Sequential posttranslational modifications regulate PKC degradation

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ABSTRACT **Cross-talk among different types of posttranslational modifications (PTMs) has emerged as an important regulatory mechanism for protein function. Here we elucidate a mechanism that controls PKCα stability via a sequential cascade of PTMs. We demonstrate that PKCα dephosphorylation decreases its sumoylation, which in turn promotes its ubiquitination and ultimately enhances its degradation via the ubiquitin-proteasome pathway. These findings provide a molecular explanation for the activation-induced down-regulation of PKC proteins.**

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INTRODUCTION

Protein kinase C (PKC) plays a crucial role in the initial events of signal transduction cascades during many important physiological and pathological processes (Parker *et al.*, 2004; Antal *et al.*, 2015). PKC isozymes consist of three serine/threonine kinase subgroups based on their distinctive structural and functional characteristics: conventional (cPKCs: α, βI, βII, and γ), novel (nPKCs: δ, ε, η, and θ), and atypical (aPKCs: ζ and λ or ι). cPKCs are activated via binding to diacylglycerol (DAG) and Ca^{2+}; nPKCs are activated only by DAG; and aPKCs are activated via neither DAG nor Ca^{2+}.

Phosphorylation is well recognized as another important mechanism that regulates PKC activation. PKC undergoes a series of ordered phosphorylations that primes the enzyme into a catalytically competent but inactive state. A pseudosubstrate segment maintains PKC in this autoinhibited conformation and also protects the phosphorylated priming sites from dephosphorylation (House *et al.*, 1987; Newton, 1997; Antal *et al.*, 2015). Activation of cPKCs and nPKCs with their agonists, such as phorbol esters, leads to their dephosphorylation and subsequently rapid degradation, a process referred to as "down-regulation" of PKC (Hansra *et al.*, 1996). Thus phosphorylation also plays a critical role in stabilizing PKC proteins. Consistent with the notion that dephosphorylated PKC is mainly degraded via the ubiquitin-proteasome pathway, PKC is ubiquitinated after the treatment of cells with phorbol esters or bryostatin (Lee *et al.*, 1996; Lu *et al.*, 1998). However, the molecular link between PKC dephosphorylation and ubiquitination remains largely unknown.

We recently reported that PKCα is also modified by sumoylation and that desumoylation of PKCα is essential for a kainate-induced endocytosis of glycine receptors in spinal cord neurons (Sun *et al.*, 2014). Sumoylation is one of the important posttranslational modifications (PTMs), which play pivotal roles in cell signaling and protein trafficking and stability, thereby regulating a plethora of biological processes, such as cell survival and neurodegeneration. Because a protein can be modified by more than one type of PTM, recent studies have provided evidence for functional cross-talk and complex interplay among sumoylation, ubiquitination, and phosphorylation for a number of proteins (Desterro *et al.*, 1998; Glotzer *et al.*, 2000; Carter *et al.*, 2007; Guo *et al.*, 2012; Luo *et al.*, 2014).

In this study, we identified a functional interplay among phosphorylation, sumoylation, and ubiquitination of PKCα. The dephosphorylation of PKCα reduces its sumoylation, which in turn promotes its ubiquitination and thus enhances its degradation via

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Abbreviations used: PKC, protein kinase C; PTM, posttranslational modification; SENP, sentrin/SUMO-specific protease; SUMO, small ubiquitin-like modifier.

the proteasome pathway. Hence this sequential cascade of post-translational modifications of PKCα represents an important molecular mechanism for the regulation of the level of PKCα proteins in cells.

RESULTS

PKCα is modified by small ubiquitin-like modifier 1 or small ubiquitin-like modifier 2/3

We showed previously that PKCα is modified by small ubiquitin-like modifier 1 (SUMO1; Sun *et al.*, 2014). To test whether PKCα also undergoes SUMO2/3 sumoylation, we transiently transfected CHO-K1 cells with hemagglutinin (HA)-tagged PKCα, either alone or together with Myc-tagged SUMO2/3. PKCα was sumoylated by SUMO2/3, and the presence of several high–molecular weight bands on Western blots corresponding to sumoylated PKCα indicates that sumoylation occurs either at multiple sites of PKCα or through the formation of SUMO1 or SUMO2/3 chains conjugated to PKCα (Figure 1, A and B, left; Sun *et al.*, 2014). To determine whether the observed bands correspond to sumoylated forms of PKCα, we also measured sumoylation of PKCα under denaturing conditions after transient transfection with HA-tagged PKCα, either alone or together with Myc-tagged SUMO2/3, in CHO-K1 cells. As expected, the results shown in Figure 1, A and B (right), further demonstrate that the observed bands were indeed sumoylated forms of PKCα. Consistent with the previous study (Sun *et al.*, 2014), overexpression of wild-type (WT) sentrin/SUMO-specific protease 1 (SENP1) completely abolished sumoylated PKCα, whereas a catalytically inactive mutant SENP1 (SENP1m) did not deconjugate sumoylated PKCα in CHO-K1 cells (Figure 1, A and B). We also confirmed the association of SENP1 and PKCα by coimmunoprecipitation assays (Figure 2A). Furthermore, PKCα was modified by endogenous SUMO1 or SUMO2/3 in CHO-K1 cells (Figure 2B), and endogenous PKC sumoylation was enhanced in SENP1$^{-/-}$ brain tissue (Figure 2C) under denaturing conditions. Taken together, these results indicate that PKCα was sumoylated by both SUMO1 and SUMO2/3 and that SENP1 acts as a major desumoylating enzyme for PKCα.

PKCα sumoylation predominately occurs at lysine 465

Our previous study identified lysine 465 (K465) as a major PKCα sumoylation site (Sun *et al.*, 2014). Based on the SUMOsp 2.0 analysis program, PKCα sumoylation could potentially occur on multiple lysine residues at amino acid positions 131, 165, 205, 304, 371, 465, and 604 (Figure 3A). To evaluate further the contribution of these lysine residues to PKCα sumoylation, we generated three PKCα constructs with lysine (K)-to-arginine (R) mutations (K465R; 6KR: K131, 165, 205, 304, 371, and 604R; and 7KR: K131, 165, 205, 304, 371, 465, and 604R) and analyzed them for their sumoylation. The K465R and 7KR mutations abolished the SUMO1 or SUMO2/3-mediated modification of PKCα, but PKCα was sumoylated when the mutated sites did not include the residue K465 (6KR) (Figure 3, B and C). Taken together, these data further confirm that K465 of PKCα is the main sumoylation site.

Conservation analysis revealed that the potential sumoylation sites, as well as the surrounding amino acid composition, are highly conserved among PKCα orthologues in different species (Figure 3D). In addition, we demonstrated that the classical and novel PKC isoforms (cPKCs and nPKCs) were sumoylated in CHO-K1 cells cotransfected with various PKC isoforms and Flag-tagged SUMO1. Among them, PKCα exhibited the strongest sumoylation, and we thus used this isoform in most of the subsequent experiments (Figure 3E).

PKCα phosphorylation promotes its sumoylation

The SUMO pathway is known to be influenced by other types of PTMs, such as phosphorylation (Müller *et al.*, 2000; Lin *et al.*, 2004; Bossis *et al.*, 2005; Hietakangas *et al.*, 2006; Guo *et al.*, 2012; Luo *et al.*, 2014). PKC is modified by three ordered priming phosphorylation sites at residues T497, T638, and S657, and phosphorylation is critical for the catalytic competence and stability of PKC (Orr *et al.*, 1994; Dutil *et al.*, 1998; Le Good *et al.*, 1998). To evaluate possible interplay between phosphorylation and sumoylation of PKCα, we cotransfected CHO-K1 cells with constructs encoding exogenous SUMO1, together with WT, phosphorylation-defective (T497A, T638A, S657A), or phosphorylation-mimetic (T497D, T638D, S657D) mutant PKCα. The phosphorylation-defective T497A, T638A, and S657A PKCα mutants exhibited significantly reduced levels of sumoylation compared with the phosphorylation-mimetic T497D, T638D, and S657D mutants (Figure 4A), suggesting that PKCα phosphorylation is required for its sumoylation. Consistently, treatment of cell extracts with lambda protein phosphatase (λ-PPase) not only decreased PKCα phosphorylation dramatically but also reduced PKCα sumoylation, further supporting the idea that PKCα sumoylation is dependent on its phosphorylation (Figure 4B).

Sumoylation entails the interaction of target proteins with the sumoylation-conjugating enzyme UBC9 (Geiss-Friedlander *et al.*, 2007). To test whether PKCα phosphorylation affects its binding to UBC9, we analyzed UBC9-PKCα coimmunoprecipitations in cotransfected CHO-K1 cells. PKCα and UBC9 association was dramatically reduced in the phosphorylation-defective PKCα mutants, whereas the phosphorylation-mimetic mutations on PKCα retained their ability to associate with UBC9 (Figure 4C). Thus inhibition of PKCα phosphorylation decreased its affinity for the UBC9 ligase, which may be accounted for the enhancing effect of PKCα phosphorylation on its sumoylation.

SUMO1 modification stabilizes PKCα

SUMO modification has been implicated in the regulation of protein degradation, a process that is vital to practically all aspects of cellular physiology (Geiss-Friedlander and Melchior, 2007; Gareau *et al.*, 2010). Here we provided several lines of evidence demonstrating that sumoylation plays an important role in stabilizing PKCα proteins by reducing its degradation in cells. First, the protein level of the sumoylation-deficient mutant K465R PKCα in transfected CHO-K1 cells was nearly twofold lower than that of WT PKCα (Figure 5A). Second, when de novo protein synthesis was inhibited by cycloheximide (CHX), the level of WT but not of K465R mutant PKCα protein was significantly higher in SUMO1-cotransfected cells than in control or SENP1-overexpressed cells (Figure 5B), indicating that the reduction of PKCα protein levels is likely due to the enhanced degradation rather than the reduced synthesis of the PKCα protein. Third, in the presence of CHX, WT PKCα was relatively stable, with a half-life of ~18 h. Overexpression of SUMO1 further enhanced the stability of PKCα, which showed little sign of degradation over a 24-h period (Figure 5, C and D). In contrast, inhibition of protein synthesis resulted in almost complete loss of K465R protein within 24 h (Figure 5, C and D). Taken together, these results further demonstrate that sumoylation stabilizes PKCα proteins by decreasing their degradation.

Consistent with previous studies (Newton, 2010; Gould *et al.*, 2011), the phosphorylation-mimetic S657D PKCα mutant was more stable than that of the phosphorylation-defective S657A PKCα (Figure 5E). However, mutation of its key sumoylation site rendered the double mutant PKCα (K465R/S657D) short-lived (Figure 5E). Thus PKCα sumoylation appears to play a key role in maintaining its stability.

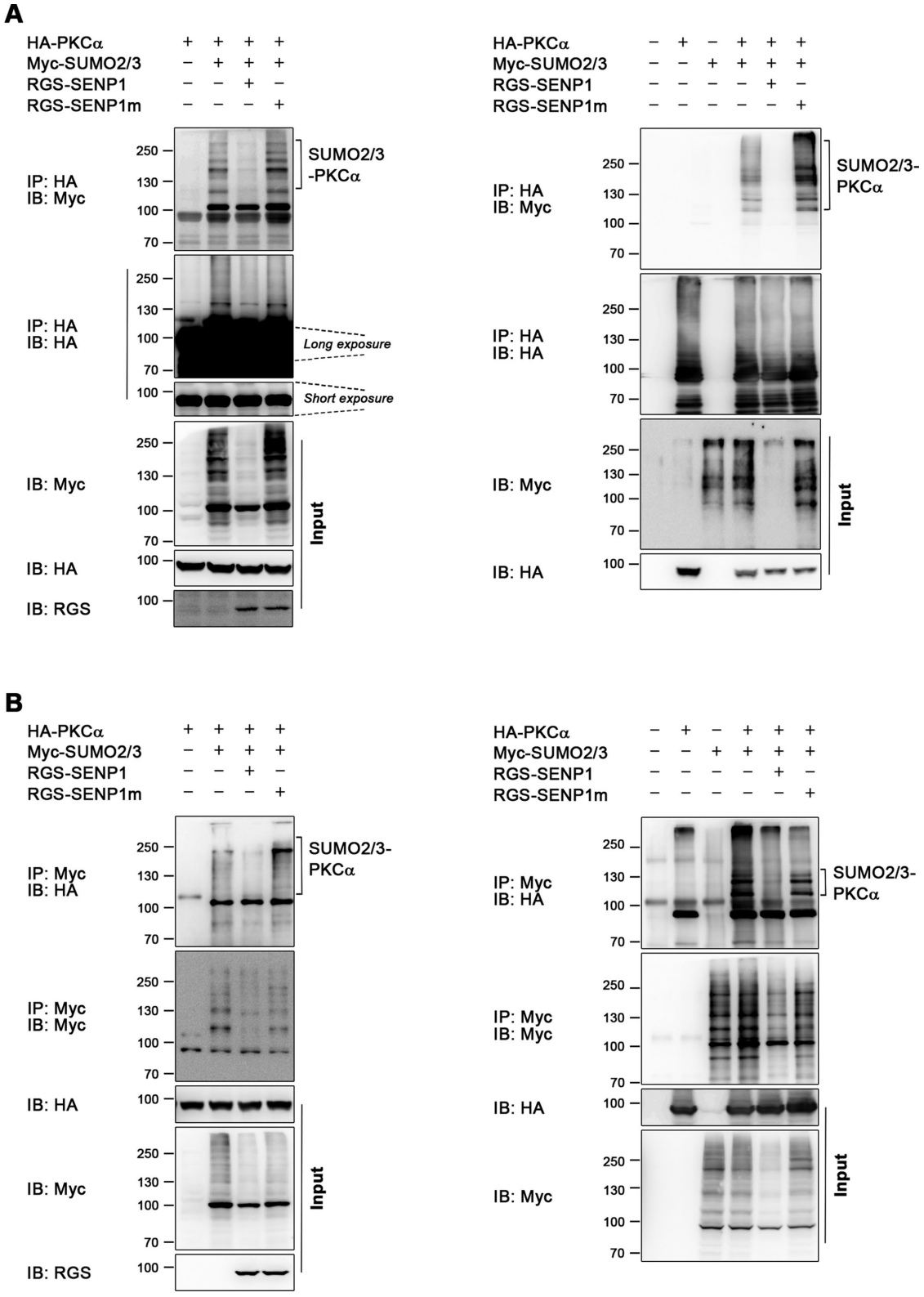

FIGURE 1: PKCα is modified by SUMO1 or SUMO2/3. (A, B) PKCα is modified by SUMO2/3 and desumoylated by SENP1. CHO-K1 cells were cotransfected with HA-tagged PKCα, Myc-tagged SUMO2/3, RGS-tagged SENP1, or RGS-tagged SENP1m as indicated. Whole-cell lysates were prepared 24 h posttransfection under nondenaturing (native; left) or denaturing (right) conditions and immunoprecipitated with anti-HA antibody (A) or anti-Myc antibody (B). SUMO-conjugated proteins were immunoblotted with anti-Myc antibody (A) or anti-HA antibody (B). The lysates were immunoblotted using anti-HA, anti-Myc, and anti-RGS antibodies as an input.

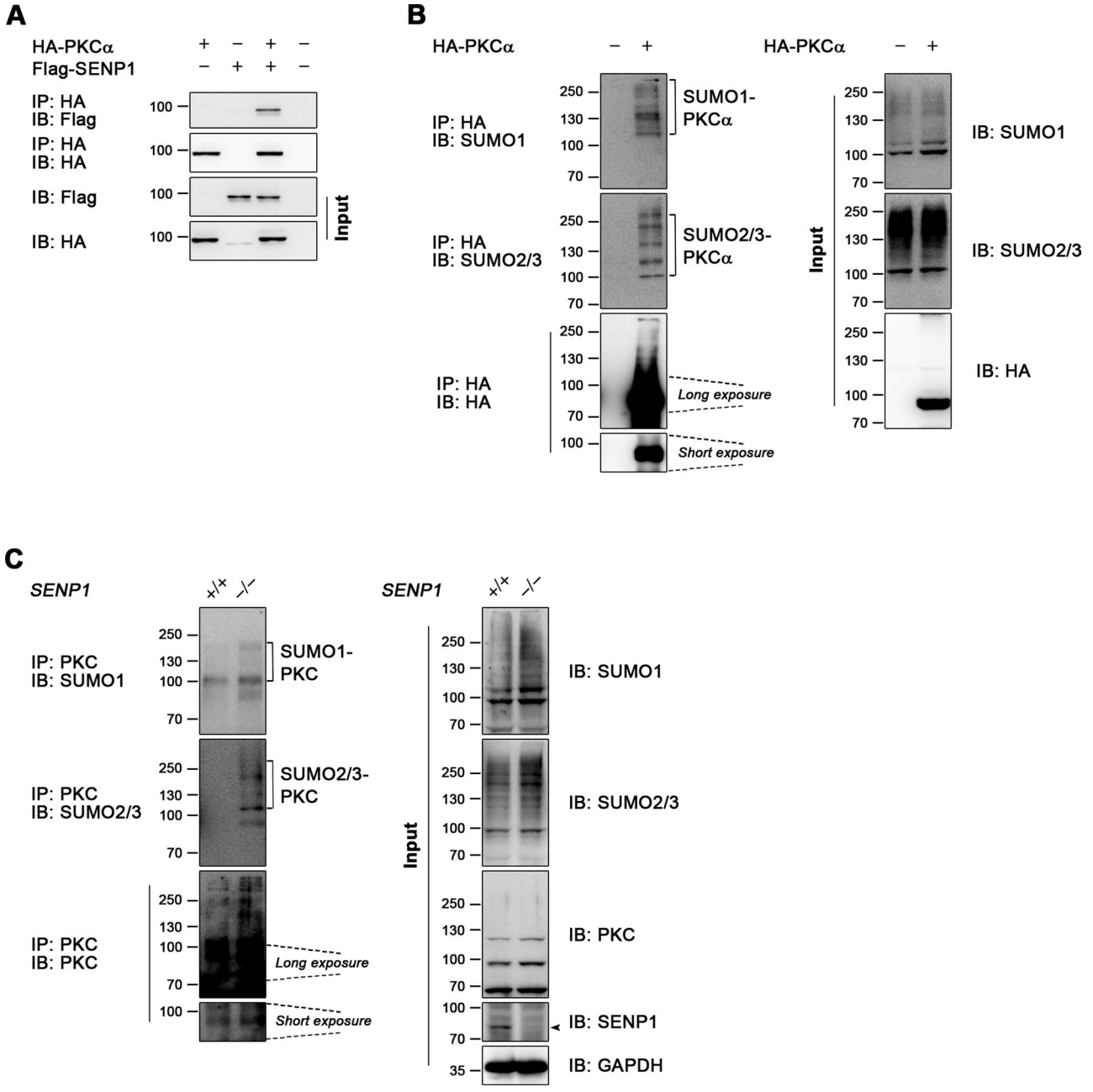

FIGURE 2: PKC is modified by endogenous SUMO1 or SUMO2/3. (A) An interaction between PKCα and SENP1. CHO-K1 cells were transfected with HA-tagged PKCα alone, Flag-tagged SENP1 alone, or both HA-tagged PKCα and Flag-tagged SENP1 together. Whole-cell lysates were prepared 24 h posttransfection and immunoprecipitated with anti-HA antibody and blotted with anti-Flag or anti-HA antibodies (top). Bottom, the lysates were blotted using anti-HA or anti-Flag antibodies as input. Data are representative of at least three independent experiments. (B) PKCα is modified by the endogenous sumoylation machinery. CHO-K1 cells were transfected with or without HA-tagged PKCα. Cell extracts were prepared under denaturing condition and immunoprecipitated with the HA antibody, followed by Western blotting with anti-SUMO1 or anti-SUMO2/3. (C) Endogenous PKC sumoylation was compared in SENP1$^{-/-}$ and SENP1$^{+/+}$ mouse brain tissue. Tissue lysates were prepared under denaturing condition and immunoprecipitated with the PKC antibody, followed by Western blotting with anti-SUMO1 or anti-SUMO2/3. Endogenous SENP1 is indicated by an arrowhead.

SUMO modification represses PKCα ubiquitination

This enhancing effect of sumoylation on PKCα prompted us to investigate the possibility that sumoylation may protect PKCα from degradation through the ubiquitin-proteasome pathway. As shown in Figure 6A, K465R protein degradation was blocked by the specific proteasome inhibitor MG132 but by not chloroquine, a lysosome inhibitor. The observation suggests that PKCα was degraded primarily via the ubiquitin-proteasome pathway, which is likely inhibited by the SUMO1 modification. To examine the potential crosstalk between SUMO1 modification of PKCα and its ubiquitination, we transfected CHO-K1 cells with plasmids expressing HA-ubiquitin (Ub), together with either WT or the sumoylation-deficient mutant PKCα. Overexpression of HA-Ub resulted in the ubiquitination of mutant K465R PKCα with a level significantly higher than that of WT

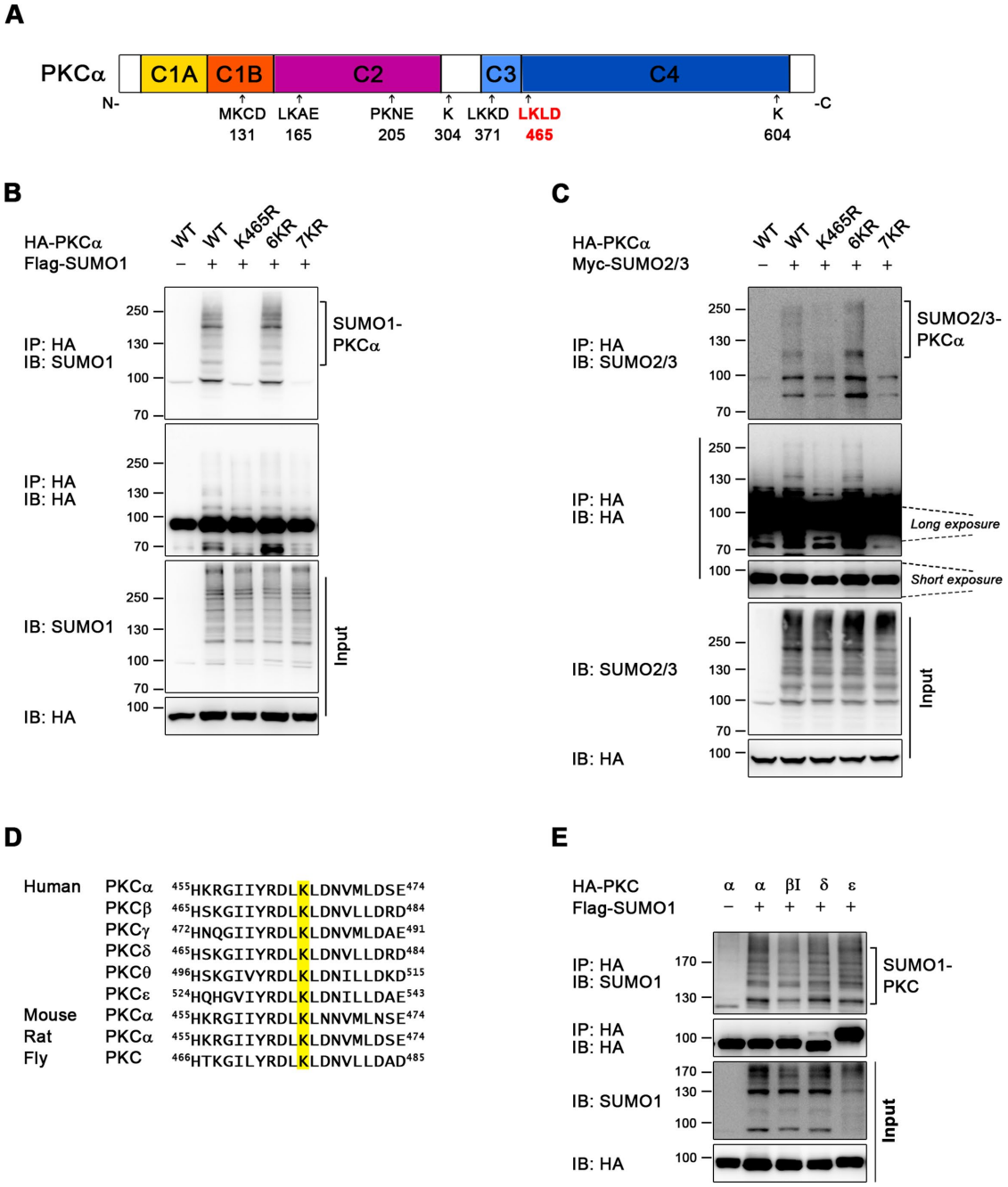

FIGURE 3: Sumoylation of PKCα occurs at lysine 465. (A) Potential SUMO modification sites in human PKCα predicted by SUMOsp 2.0 software. (B, C) Mutation of Lys-465 abolishes PKCα sumoylation. CHO-K1 cells were cotransfected with Flag-tagged SUMO1 (B) or Myc-tagged SUMO2/3 (C) with the indicated HA-tagged PKCα, K465R, 6KR (K131, 165, 205, 304, 371, 604R), or 7KR (K131, 165, 205, 304, 371, 465, 604R) PKCα. Cell lysates were prepared under denaturing condition and subjected to immunoprecipitation with anti-HA antibody, followed by Western blotting using anti-SUMO1 (B) or anti-Myc (C) antibodies. (D) Alignment of various PKC sequences and PKCα sequences from different species (indicated). The sumoylation consensus sites are shown in yellow. (E) cPKCs and nPKCs are modified by SUMO1. CHO-K1 cells were cotransfected with plasmids encoding Flag-tagged SUMO1 and various HA-tagged PKC isoforms as indicated. Cell lysates were immunoprecipitated by anti-HA antibody and analyzed by Western blotting with anti-SUMO1 or anti-HA antibodies. The lysates were blotted using anti-HA or anti-SUMO1 antibodies.

PKCα (Figure 6B), indicating that SUMO1 modification represses PKCα ubiquitination.

Further supporting the inhibitory effect of PKCα sumoylation on its ubiquitination, overexpression of SUMO1 attenuated the ubiquitination of PKCα in cotransfected CHO-K1 cells, an effect that was reversed by coexpression of the desumoylating enzyme SENP1 (Figure 6C). In contrast, PKCα sumoylation was not significantly altered by the treatment of the proteasome inhibitor MG132 (Figure 6D), indicating that PKCα ubiquitination may have minimal effect on its sumoylation. Collectively these results demonstrate that PKCα

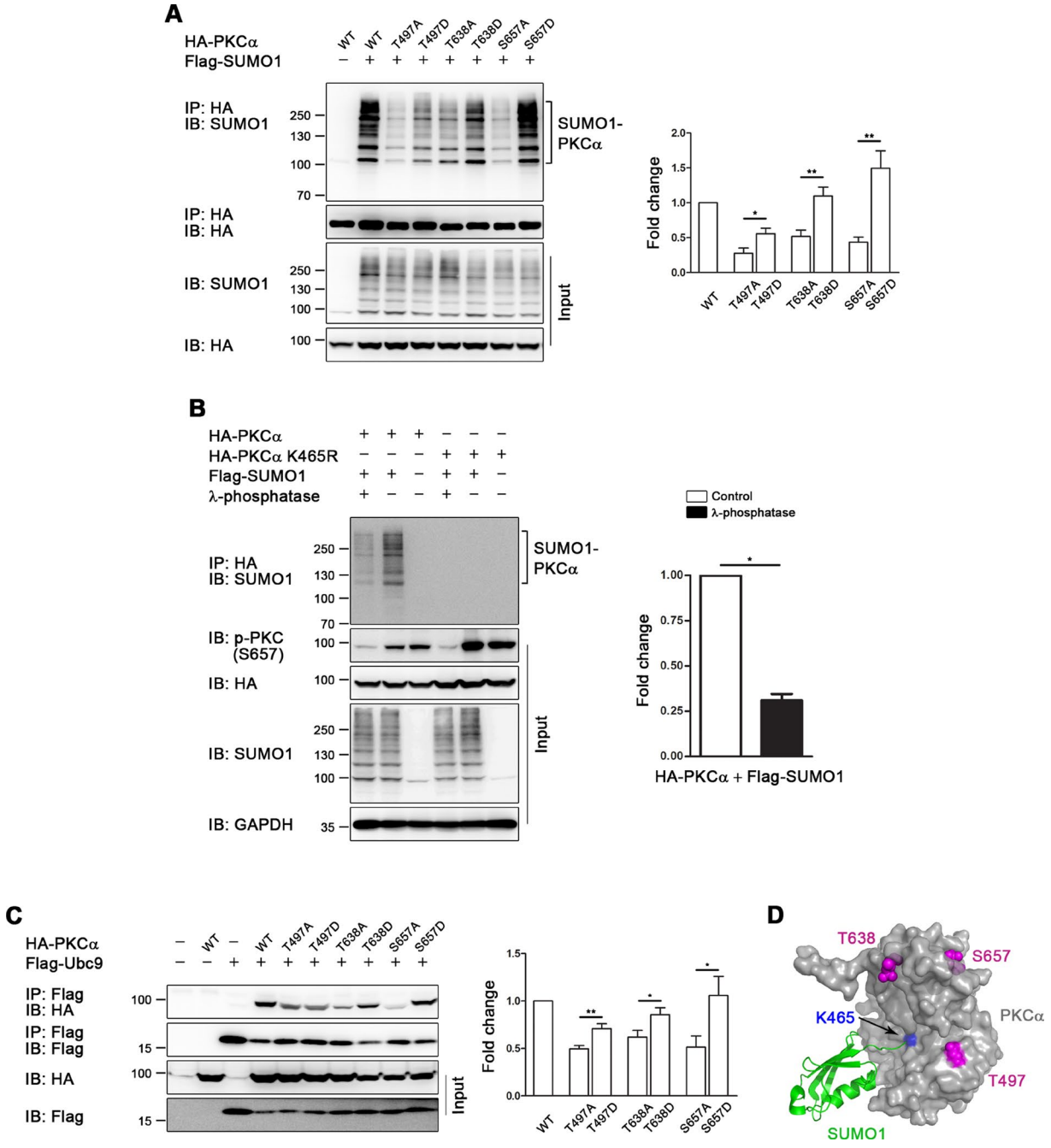

FIGURE 4: SUMO modification of PKCα is phosphorylation dependent. (A) PKCα phosphorylation promotes its sumoylation. CHO-K1 cells were transfected with HA-tagged PKCα or phosphorylation-defective (T497A, T638A, S657A) or phosphorylation-mimetic (T497D, T638D, or S657D) mutants and Flag-tagged SUMO1 as indicated. Cell lysates were prepared 24 h posttransfection and immunoprecipitated with anti-HA antibody, followed by Western blot with anti-SUMO1 or anti-HA antibodies. The lysates were immunoblotted using anti-HA or anti-SUMO1 antibodies as an input. (B) Dephosphorylation of PKCα decreases its sumoylation. CHO-K1 cells overexpressing HA-tagged WT or K465R PKCα with or without Flag-SUMO1. Cell lysates were treated with or without λ-PPase for 2 h before coimmunoprecipitation assays as indicated. Cell lysates were subjected to immunoprecipitation by anti-HA antibody, followed by Western blotting with an anti-SUMO1 antibody. The lysates were immunoblotted by anti–phospho-PKC, anti-HA, and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. (C) The phosphorylation-mimetic mutants had higher binding affinity for UBC9. CHO-K1 cells overexpressing Flag-tagged UBC9 and HA-tagged WT or phosphorylation-defective (T497A, T638A, S657A) or phosphorylation-mimetic (T497D, T638D, or S657D) PKCα mutants as indicated. Cell lysates were prepared 24 h posttransfection and immunoprecipitated with anti-Flag antibody, followed by Western blotting for anti-HA antibody. Data are representative of at least three independent experiments and are means ± SEM; *$p < 0.05$, **$p < 0.01$. (D) Model for sumoylated PKCα C-terminal domain. PKCα C-terminal domain is shown as gray, space-filling model and SUMO1 as a green ribbon. The phosphorylation sites Thr-497, Thr-638, and Se-r657 of PKCα are shown in magenta. The sumoylation site Lys465 is shown in blue. The model is based on structures from PDB1A5R and 3IW4.

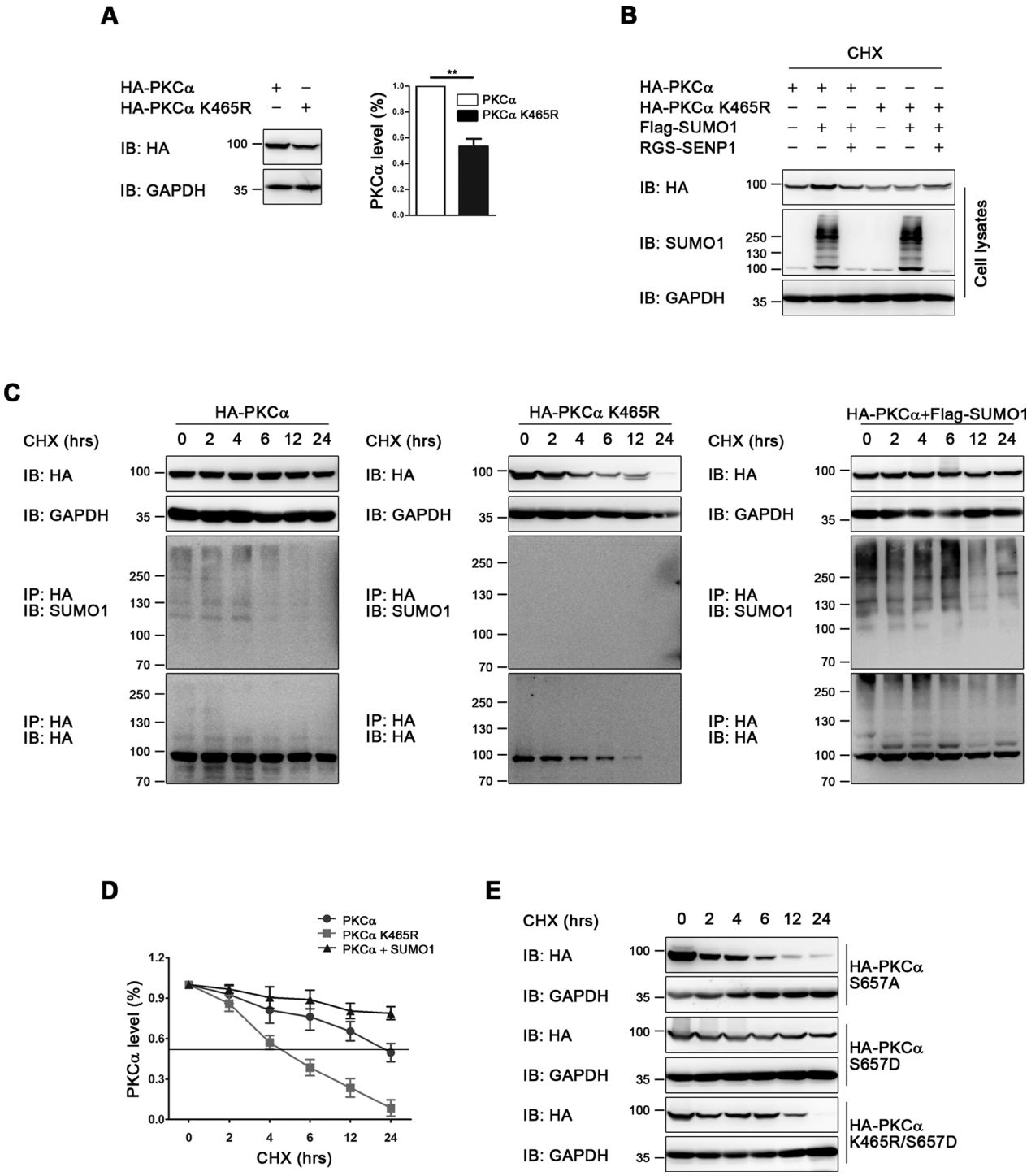

FIGURE 5: The sumoylation-deficient K465R PKCα becomes unstabilized. (A) Decreased protein levels of K465R PKCα. CHO-K1 cells were transfected with either HA-tagged WT or K465R PKCα. Cell lysates were analyzed by Western blotting using anti-HA and anti-GAPDH antibodies. (B) SUMO1 modification increases the level of WT but not K465R mutant PKCα. The expression of HA-tagged PKCα or K465R PKCα was analyzed by blotting with anti-HA antibody in CHO-K1 cells transfected with HA-tagged PKCα, K465R Flag-SUMO1, or RGS-SENP1 as indicated. CHX (20 μg/ml) was added to prevent de novo protein synthesis. (C) SUMO1 modification of PKCα enhances its stability. CHO-K1 cells were transfected with HA-tagged PKCα, K465R PKCα, or HA-tagged PKCα together with Flag-SUMO1 for 24 h and then treated with 20 μg/ml CHX for additional 0, 2, 4, 6, 12, or 24 h as indicated. Cells were lysed under denaturing condition and immunoprecipitated by anti-HA antibody. (C) Western blotting with anti-HA, anti-SUMO1, and anti-GAPDH antibodies. (D) Quantitative analysis. (E) The phosphorylation-mimic mutant is more stable than the phosphorylation-defective mutant. CHO-K1 cells were transfected with S657A, S657D, or K465R/S657D PKCα for 24 h, and then the cells were treated with 20 μg/ml CHX at indicated times. The degradation of PKCα was detected by Western blotting with anti-HA and anti-GAPDH antibodies. Data presented in A and D are expressed as means ± SEM and are representative of at least three independent experiments; $^{**}p < 0.01$ by Student's *t* test.

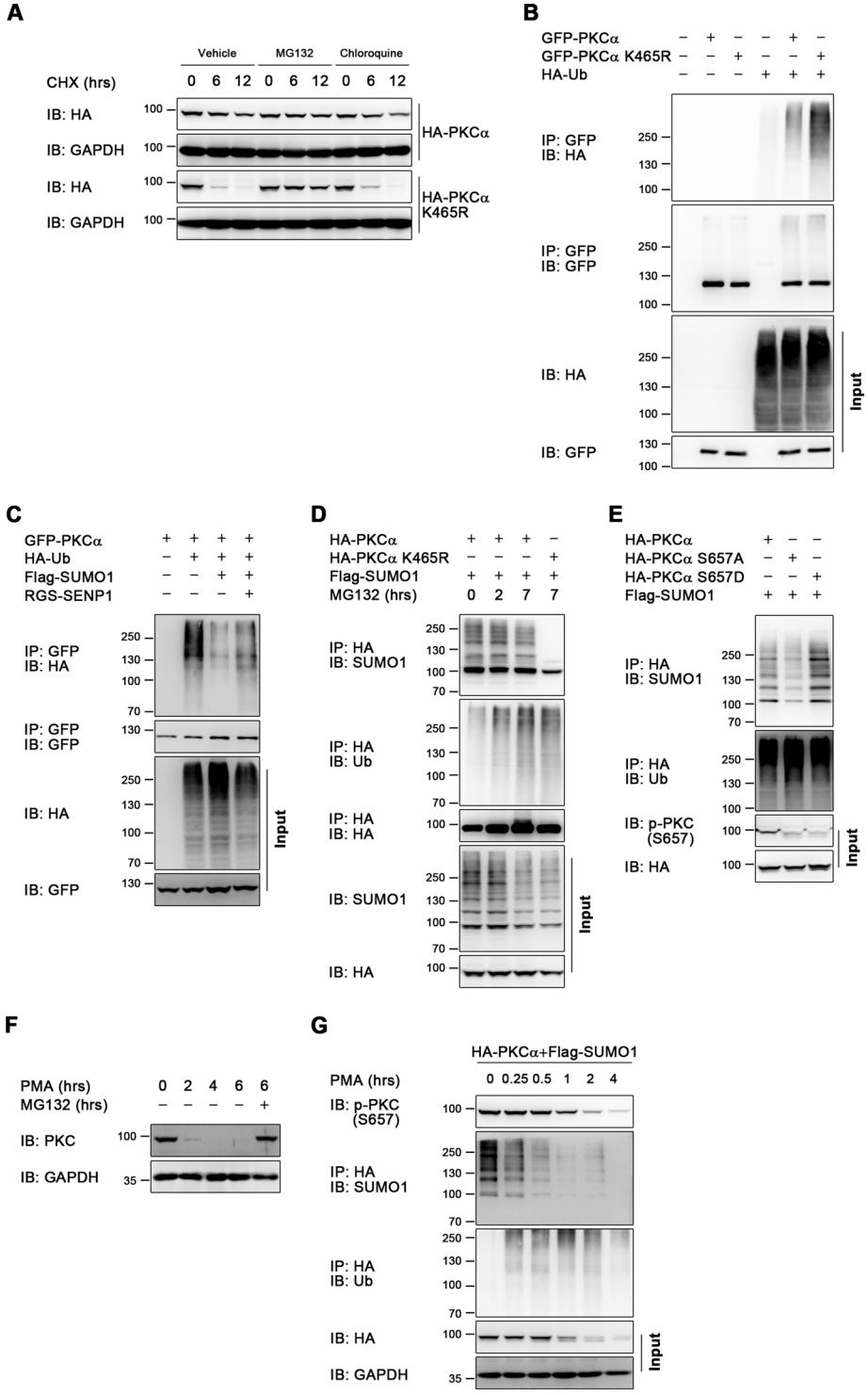

FIGURE 6: SUMO1 modification enhances PKCα stability and down-regulation, of PKC induced by PMA is mediated by the interplay among diverse PTMs. (A) The sumoylation-deficient K465R PKCα is degraded via the proteasome pathway. CHO-K1 cells overexpressing either HA-tagged PKCα or K465R PKCα were treated with dimethyl sulfoxide (vehicle control), proteasome inhibitor MG132 (20 μM), or lysosome inhibitor chloroquine (100 μM) for 1 h before CHX (20 μg/ml) addition, and cells were harvested at indicated time points. Whole-cell lysates were immunoblotted with anti-HA and anti-GAPDH antibodies. (B) The sumoylation-deficient K465R PKCα has enhanced ubiquitination. CHO-K1 cells were cotransfected with either GFP-tagged PKCα or K465R PKCα with or without HA-tagged Ub. Cell lysates were prepared 24 h posttransfection under denaturing condition and immunoprecipitated with anti-GFP antibody, followed by Western blot with anti-GFP or anti-HA antibodies. The lysates were immunoblotted using anti-HA or anti-GFP antibodies as an input. (C) SUMO1 modification of PKCα decreases its ubiquitination. CHO-K1 cells were transfected with GFP-tagged PKCα, Flag-SUMO1, and/or RGS-SENP1 as indicated. PKCα ubiquitination was measured by immunoprecipitation with anti-GFP antibody under denaturing condition, followed by immunoblotting anti-HA antibody. The lysates were immunoblotted using anti-HA or anti-GFP antibodies as an input. (D) The ubiquitination of PKCα had no influence on its sumoylation. CHO-K1 cells were cotransfected with Flag-tagged SUMO1 and either HA-tagged PKCα or K465R PKCα and treated with CHX (20 μg/ml) at the indicated time before harvesting of cells. Cell lysates were prepared 24 h posttransfection under denaturing condition, immunoprecipitated with anti-HA antibody, and immunoblotted with anti-SUMO1, anti-Ub, and anti-HA antibodies. (E) Coordination among diverse PTMs of PKCα. Immunoprecipitated HA-tagged PKCα from CHO-K1 cells coexpressing HA-tagged PKCα, S657A, or S657D PKCα with Flag-tagged SUMO1 was probed for ubiquitination with anti-Ub antibody under denatured conditions. (F) Degradation of endogenous PKCα induced by PMA is blocked by MG132. Cells were treated with 100 nM PMA at indicated time points and pretreated with 20 μM MG132 for 1 h as indicated time point. Subsequently, endogenous PKCα expression levels were analyzed by Western blot using anti-PKC and anti-GAPDH antibodies. (G) The steady-state "down-regulation" induced by PMA correlates with sequential modification of PKCα. CHO-K1 cells were cotransfected with Flag-tagged SUMO1 and HA-tagged PKCα for 24 h and then treated with PMA (100 nM) at indicated times before harvesting of cells. Sumoylation and ubiquitination of PKCα were detected with anti-SUMO1 and anti-Ub antibodies after immunoprecipitating with anti-HA antibody under denature conditions. Whole-cell lysates were immunoblotted with anti-phospho-PKC, anti-HA, and anti-GAPDH antibodies. Data have been reproduced at least three times.

sumoylation represses its ubiquitination, thereby leading to reduced degradation of PKCα proteins by the proteasome.

The interplay among diverse PTMs determines "down-regulation" of PKCα induced by phorbol esters

Previous studies showed that phosphorylated PKC proteins are more stable than the nonphosphorylated forms (Newton, 2010; Gould *et al.*, 2011). This raises the possibility that PKC degradation is controlled by interplay among phosphorylation, sumoylation, and ubiquitination. To test this, we further examined interactions of these PTMs in cells expressing WT, phosphorylation-defective,

or phosphorylation-mimetic PKCα. As expected, the S657A mutant PKCα exhibited reduced sumoylation, but the level of its ubiquitination was considerably higher than that of WT. Correlatively, the S657D mutant PKCα displayed enhanced sumoylation and decreased ubiquitination (Figure 6E). Thus PKCα phosphorylation appears to interact with its sumoylation and ubiquitination in a sequentially regulated manner.

Activation of PKCs by second messengers is known to promote "down-regulation" of PKC proteins (Gould *et al.*, 2011). Consistently, we showed that the level of endogenous PKC proteins rapidly declined in response to activations by the phorbol ester phorbol 12-myristate 13-acetate (PMA; Figure 6F). This down-regulation of PKC was likely due to enhanced PKC degradation by the ubiquitin-proteasome system, as the effect of PMA was abrogated by MG132 (Figure 6F). To determine whether PMA-induced degradation of PKCα is the consequence of interplay among PKCα phosphorylation, sumoylation, and ubiquitination, we examined the time course of different PTMs on PKCα in transfected CHO-K1 cells. After PMA stimulation, PKCα became gradually dephosphorylated, which was correlated with reduced sumoylation, enhanced ubiquitination, and decreased PKCα protein level (Figure 6G). Taken together, these results provide a potential molecular mechanism for activation-promoted down-regulation of PKCα proteins.

DISCUSSION

We demonstrated a complex interplay among various PTMs that finely tune PKCα functions and ultimately regulate its degradation via the proteasome pathway. We showed that PKCα is sumoylated not only by SUMO1, but also by SUMO2/3. PKCα dephosphorylation decreases its sumoylation and consequently enhances its ubiquitination and degradation. These results provide evidence that down-regulation of PKCα is mediated by a novel regulatory mechanism through functional cross-talk between different types of PTM.

Based on previous reports, phosphorylation can either facilitate or repress sumoylation within the same protein (Hietakangas *et al.*, 2003, 2006; Grégoire *et al.*, 2006). The presence of a phosphorylation-dependent sumoylation motif (PSDM) in target proteins has been proposed as a common mechanism for phosphorylation-mediated enhancement of substrate sumoylation (Müller *et al.*, 2000; Yang *et al.*, 2003; Bossis *et al.*, 2005). The PSDM is defined as ψKxExxSP, where ψKxE conforms to the sumoylation consensus motif, followed by any two residues and then a proline-directed phosphorylatable serine residue. A variation of the PDSM is the negative charge–dependent sumoylation motif, ΨKxExxEEEE, which comprises ψKxE followed by at least two acidic amino acids, one of which has to be located between three and six residues from the C-terminus of the target lysine (Yang *et al.*, 2006). In this study, we showed that PKCα sumoylation is positively regulated by its phosphorylation (Figure 4), but the three critical PKCα phosphorylation sites (T497, T638, and S657) do not appear to fall into a consensus PDSM. This is not completely surprising, since other studies identified the phosphorylation-promoted SUMO modification that occurs outside of canonical PDSMs or phospho–SUMO-interaction motifs (Hayakawa *et al.*, 2004; Gresko *et al.*, 2009). Based on our analyses, the PKCα sumoylation and phosphorylation sites likely localize in structural proximity (Figure 4D). Thus phosphorylation of PKCα might lead to conformational changes that expose the SUMO modification site to the SUMO conjugation apparatus. In support of this hypothesis, binding affinity to the UBC9 sumoylation ligase is significantly reduced in phosphorylation-defective PKC mutants (Figure 4C).

The cross-talk between the SUMO and ubiquitin pathways is not surprising because ubiquitination and sumoylation on the same Lys residue can differentially modulate the activity and fate of the substrate proteins. This becomes even more complicated when we consider the fact that the enzymes within one pathway can be regulated by those in the other (Lee *et al.*, 2006; Carbia-Nagashima *et al.*, 2007; Carter *et al.*, 2007). Previous studies showed that sumoylation can compete against or cooperate with ubiquitination and proteasomal degradation by attachment to the same lysine residues within substrate proteins (Buschmann *et al.*, 2000; Guzzo *et al.*, 2012). The equilibrium between sumoylation and ubiquitination can influence the balance between p53 nuclear localization and stabilization and between cytoplasmic export and degradation, as well as regulate the activity and stability of hypoxia-induced factor (Lee *et al.*, 2006; Carter *et al.*, 2007). Sumoylation of E2-25k, an E2 enzyme in the ubiquitin pathway, down-regulates the ubiquitination system by inhibiting its capability to conjugate ubiquitin (Pichler *et al.*, 2005). In this study, we confirmed that the degradation of PKCα is mainly mediated through the ubiquitin-proteasome pathway. Of greater importance, we determined that SUMO modification of PKCα suppresses its ubiquitination and thus inhibits proteasome-dependent PKCα degradation. Of interest, we found that the sumoylation-deficient mutant K465R PKCα exhibited a higher level of ubiquitination, as well as enhanced protein degradation (Figure 6B). This suggests that sumoylation and ubiquitination do not occur on the same lysine residue of PKCα. Future studies are needed to further determine how sumoylation of PKCα might influence its ubiquitination.

Of interest, we previously showed that PKCα sumoylation suppressed its activity both in vivo and in vitro (Sun *et al.*, 2014). Taking this together with our present findings, we propose that both sumoylation and desumoylation of PKCα may play a significant role in inactivating PKCα activity. However, the analogous functional outcomes are likely mediated by distinct molecular mechanism and cellular pathways: sumoylation of phosphorylated PKCα maintains the enzyme at its inactive state, whereas desumoylation of nonphosphorylated/active PKCα promotes its ubiquitination and subsequent degradation.

On the basis of the findings reported here, we propose a model for the interplay among phosphorylation, sumoylation, and ubiquitination of PKCα (Figure 7). Phosphorylation of PKCα at the priming sites promotes its sumoylation, which inhibits its ubiquitination and thus stabilizes the PKCα proteins. Activation of PKCα by phorbol esters leads to its dephosphorylation and desumoylation, which enhances its ubiquitination and subsequent degradation by the proteasome. Thus the cellular levels of PKCα are regulated by intricate cross-talk among these three different types of PTM. In particular, the newly discovered sumoylation of PKCα seems to play an important role in bridging the PKCα phosphorylation and ubiquitination pathways.

Regulation of PKC levels sets the gain of the PKC signaling pathway. We showed here that phosphorylation-dependent sumoylation of PKCα maintains its protein levels by inhibiting proteasome-mediated degradation. Given the well-defined role of the PKC signaling pathway in various cellular processes, therapeutic targeting of PKC signaling components is an area of intense investigation. Thus it is conceivable to attenuate PKC signaling via reduction of PKC sumoylation. In the future, this strategy might be applicable to the treatment of human diseases such as neurodegeneration and cancer metastasis.

MATERIALS AND METHODS

Cell culture and transfection

CHO-K1 cells were cultured and maintained at 37°C in a 5% CO_2 humidified incubator supplemented with Ham's F-12 medium

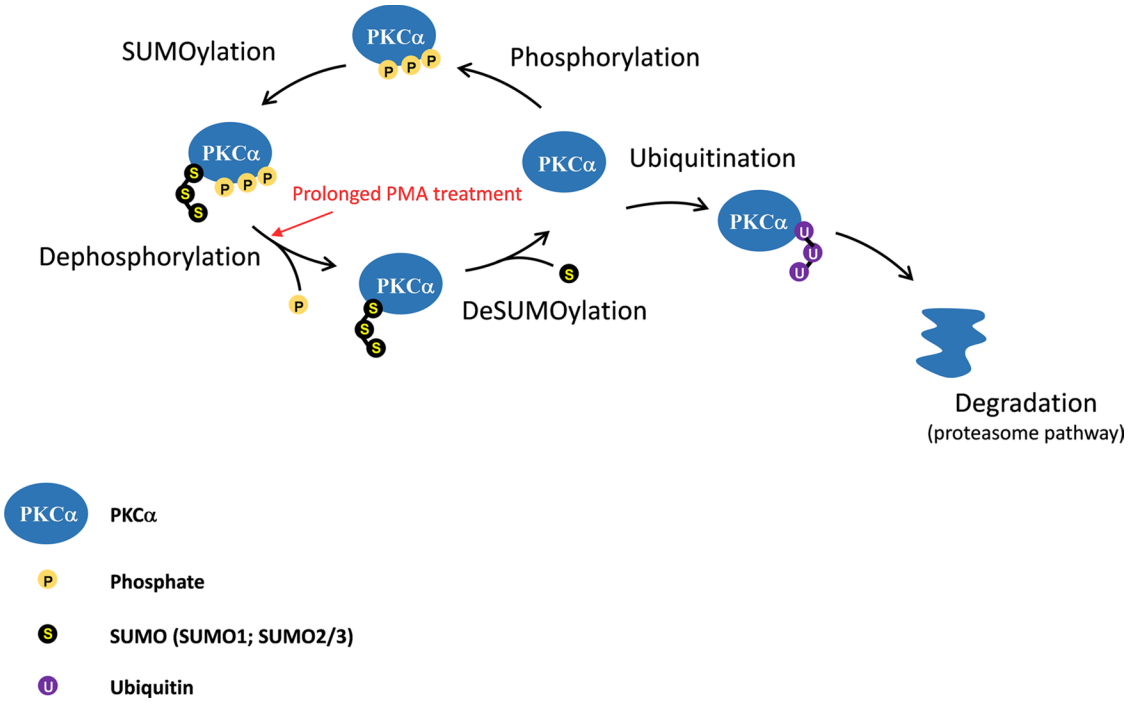

FIGURE 7: Model of the interplay among phosphorylation, sumoylation, and ubiquitination of PKC. Phosphorylation of PKC at the priming sites promotes its sumoylation, which inhibits its ubiquitination and thus stabilizes the PKC proteins. Activation of PKC by phorbol esters (e.g., PMA) leads to its dephosphorylation and desumoylation, which enhances its ubiquitination and subsequent degradation by the proteasome.

containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA). Cells were grown to 60–80% confluency and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with manufacturer's instructions. In most experiments, equimolar ratios of DNAs were used for cotransfection experiments. The total amount of plasmid DNA was adjusted with empty vector. Note that to ensure that the various transfectants expressed comparable levels of protein, a twofold amount of HA-tagged or green fluorescent protein (GFP)–tagged K465R PKCα plasmids was used for transfection in some of the corresponding experiments.

Plasmids, antibodies, and reagents

GFP-PKCα was a gift from Gerald W. Zamponi (University of Calgary, Calgary, Canada), Myc-SUMO2/3 was kindly provided by Jinke Cheng (Shanghai Jiao Tong University School of Medicine, Shanghai, China), and HA-Ub was a gift from Guanghui Wang (Soochow University, Suzhou, China). HA-PKCβI, HA-PKCδ, and HA-PKCε were purchased from Addgene (Cambridge, MA). Flag-SUMO1, RGS-SENP1, and RGS-SENP1m plasmids were previously described (Sun *et al.*, 2014). GFP-K465R PKCα and various HA-PKCα mutations (HA-6KR, 7KR, T497A, T497D, T638A, T638D, S657A, S657D, and K465R/S657D PKCα) were generated by a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Anti–phospho-PKC (pan; γThr-514), PKC (α/βII) (Thr-638/641), and PKC (pan; βII Ser-660; Cell Signaling Technology, Danvers, MA) were used to detect the phosphorylation of PKCα T497, T638, and S657. SENP1 antibody was from GeneTex (Irvine, CA). All other antibodies were as described previously (Sun *et al.*, 2014). CHX, MG132, chloroquine, and N-ethylmaleimide (NEM) were purchased from Sigma-Aldrich (St. Louis, MO). PMA was from Ascent Scientific (Cambridge, MA). Lambda protein phosphatase was from New England Biolabs (Ipswich, MA).

Immunoprecipitation and Western blot analysis

Immunoblotting was performed as described previously (Sun *et al.*, 2014) with modifications. After 24 h of transfection, CHO-K1 cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor cocktail (Sigma-Aldrich). NEM, 20 mM, and phosphatase inhibitor cocktail (Roche, Indianapolis, IN) were added to cell lysates for the detection of sumoylated and phosphorylated PKCα. The lysates were incubated with 2 μg of antibody for 3 h at 4°C and subsequently with 20 μl of protein A/G agarose beads (Pierce Biotechnology, Rockford, IL) for an additional 1 h. HA-tagged or Flag-tagged proteins were immunoprecipitated using 20 μl of anti–HA-agarose (Pierce Biotechnology) or anti-Flag M2 affinity gel for 3 h at 4°C. The beads were subsequently washed three times with lysis buffer and then boiled for 10 min in sample buffer and further analyzed by immunoblotting. The λ-PPase assay was performed as described previously (Huntwork-Rodriguez *et al.*, 2013) with modifications. Lysates were incubated with 1× phosphatase buffer, 2 mM $MnCl_2$, and 1600 U of λ-PPase (to dephosphorylate proteins) at 30°C for 2 h.

Denaturing immunoprecipitations were performed as previously described with minor modifications (Becker *et al.*, 2013). Briefly, cells were washed with ice-cold phosphate-buffered saline and lysed with denatured lysis buffer I (50 mM Tris-HCl, pH 6.8, 2% SDS, 40 mM dithiothreitol, and 5% glycerol) and boiled at 95°C for 15 min. The lysates were diluted 10-fold with denatured lysis buffer II (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% Nonidet P-40) and centrifuged at 13,000 × *g* for 8 min at 4°C. Then the supernatant was collected and incubated with the indicated primary antibodies at 4°C overnight. The immunoprecipitates were collected on protein A/G–Sepharose beads, followed by washing with washing buffer three times and boiling in sample buffer. The

bound proteins were separated by SDS–PAGE and subjected to Western blotting.

Cycloheximide chase assays

Protein degradation was assessed by CHX chase assays as described previously with minor modifications (Wang *et al.*, 2010). CHX was added to the culture (20 μg/ml final concentration) 24 h posttransfection, samples were taken at the indicated time points, and steady-state levels of protein of interest were visualized by Western blotting with appropriate antibodies as indicated.

Statistical analysis

Data are expressed as means ± SEM with statistical significance assessed by Student's *t* test for two-group comparison. $p < 0.05$ was considered a statistically significant difference.

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