28 antibodies. Surface expression of CD25 and CD69 on activated Jurkat T cells was analyzed by flow cytometry. (B) Jurkat T cells were stimulated with anti-CD3 and anti-CD28 antibodies, and PDK1 was analyzed by immunoblot analysis with anti-PDK1 antibody. (C) Binding of HA-tagged PKCθ with Myc-tagged PDK1 was analyzed by co-immunoprecipitation analysis. (D) Changes in the mobility of Myc-tagged PDK1 co-immunoprecipitated with HA-tagged PKCθ or HA-tagged PKCθ (K409W) were analyzed by immunoblot analysis with or without λ protein phosphatase treatment. Data are representative of three (A) or two (B-D) independent experiments.

shown that PDK1 phosphorylation at Thr-513 was induced during T cell receptor signaling by autophosphorylation and this phosphorylation increased binding of PDK1 to PKCθ. Thus, in this analysis, we used the PDK1(T513D) mutant instead of wild-type PDK1. This experimental condition enables more feasible analysis of PKCθ-induced PDK1 phosphorylations. In addition, prior to mass spectrometry analysis, phosphopeptides were selectively enriched by IMAC to increase the identification of low stoichiometry phosphopeptides (Fig. 2A). Previous studies showed that PDK1 can autophosphorylate at Ser-241, leading to its own activation. As expected, Ser-241 phosphorylation was detected in both

PKCθ-bound PDK1 and PKCθ(K409W)-bound PDK1 (Figs. 2B and 2C). In addition to the Ser-241 phosphorylation site, we identified a new phosphorylation site, Ser-64, which was phosphorylated in PKCθ-bound PDK1 but not in kinase activity deficient PKCθ(K409W)-bound PDK1 (Figs. 2B and 2C). Thus, phosphorylation of Ser-64 on PDK1 is dependent on PKCθ kinase activity.

Phosphorylation of Ser-64 on PDK1 enhanced TCR/CD28-mediated NF-κB activation

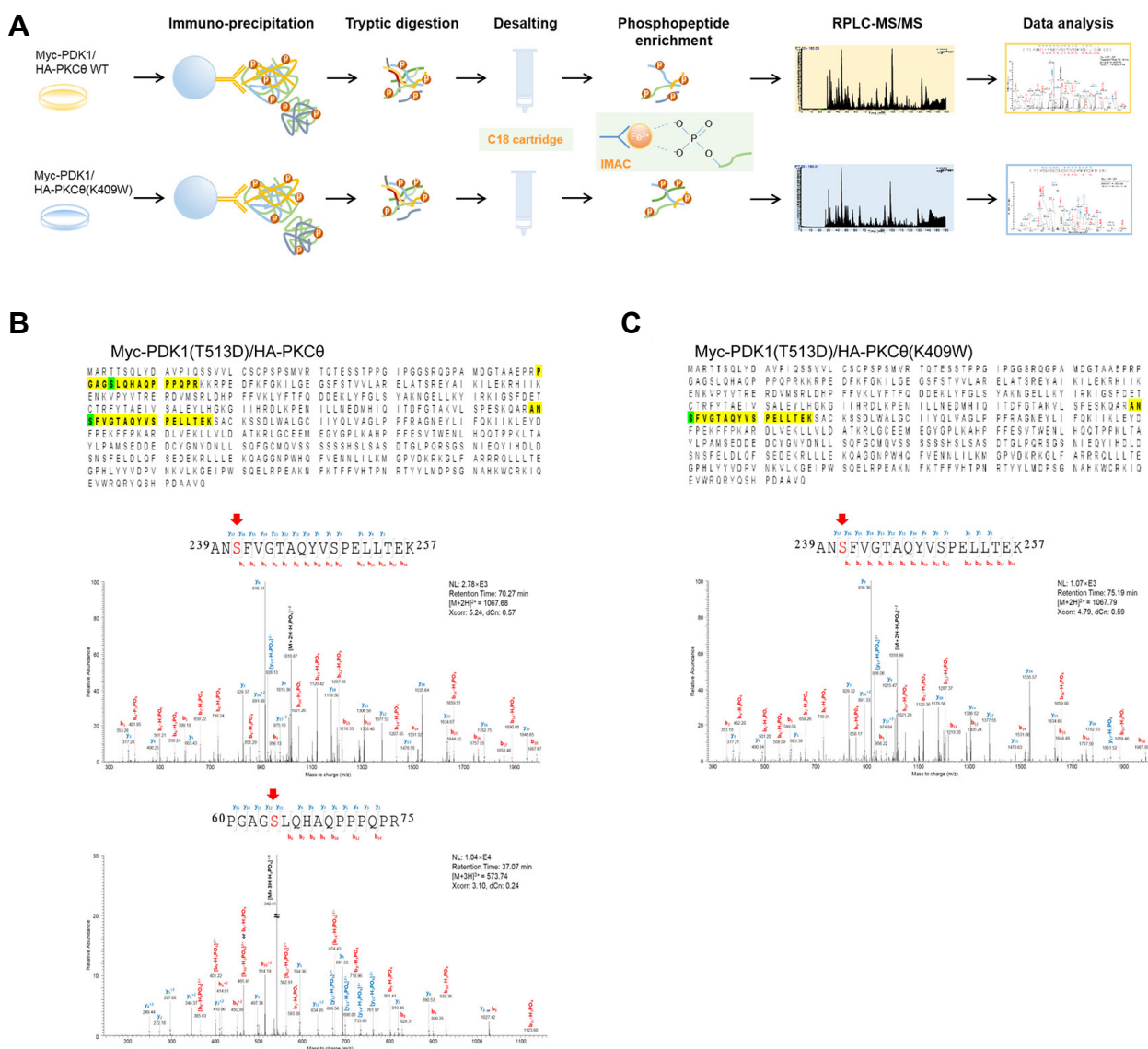PDK1 is crucial for TCR/CD28-mediated NF-κB activation. Thus, we determined whether the newly identified phos-

Fig. 2. Ser-64, a novel phosphorylation site on PDK1, is phosphorylated by PKCθ. (A) Experimental scheme of phosphopeptide-specific mass spectrometry analysis. (B) Results of mass spectrometry analysis of PDK1 with PKCθ. Arrows indicate phosphorylated serine. (C) Results of mass spectrometry analysis of PDK1 with kinase activity-deficient PKCθ(K409W). Data are representative of two (B, C) independent experiments.

phorylation site at PDK1 Ser-64 affects TCR/CD28-mediated NF-κB activation. To examine the effect of mutating the site to generate a phosphomimetic (S64D) or phosphorylation-deficient (S64I) mutant on NF-κB activation, we analyzed NF-κB reporter gene activity (to assess long-term effects, 12-24 h) or phospho-IκBα levels (to assess short-term effects, 0-30 min). During TCR/CD28-mediated NF-κB activation, PDK1, PKCθ, and the CBM complex are core components. Figure 3A shows that PDK1(S64D) enhanced PKCθ-CARMA1-Bcl10-MALT1-mediated NF-κB activity in HEK293 cells compared to PDK1(WT). Interestingly, PDK1(S64D) protein levels were higher than PDK1(WT) in HEK293 cells (Fig. 3B), but mRNA levels were not different from each other(Supplementary Fig. S1). In addition, we generated Myc-tagged PDK1(WT), PDK1(S64I), or PDK1(S64D) constructs and expressed them in Jurkat T cells using a recombinant lentiviral system. Interestingly, PDK1(S64D) protein levels in these cells were also higher than PDK1(WT) or PDK1(S64I) protein levels (Fig. 3C), but also mRNA levels were not different from each other(Supplementary Fig. S1). Moreover, TCR/CD28-mediated NF-κB activation was enhanced in PDK1(S64D)-expressing

Jurkat T cells compared to PDK1(WT)-expressing Jurkat T cells. NF-κB activity in Jurkat T cells expressing phosphorylation-deficient PDK1(S64I) was also decreased compared to the activity in PDK1(WT)-expressing Jurkat T cells (Fig. 3D). However, IκBα phosphorylation levels, which are an indicator of NF-κB activation, were different between cells expressing PDK1(S64D) and PDK1(WT), but not between cells expressing PDK1(WT) and PDK1(S64I) (Fig. 3E). Thus, our data indicate that changes in PDK1 protein levels induced by the Ser-64 phosphomimetic mutation possibly affect TCR/CD28-mediated NF-κB activation.

Ser-64 on PDK1 is important for protein stability

Our data showed that PDK1(S64D) protein levels were higher than those of PDK1(WT) in HEK293 cells and Jurkat T cells. Thus, we hypothesized that phosphorylation of PDK1 at Ser-64 affects the stability of PDK1 protein. To assess the stability of expressed PDK1, we monitored protein half-life by treating the cells with cycloheximide, which blocks protein synthesis. PDK1(S64D) expressed in HEK293 cells exhibited a significantly increased protein half-life (2.5 ± 0.1h) compared
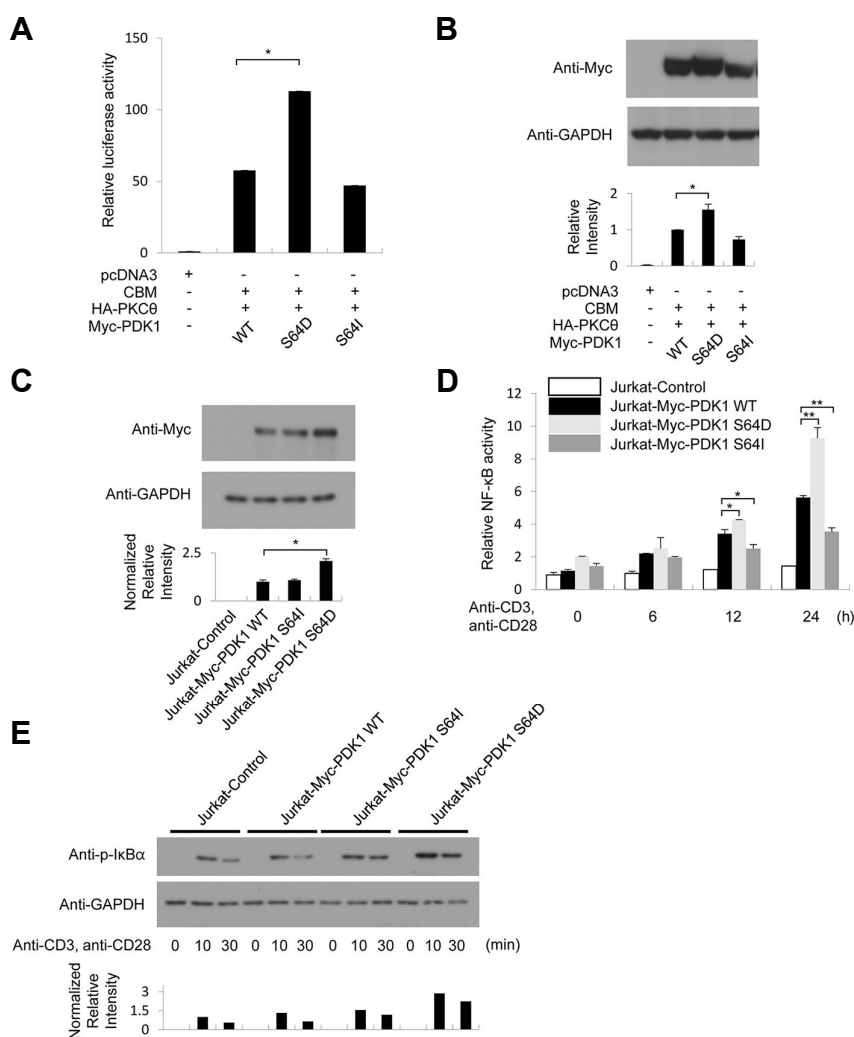

Fig. 3. Phosphorylation of PDK1 at Ser-64 regulates TCR/CD28-mediated NF-κB activation. (A) NF-κB reporter luciferase assay with wild-type (WT), phosphomimetic (S64D), or phosphorylation-deficient (S64I) PDK1 in HEK293 cells. (B) Protein expression levels of wild-type (WT), phosphomimetic (S64D), or phosphorylation-deficient (S64I) PDK1 in HEK293 cells were assessed by immunoblot analysis. (C) Protein expression levels of wild-type (WT), phosphomimetic (S64D), or phosphorylation-deficient (S64I) PDK1 in Jurkat T cells were assessed by immunoblot analysis. (D) NF-κB reporter luciferase assay with wild-type (WT), phosphomimetic (S64D), or phosphorylation-deficient (S64I) PDK1 in Jurkat T cells after stimulation with anti-CD3 and anti-CD28 antibodies. (E) After stimulation with anti-CD3 and anti-CD28 antibodies, IκBα phosphorylation levels in Jurkat T cells expressing wild-type (WT), phosphomimetic (S64D), or phosphorylation-deficient (S64I) PDK1 were assessed by immunoblot analysis. Data are representative of three (A-E) independent experiments. Results are expressed as the mean ± SD. *$p < 0.05$; **$p < 0.01$ (Student's *t*-test).
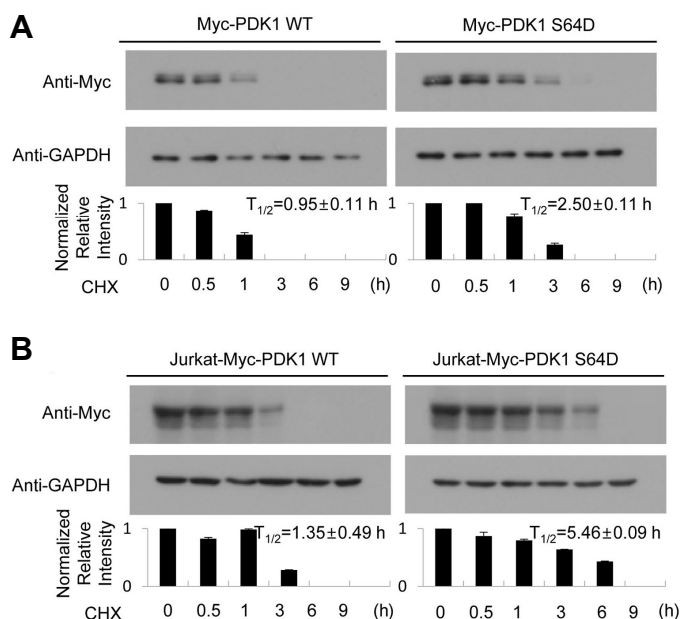
A

B

Fig. 4. Ser-64 on PDK1 is important for regulation of protein stability. (A) After treatment with cycloheximide, the protein half-life of wild-type (WT) and phosphomimetic (S64D) PDK1 in HEK293 cells was assessed by immunoblot analysis. (B) After treatment with cycloheximide, the protein half-life of wild-type (WT) and phosphomimetic (S64D) PDK1 in Jurkat T cells was assessed by immunoblot analysis. PDK1 half-life values were calculated by nonlinear regression analysis with GraphPad Prism software. Data are representative of three (A, B) independent experiments.

to the half-life of PDK1(WT) (1.0 ± 0.1 h; Fig. 4A). PDK1(S64D) expressed in Jurkat cells also exhibited a significantly increased protein half-life (5.5 ± 0.1 h) compared to the half-life of PDK1(WT) (1.4 ± 0.5 h) (Fig. 4B). Thus, our results suggest that phosphorylation of PDK1 at Ser-64 by PKCθ enhances PDK1 protein stability in T cells. Interestingly, the Ser-64 site is not found in rodent PDK1, including mouse and rat, while the site is found in primates, canine, and even chicken (Supplementary Fig. S2). In rodents, proline is found at the residue in position 64 instead of serine. There is a serine residue near this proline, at position 62. We substituted this residue to the phosphomimetic form, S62D; however, the substitution did not affect the stability of the mouse PDK1 protein (Supplementary Fig. S3). S64 may be one reason the properties of human T cells are different from those of mouse T cells.

Ser-64 on PDK1 contributes to T cell activation

We next sought to evaluate the effect of PDK1(S64D) on T cell activation. Because *Pdk1* gene deficiency in T cells affects IL-2 production and expression of activation surface markers, we analyzed IL-2 production and the expression of the activation surface markers CD69 and CD25. Expression of IL-2, which is an important target of NF-κB during T cell activation, was increased in a time-dependent manner during T cell stimulation. We found that expression of PDK1(S64I) decreased IL-2 mRNA expression compared to cells expressing PDK1(WT), whereas expression of PDK1(S64D) increased IL-2 mRNA compared to cells expressing PDK(WT) (Fig. 5A). Consistently, secreted IL-2 levels were increased in PDK(S64D)-expressing Jurkat T cells while the levels were decreased in PDK1(S64I)-expressing Jurkat T cells compared to PDK1(WT)-expressing Jurkat T cells (Fig. 5B). In addition to IL-2 production, expression levels of activation surface markers were also increased in PDK1(S64D)-expressing

Jurkat T cells and decreased in PDK1(S64I)-expressing Jurkat T cells compared to PDK1(WT)-expressing Jurkat T cells (Fig. 5C). Thus, our data show that phosphorylation of PDK1 at S64 also affects T cell activation.

DISCUSSION

PDK1 is essential for TCR-mediated NF-κB activation and T cell activation (Park et al., 2009; 2013). In this pathway, PDK1-induced phosphorylation of PKCθ is important for TCR-induced NF-κB activation. However, inverse regulation via phosphorylation of PDK1 by PKCθ has not been investigated. Our study showed that PKCθ has a role in human PDK1 phosphorylation and that its kinase activity is crucial for human PDK1 phosphorylation. Using mass spectrometry, we found that PKCθ induced PDK1 phosphorylation at Ser-64. We then hypothesized that PKCθ-induced phosphorylation of PDK1 on Ser-64 plays a role in TCR/CD28-induced NF-κB pathway activation and T cell activation because PDK1 is an important regulator for this pathway. To verify this hypothesis, we constructed PDK1 phosphomimetic (S64D) and phosphorylation-deficient (S64I) mutants and assessed NF-κB activity and T cell activation marker expression in cells expressing these constructs. Our results showed that PDK1 phosphorylation on Ser-64 promotes TCR/CD28-mediated NF-κB activation and T cell activation. Furthermore, we found that PDK1 phosphorylation on Ser-64 increases the stability of the protein. Previous papers have reported that phosphorylation can regulate protein stability through inhibition or promotion of ubiquitination-mediated protein degradation. For example, phosphorylation of Pin1 by polo-like kinase 1 inhibits ubiquitination-dependent degradation (Eckerdt et al., 2005) and phosphorylation of IκBα by IκB kinase induces ubiquitination-dependent degradation (Bhatt and Ghosh, 2014). Thus, one possible effect of increased
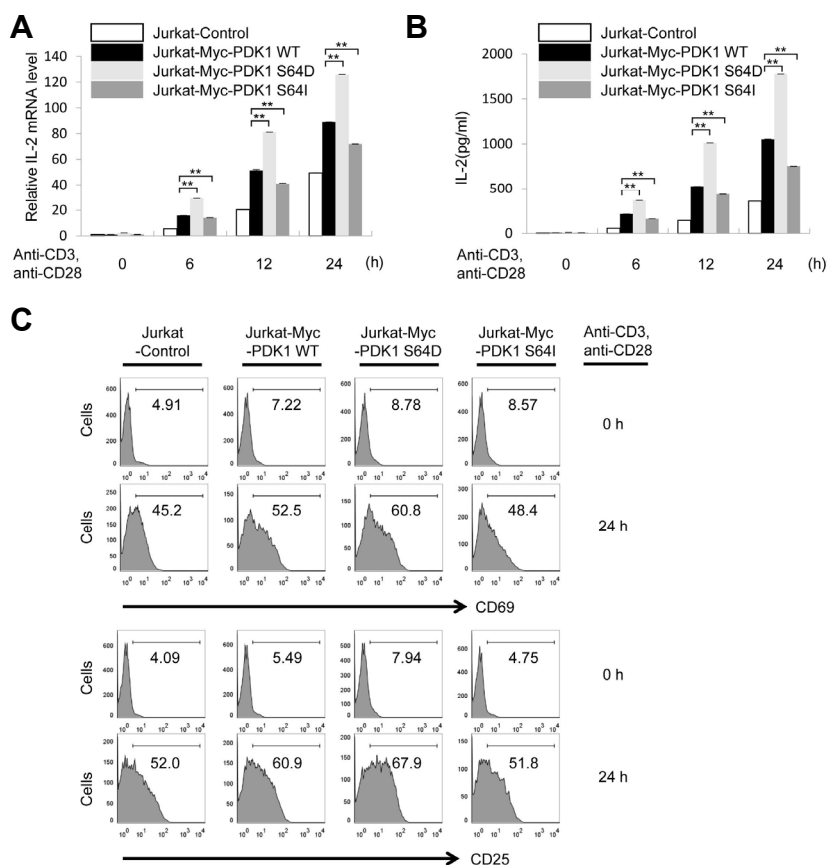
Fig. 5. Ser-64 on PDK1 is important for regulation of T cell activation. (A) After stimulation with anti-CD3 and anti-CD28 antibodies, IL-2 mRNA expression levels in Jurkat T cells expressing wild-type (WT), phosphomimetic (S64D), or phosphorylation-deficient (S64I) PDK1 were assessed by quantitative RT-PCR. (B) After stimulation with anti-CD3 and anti-CD28 antibodies, IL-2 secretion levels in Jurkat T cells expressing wild-type (WT), phosphomimetic (S64D), or phosphorylation-deficient (S64I) PDK1 were assessed by ELISA. (C) After stimulation with anti-CD3 and anti-CD28 antibodies, surface expression levels of the T cell activation markers CD69 and CD25 in Jurkat T cells expressing wild-type (WT), phosphomimetic (S64D), or phosphorylation-deficient (S64I) PDK1 were assessed by flow cytometry. Data are representative of three (A-C) independent experiments. Results are expressed as the mean ± SD. $*p < 0.05$; $**p < 0.01$ (Student's t-test).

PDK1 protein stability through phosphorylation is an inhibition of ubiquitination-dependent PDK1 degradation. Interestingly, the Ser-64 site is not found in rodents, while it is found in primates, canines, and chickens. There are significant differences between mice and humans in immune system development, activation, and response to challenges, in both the innate and adaptive arms (Mestas and Hughes, 2004). Thus, it is possible that Ser-64 contributes to the differences between human and mouse T cells. However, extensive testing of the role of the Ser-64 site in T cell functions is needed to answer this question. In addition, previous a report has shown that palmitate induced PDK1 phosphorylation at Ser-504 and Ser-532 by PKCθ and these phosphorylations inhibited insulin-mediated signaling cascades (Wang et al., 2012). However, even though those phosphorylations reduced PDK1 kinase activity, the mechanism has not been resolved. Thus, the previous report and our data suggest that PKCθ can phosphorylate PDK1 at specific sites under specific conditions.

In conclusion, our findings reveal a new interaction between PDK1 and PKCθ that has not been investigated and suggest a new function for PKCθ in inducing PDK1 phosphorylation at Ser-64. These findings further our understanding of T cell activation through the PDK1 pathway, which is one of the major T cell activation pathways.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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