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# CK2 phosphorylation of SAG at Thr10 regulates SAG stability, but not its E3 ligase activity

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## Abstract

Sensitive to Apoptosis Gene (SAG), a RING component of SCF E3 ubiquitin ligase, was shown to be phosphorylated by protein kinase CK2 at the Thr10 residue. It is, however, unknown whether this phosphorylation is stress-responsive or whether the phosphorylation changes its E3 ubiquitin ligase activity. To address these, we made a specific antibody against the phosphor-SAG$^{Thr10}$. Transient transfection experiment showed that SAG was phosphorylated at Thr10 which can be significantly inhibited by TBB, a relatively specific inhibitor of protein kinase CK2. To determine whether this SAG phosphorylation is stress-responsive, we defined a chemical-hypoxia condition in which SAG and CK2 were both induced. Under this condition, we failed to detect SAG phosphorylation at Thr10, which was readily detected, however, in the presence of MG132, a proteasome inhibitor, suggesting that the phosphorylated SAG has undergone a rapid degradation. To further define this, we made two SAG mutants, SAG-T10A which abolishes the SAG phosphorylation and SAG-T10E, which mimics the constitutive SAG phosphorylation. The half-life study revealed that indeed, SAG-T10E has a much shorter protein half-life (2 h), as compared to wild-type SAG (10 h). Again, rapid degradation of SAG-T10E in cells can be blocked by MG132. Thus, it appears that CK2-induced SAG phosphorylation at Thr10 regulates its stability through a proteasome-dependent pathway. Immunocytochemistry study showed that SAG as well as its phosphorylation mutants, was mainly localized in nucleus and lightly in cytoplasm. Hypoxia condition did not change their sub-cellular localization. Finally, an *in vitro* ubiqutination assay showed that SAG mutation at Thr10 did not change its E3 ligase activity when complexed with cullin-1. These studies suggested that CK2 might regulate SAG-SCF E3 ligase activity through modulating SAG's stability, rather than its enzymatic activity directly.

*Key words*: protein stability, CK2, SAG, E3 ubiquitin ligase, phosphorylation, apoptosis

*Abbreviations*: CHX, cycloheximide; CK2, protein kinase CK2; ROC1, Regulator of Cullin-1; Rbx1, RING box protein-1; SAG, Sensitive to Apoptosis Gene; SCF, Skp1, cullin, F-box protein complex.

## Introduction

The SAG/ROC-SCF E3 ubiquitin ligase, consisting of Skp1, Cullins, F-box proteins, and a RING domain containing protein, ROC/Rbx/SAG, is one of the well-defined E3 ubiquitin ligases in eukaryotes that mainly controls cell cycle progression [1, 2]. Sensitive to Apoptosis Gene (SAG) or Rbx2/ROC2, was originally identified as a redox inducible

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antioxidant protein that when over-expressed protected cells from apoptosis induced by redox agents [3, 4]. Indeed, in an *in vitro* system, bacterially expressed and purified SAG was found to be a redox sensitive protein that formed oligomers to buffer hydrogen peroxide. SAG binds to metal ions, zinc and copper, scavenges reactive oxygen species (ROS) and inhibits ROS-induced lipid peroxidation [4, 5]. Consistent with this, others have reported that SAG is a thiol-dependent peroxidase [6] that inhibited peroxynitrite-induced DNA damage [7]. In mammalian cells, SAG was inducible by redox agents as well as hypoxia and inhibited apoptosis induced by these stress conditions in human cancer cells [4, 8], in *in vivo* mouse brain [9], and in rat cardiomyocytes [10]. On the other hand, SAG was found to promote cell growth under serum starvation, possibly by promoting p27 degradation, whereas SAG down-regulation by antisense transfection inhibits cancer cell growth both in monolayer culture and in soft agar [11, 12]. Furthermore, in yeast, SAG is a growth essential gene. Knocking out ySAG induced cell death that can be fully rescued by human SAG [13]. Consistent to its anti-apoptotic and growth-promoting activity, SAG was found to be over-expressed in a subset of colon carcinoma tissues and in non-small cell lung carcinomas and a high expression of SAG correlates well with a poor patient survival [12, 14]. Besides its antioxidant feature derived from its high content of cysteine residues, SAG is the second member of Rbx/ROC/Hrt family, a key component of E3 ubiquitin ligase. Although the overall sequence identity is 53% between SAG and ROC1/Rbx1, seven out of eight cysteine or histidine residues that constitute the $C_3H_2C_3$ RING finger motif are identical [2]. Like ROC1/Rbx1, when complexed with Cul1, SAG shows ubiquitin ligase activity [13]. Thus, it appears that SAG is a dual function molecule that is an antioxidant when acting alone, but when accessible and complexed with other components of SCF acts as an E3 ligase by recruiting E2 and facilitating ubiquitin transfer from E2 to substrates [15].

Protein kinase CK2 (formerly casein kinase II or 2, CK2) consists of two catalytic (α and α') subunits and two regulatory $\beta$ subunits [16]. Evolutionarily conserved CK2 is a ubiquitously expressed and constitutively active Ser/Thr protein kinase [17]. Through phosphorylating protein substrates, CK2 regulates many biological processes including cell viability, cell cycle progression, neoplasia, and viral infection (for review, see [18, 19]). Recently, CK2 was found to be implicated in cell proliferation and cell survival or anti-apoptosis in response to diverse stress inducers as a cell protective mechanism. Over-expression of CK2α or CK2$\alpha\beta$ protected cancer cells from apoptosis induced by etoposide [20], whereas over-expression of a CK2α kinase-deficient mutant, CK2α-K68A, induced a marked inhibition of cell proliferation [21]. Likewise, inhibition of CK2 activity by CK2 inhibitor or decrease of CK2 concentration via antisense oligonucleotides induced apoptosis [22, 23] and sensitized tumor cells to TRAIL-induced apoptosis [24, 39]. Over-expression of CK2 was found in many human cancers including prostate [25], lung [26], head and neck [27], kidney [28], and breast [29], as well as experimental tumors [30]. All these make CK2 an appealing anti-cancer target. Indeed, several classes of CK2 inhibitors have been discovered and are being optimized [31], and approaches to molecular down-regulation of CK2 are being investigated to target CK2 in cancer cells [32].

Most recently, CK2 was found to bind to and phosphorylate SAG at the Thr10 residue [33, 34]. This phosphorylation appears to be important in regulating SCF E3 ligase activity, as evidenced by the change of the levels of its substrates IκBα and p27 by over-expression of SAG phosphorylation mutants [34]. However, it is unknown whether SAG phosphorylation by CK2 is a stress-responsive event and whether it directly induces ligase activities of SAG-SCF. To address these questions, we generated a specific antibody against phosphor-$SAG^{Thr10}$ and found that the $SAG^{Thr10}$ phosphorylation can be inhibited by a specific CK2 inhibitor, but rapidly degraded through a proteasome-dependent pathway. Furthermore, SAG phosphorylation did not change its E3 ubiquitin ligase activity.

## Materials and methods

### *Cell culture and drug treatment*

Human 293, HeLa, and DLD1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum at 37 °C in humidified $CO_2$/95% air. For CK2 inhibitor treatment, 293 cells were treated with 60 $\mu$M TBB [4,5,6,7-Tetrabromo-2-azabenzimidazole) (CalbioChem) for various periods of time 24 h post-transient transfection. For chemical-hypoxia exposure, subconfluent DLD1 cells were treated with 150 $\mu$M $CoCl_2$ for various periods of time up to 24 h and subjected to western blotting, CK2 activity assay, and immunofluorescent analysis. To determine proteasome-mediated degradation of SAG, cells were treated with MG132 (10 $\mu$M) for 4 or 8 h prior to being harvested for western blotting analysis.

### *Generation of phosphor-$SAG^{Thr10}$ antibody*

The anti-phospho-$SAG^{Thr10}$ antibody was produced by Zymed Inc. Briefly, the SAG peptide, *K*EDGEE<u>T(P)</u>CAL, corresponding to SAG residue 5–13, in which the threnine 10 is phosphorylated was synthesized by Tuft University

Core Facility. After HPLC purification, the phospho-peptide was coupled to KHL and injected into rabbits. The antibody generated was affinity purified first through a phosphorylated peptide column, then absorbed through a non-phospho-petide column.

*Generation of SAG mutants and DNA transfection*

Two SAG mutants, SAG-T10A and SAG-T10E were generated by PCR using FLAG-tagged or HA-tagged wild-type SAG as template. The primer pair used for FLAG-SAG-T 10A was FLAG-SAG-T10A1: 5′-GGGGTACCGCCACC-ATGGACTACAAGGACGACG ATGACAAGGCCGAC G-TGGAAGACGGAGAGGAAGCCTGCGCC-3′ and Xho-SAG02: 5′-CCGCTCGAGTCATTTGCCGATTCTTTGGA C-3′. The primer pair used for FLAG-SAG-T10E was FLAG-SAG-T10E1: 5′- GGGGTACCGCCACCATGGAC-TACAAGGACGACGATGACAAGGCCGACGTGGAAG-ACGGAGAGGAAGAATGCGCC-3′ and Xho-SAG02. The primer pair used for HA-SAG-T10A was HA-SAG-T10A1: 5′-GGGGTACCGCCACCATGTATCCATATGATGTTCCA-GATTATGCTGCCGACGTGGAAGACGGAGAGGAAGC-CTGCGCC-3′ and Xho-SAG02. The primer pair used for HA-SAG-T10E was HA-SAG-T10E1: 5′-GGGGTACCGC-CACCATGTATCCATATGATGTTCCAGATTATGCTGCC-GACGTGGAAGACGGAGAGGAAGAATGCGCC-3′ and Xho-SAG02. PCR fragments were digested with KpnI and XhoI and subcloned into pcDNA3, followed by DNA sequence confirmation. DNA tranfection was conducted using Lipofectamine 2000, according to manufacturer's instruction (Invitrogen).

*Western blotting and immunoprecipitation*

After drug treatment, cells were lysed using lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton, 5 mM EGTA, 5 mM EDTA, 1 mM NaF, 1 mM $Na_3VO_4$, freshly added protenase inhibitor tablet) and supernatants were collected by centrifugation. Proteins were separated on polyacrylamide gels in the presence of SDS and electrophoretically transferred onto nitrocellulose membrane. The membranes were blocked with 5% Blotto in TBST (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 1 hr at room temperature and probed with various antibodies against SAG, phosphor-$SAG^{Thr10}$, CK2$\alpha$, CK2$\beta$ (BD Transduction labs). Specific proteins were visualized by ECL (Amersham Biosciences) detection. For immunoprecipitation, cell lysates were prepared after drug treatment and subjected to immunoprecipitation using anti-SAG antibody by rotating at 4 °C overnight, followed by precipitation using protein agarose bead A/G (Santa Cruz) and subjected to western blotting using antibody against phosphor-$SAG^{Thr10}$.

*CK2 activity assay*

Human colon cancer DLD1 cells were treated with 150 $\mu$M $CoCl_2$ for various periods of time and cells pellets were washed with phosphate buffered saline (PBS) and subjected to cytoplasm and nucleus fractionation and CK2 activity assay as described [20, 35]. Briefly, cells are washed twice by suspension in ice-cold PBS and centrifugation at $100 \times g$. The pellet is then suspended in CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 0.3 M sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 4 mM vanadyl ribonucleoside complex, 1 mM phenylmethylsulfonyl fluoride (PMSF), and leupeptin 2 $\mu$g/ml. Following homogenization, the suspension is centrifuged for 5 min at $600 \times g$ at 4 °C. The sedimented material is kept for isolation of nuclear matrix. The cytoplasmic fraction was prepared from the supernatant fraction by centrifugation at $10{,}000 \times g$. For nuclear matrix Method C was followed (Tawfic *et al.* 1997 above) by suspending the initial pellet in ''extraction buffer'' consisting of 10 mM Tris–HCl, pH 7.4 at 25 °C, 10 mM NaCl, 3 mM $MgCl_2$, 1% Tween-40, 0.5% sodium deoxycholate, 4 mM vanadyl ribonucleoside complex, 1 mM PMSF, and leupeptin 2 $\mu$/ml. The mixture is incubated at 4 °C for 5 min, centrifuged at $600 \times g$ for 5 min, and the pellet is collected. It is resuspended in digestion buffer containing RNase A 100 $\mu$g/ml and DNase I 100 $\mu$g/ml. After incubation at 25 °C for 30 min, a solution of 1 M $(NH_4)_2SO_4$ is added to a final concentration of 0.25 M. The material is centrifuged at $600 \times g$ for 5 min, and the residue representing the nuclear matrix fraction is collected and suspended in TMED buffer. The CK2 activity assay for cytoplasmic or nuclear matrix fraction, and immunoblot analysis, was carried out as detailed previously [20].

*Immunoflurescence assay for SAG sub-cellular localization*

Subconfluent HeLa cells in 8-well glass chamber were transfected with HA-tagged wild-type SAG and two SAG mutants (SAG-T10A and SAG-T10E). After 24 h post transfection, the cells were treated with DMSO control, 150 $\mu$M $CoCl_2$, or 60 $\mu$M TBB for 16 h. The cells were fixed with 4% paraformaldehyde for 10 min at RT and permeabilized with 0.5% Triton X-100 for 2 min. The slides were blocked with 1% BSA in PBS for 1 h at RT; Primary antibody against HA-tag (HA.11 McAb, 1:1000, Covance, California, Cat #MMS-101P) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (1:100

dilution, Jackson, #115-095-146) in PBS containing 1% BSA and 0.05% Tween-20 were applied for 2 and 1 h, respectively; Nucleus was stained with DAPI (1 $\mu$M; Molecular Probes, D3571) in PBS for 10 min at RT. The slides were mounted with 120 $\mu$l of Prolong antifade reagent (Molecular probes, #p-7481).

*SAG half-life determination*

HeLa cells were transiently transfected with plasmids expressing SAG-wild type, SAG-T10A or SAG-T10E. In 38 h post-transfection, cells were treated with 10 $\mu$g/ml cycloheximide for various periods of time and subjected to western blotting analysis. In some experiment, proteasome inhibitor, MG132 (10 $\mu$M) was included.

*In vitro ubiquitination assay*

Ubiquitination activity assay was performed as described previously [13, 36]. First, bacterially expressed PK-ubiquitin was prepared using $Ni^{2+}$-NTA based affinity purification procedures (Qiagen). The recombinant protein, eluted with 0.5 M imidazole, was dialyzed against PBS for 4 h at 4 °C. PK-ubiquitin (7 $\mu$g) was then radio-labeled in a reaction mixture (20 $\mu$l) containing 20 mM Tris–HCl pH7.4, 12 mM $MgCl_2$, 2 mM NaF, 50 mM NaCl, 25 $\mu$M ATP, 5 $\mu$Ci of [$\gamma^{32}$P] ATP, 0.1 mg/ml BSA, and 1 unit of cAMP kinase (Sigma). The reaction mixture was incubated at 37 °C for 30 min, followed by heat-inactivation of kinase at 70 °C for 3 min.

To prepare cullin1-SAG E3 ubiquitin ligase, 293 cells were transiently co-transfected with cullin-1 in combination with HA-tagged wild-type SAG and SAG mutants. The cell pellet was resuspended in buffer A (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 0.5% NP40, 1 mM PMSF, 1 $\mu$g/ml antipain, and 1 $\mu$g/ml leupeptin) at 0.2 ml per plate and lysed by sonication (6 repetitive 20-s treatments). Buffer B (equal volume; 20 mM Tris–HCl, pH 7.4, 1 M NaCl, 0.2% NP40, 1 mM PMSF, 1 $\mu$g/ml antipain and 1 $\mu$g/ml leupeptin) was then added and the resulting mixture was rocked for 60 min at 4 °C prior to centrifugation at 14,000 rpm. To isolate the recombinant HA-SAG/CUL1, 293T cell extracts (1 mg of total protein) were then incubated with 10 $\mu$g of purified anti-HA monoclonal antibody (Sigma) for 1 h at 4 °C. Approximately 10–15 $\mu$l (packed volume) of protein A agarose (Santa Cruz) was added and the resulting mixture was rocked for 1 h at 4 °C. The beads were then washed sequentially three times with buffer C (mixture of buffer A plus B in equal volume), and three times with buffer D (25 mM Tris–HCl, pH7.5, 1 mM EDTA, 0.01% NP-40, and 10% glycerol) plus 0.05 M NaCl. The washed beads were used directly in the Ub ligation assay.

Ubiquitin ligase assay was performed by adding immunopurified recombinant HA-SAG-Cullin1, prepared as described above to a Ub ligation reaction mixture (30 $\mu$l) that contained 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM NaF, 10 nM Okadaic Acid, 2 mM ATP, 0.6 mM DTT, 1 $\mu$g $^{32}$P-Ub, 60 ng E1, and 300 ng E2 (Boston Biochem). The mixture was incubated at 37 °C for 60 min. The reaction mixture was then treated with 20 $\mu$l 4× concentrated Laemmli loading buffer and boiled for 3 min prior to 7.5% SDS-PAGE analysis.

# Results

*Characterization of a specific antibody against phosphor-SAG$^{Thr10}$*

Sensitive to Apoptosis Gene has been shown to be phosphorylated at Thr10 by CK2 protein kinase [33, 34]. To determine its physiological significance, we generated a phosphor-antibody against phosphor-SAG$^{Thr10}$ using a specific phospho-SAG peptide (at the Thr10 residue) by Zymed Laboratories, as described under the Materials and Methods. As an initial characterization, the 293 cells were transiently transfected with FLAG-tagged wild-type SAG or FLAG-tagged SAG-T10A mutant in which the Thr10 CK2 phosphorylation site [34] was mutated to alanine. The cell extracts were prepared and immunoprecipitated by beads-conjugated anti-FLAG antibody, followed by immunoblotting with this specific phospho-SAG$^{Thr10}$ antibody. As shown in Fig. 1, top panel, the antibody readily detected wild-type SAG (left lane), but barely detected SAG-T10A (right lane). Detection of phospho-SAG$^{Thr10}$ was only slightly blocked by a 100-fold excess of non-phospho-peptide (the second panel), but was completely blocked by a 100-fold excess of phospho-peptide (the third panel). Both wildtype SAG and SAG mutant were expressed equally well (the fourth panel) and an equal amount of protein (the bottom panel) was used for immunoprecipitation. The results indicated that this antibody detected phospho-SAG at the residue Thr10.

*Phosphor-SAG$^{Thr10}$ inhibition by CK2 inhibitor, TBB*

We next determined whether the level of phosphor-SAG$^{Thr10}$ would be inhibited by TBB, a specific CK2 inhibitor [37]. The 293 cells were transiently transfected with wild-type SAG (Fig. 2, lanes 2–4), along with pcDNA3 vector control (lane 1) and treated, 24 h post-transfection, with DMSO control (lanes 1 and 2) or TBB

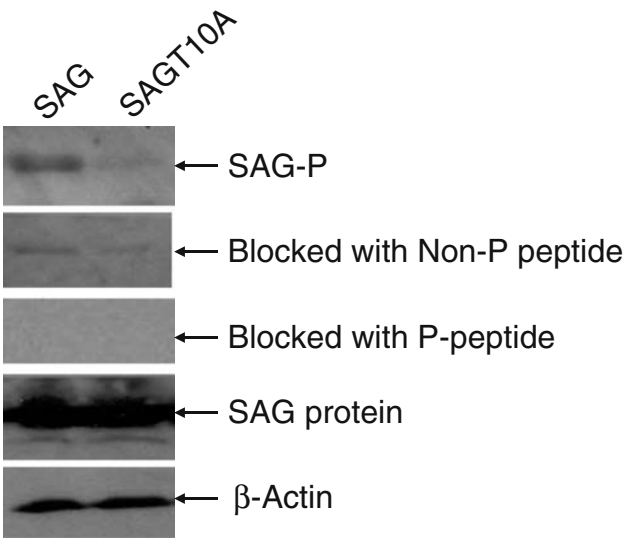


*Fig. 1.* Characterization of a phospho-SAG$^{Thr10}$ antibody: The antibody was raised against a phospho-SAG peptide (*k*EDGEET(P)CAL, corresponding to SAG residue 5–13) and affinity purified by Zymed laboratories. The 293 cells were transiently transfected with FLAG-tagged wild-type SAG and SAG-T10A mutant. After 36 h of transfection, cells were lysed with RIPA buffer containing phosphatase inhibitors and immunoprecipitated with bead-conjugated FLAG antibody. After 3X wash with RIPA buffer, beads were subjected to SDS-PAGE and immunoblotted with phospho-SAG$^{Thr10}$ antibody (at a 1:200 dilution). Peptide competition was carried out by incubating antibody (5 $\mu$g) with either non-phospho- or phospho-peptide (500 $\mu$g) for 30 min prior to being applied to immunoblot. The immunoblots were developed using Amersham ECL kit. To detect SAG expression and to ensure an equal amount of protein used for immunoprecipitation, an aliquot of cell lysate from each transfection prior to FLAG immunoprecipitation was subjected to a direct western analysis, probed with antibody against FLAG tag [4] and $\beta$-actin.

(60 $\mu$M) for a period of 2 or 4 h (lanes 3 and 4), followed by western blotting analysis. As shown in Fig. 2, while the levels of total transfected SAG and endogenous CK2$\alpha$ or CK2$\beta$ remained unchanged, a significant reduction of phosphor-SAG$^{Thr10}$ was observed 4 h after TBB treatment (lane 4). The results strongly suggest that when SAG is over-expressed, CK2 indeed phosphorylates SAG at Thr10 *in vivo*, which is blocked by a CK2 inhibitor.

### *Phosphor-SAG$^{Thr10}$ was detectable under chemical-hypoxia condition in the presence of proteasome inhibitor*

To determine the physiological significance of SAG phosphorylation by CK2, we next determined whether this phosphorylation occurs *in vivo* under physiological condition or in response to stress stimuli. After multiple unsuccessful attempts to detect phosphor-SAG$^{Thr10}$ in unstressed human tumor cells by a direct western blotting analysis or immunoprecipitation-coupled western blotting analysis

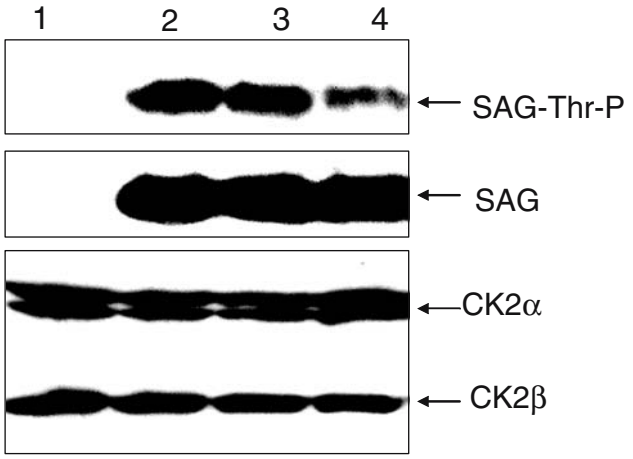


*Fig. 2.* SAG$^{Thr10}$ phosphorylation was inhibited by CK2 inhibitor: Human kidney embryonic 293 cells were transiently transfected with pcDNA3 control (lane 1), or wild-type SAG (lanes 2–4). After 24 h transfection, cells were treated with DMSO control (lanes 1 & 2), or CK2 inhibitor, TBB for 2 h (lane 3) or 4 h (lane 4), respectively. After drug treatment, cells were harvested and cell lysates prepared for immunoblotting analysis using antibodies against phosphor-SAG$^{Thr10}$, total SAG, CK2$\alpha$, or CK2$\beta$.

(data not shown), we went on to determine whether SAG phosphorylation occurs in responsive to stress condition such as hypoxia which has been previously shown to induce SAG in *in vivo* mouse brain tissues as well as in rat heart tissues and cultured cardiomyocytes [9, 10]. To confirm SAG induction by hypoxia in DLD1 colon cancer cells, we treated cells with a hypoxia-inducing agent $CoCl_2$ for various time periods. Cell lysates were prepared for western blotting analysis. As shown in Fig. 3A, SAG expression was relatively low at the basal level, started to increase 1 h post-$CoCl_2$ exposure and reached a 2–3-fold induction level throughout a period of up to 16 h. We next determined whether CK2 activity would be induced upon $CoCl_2$ hypoxia. It has been previously shown that CK2 was subjected to sub-cellular re-distribution in response to stress [20]. The total cell lysates, prepared from cells exposed to $CoCl_2$ for a variety of time periods, were fractionated into cytoplasm and nuclear matrix fractions. The CK2 enzymatic activity in the two fractions was measured as described [20]. Indeed, as shown in Fig. 3B, $CoCl_2$ treatment of DLD1 colon carcinoma cells induced the CK2 activity in cytoplasmic fraction. CK2 activity began to increase 3 h post-treatment, and reached the peak at 12–16 h. Thereafter, the activity decreased toward the basal level at 24 h post-treatment. No CK2 activity change was detected in nuclear matrix fraction at any time point (data not shown). Thus, CK2 activity in a particular cellular compartment, namely cytoplasm, was induced by $CoCl_2$.

Having established the induction of both SAG level and CK2 activity by $CoCl_2$, we went on to determine potential

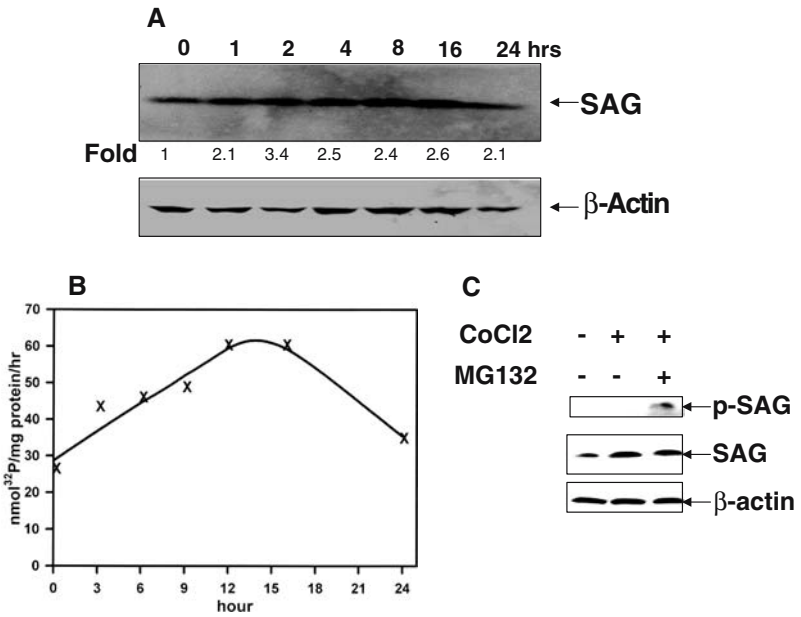


*Fig. 3.* Induction of SAG and activation of CK2 activity by $CoCl_2$: (A) SAG induction: Subconfluent DLD1 cells were subjected to serum starvation for 16 h prior to $CoCl_2$ (150 $\mu$M) treatment in the absence of serum for indicated times up to 24 h. Cells were harvested, lysed in Triton lysis buffer and subjected (150 $\mu$g) to immunoblotting analysis using antibodies against SAG or $\beta$-actin as a loading control. The fold-induction relative to the untreated control was shown after densitometry quantitation and $\beta$-actin normalization. (B) Induction of cytoplasmic CK2 activity by $CoCl_2$: DLD-1 colon carcinoma cells were plated into T150 flask on day 1 and subjected to 16 h serum starvation on day 2, followed by $CoCl_2$ (150 $\mu$M) treatment on day 3 for 3, 6, 9, 12, 16, and 24 h. Controls were left untreated (0 h time point). Cytoplasmic and nuclear matrix fractions were prepared and CK2 enzymatic activity measured as described previously [20]. The results are expressed as the rate of phosphorylation of a synthetic dodecapeptide substrate by CK2 and are described as nmol $^{32}$P/mg protein/hr. (C) SAG phosphorylation subjected to proteasome-dependent degradation: DLD1 cells were left untreated or treated with $CoCl_2$ alone or in combination with MG132 for 8 h. Cell lysates were prepared and subjected to western blotting analysis using antibodies against phosphor-SAG$^{Thr10}$, total SAG or $\beta$-actin for loading control.

SAG phosphorylation by CK2 under this hypoxia condition. DLD1 cells were left untreated or treated with $CoCl_2$ for 8 h in the absence or presence of MG132, a proteasome inhibitor. As shown in Fig. 3C, SAG phosphorylation was not detectable under the basal or $CoCl_2$-treated conditions, even though the SAG protein level was induced by $CoCl_2$ (lanes 1 and 2). However, in the presence of MG132, phosphorylated SAG is readily detectable upon $CoCl_2$ treatment (lane 3). This result suggested that a very small fraction of total SAG protein was phosphorylated and upon phosphorylation SAG is very unstable and undergoes a rapid degradation via a proteasome-dependent pathway.

### *SAG phosphorylation at Thr10 significantly shortened its protein half-life*

To further define this SAG phosphorylation-mediated SAG degradation, we made two SAG mutants, SAG-T10A (Thr → Ala) which abolished SAG phosphorylation or SAG-T10E (Thr → Glu) which mimicked a constitutive SAG phosphorylation and determined the protein half-life of wild-type SAG and SAG phosphor mutants. Three SAG expressing constructs were transiently transfected into HeLa cells in an individual basis. After 24 h post-transfection, cells were treated with 10 $\mu$M cycloheximide (CHX) to block new protein synthesis. Cells were harvested at various periods of time post-CHX treatment and cell lysates were subjected to western blotting analysis to determine the decay of SAG protein as a measurement of the protein half-life. As shown in Fig. 4 with densitometry quantitation data presented in Fig. 4D, the protein half-life was 10 h for wild-type SAG (A), as well as for SAG-T10A (B), indicating that abolishing SAG CK2 phosphorylation site had no effect on its protein stability. Interestingly, the protein half-life was significantly shortened to about 2 h in SAG-T10E mutant (C), which mimicked constitutive phosphor-SAG$^{Thr10}$. To determine whether shortened protein half-life was derived from an enhanced degradation through a proteasome-dependent pathway, we treated SAG-T10E transiently transfected cells with CHX for 4 h in the presence of MG132. Whereas a complete degradation of SAG-T10E was observed by CHX alone treatment, inclusion of MG132 significantly blocked its degradation (C). The results strongly suggested that SAG phosphorylation at Thr10 dramatically shortened its protein half-life, consistent with our observation made in DLD-1 cells with endogenously phosphorylated SAG (Fig. 3C).

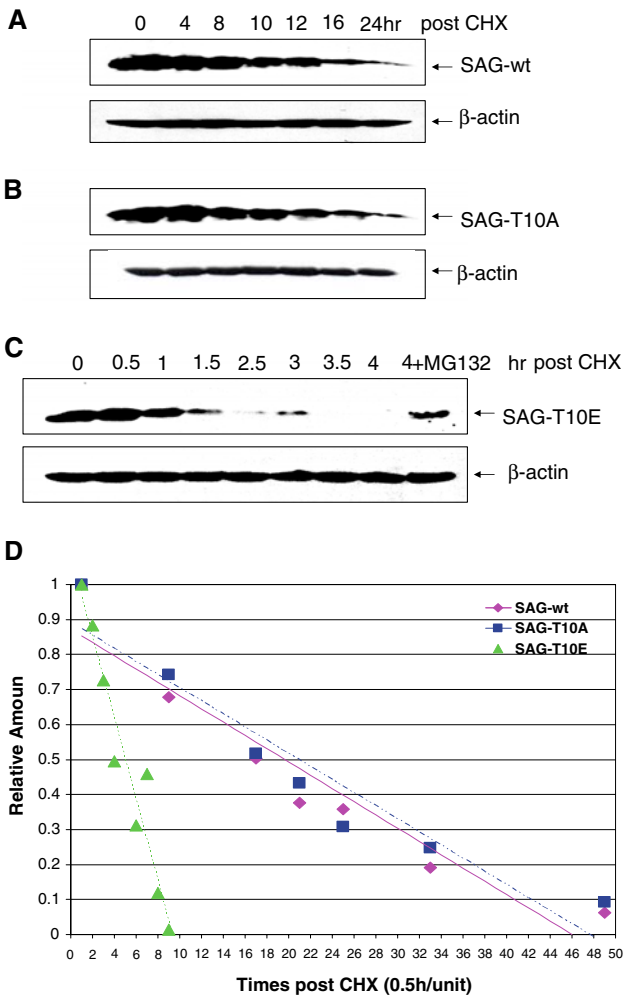


*Fig. 4.* SAG phosphorylation shortened its protein half-life: HeLa cells were transiently transfected with plasmid constructs expressing SAG wild type (A), SAG-T10A (B), or SAG-T10E (C), respectively. After 24 h post-transfection, cells were treated with 10 $\mu$M cycloheximide (CHX) for indicated periods of time. Cells were harvested and subjected to immunoblotting analysis using antibodies against FLAG tag or $\beta$-actin as loading control. Densitometric quantification was performed. The data after normalization with $\beta$-actin were plotted (D).

### *SAG phosphorylation at Thr10 did not change its sub-cellular localization*

We next determined whether the failure to detect SAG phosphorylation by CK2 in response to chemical-hypoxia can also be partly attributable to sub-cellular re-localization of SAG into nucleus. We have previously shown that SAG is localized to both the cytoplasmic and nuclear compartment in the cell [4]. If hypoxia induced a nuclear import of SAG, activated cytoplasmic CK2 would fail to phosphorylate nuclear SAG. To determine this, HA-tagged wild-type SAG, along with two SAG mutants, were transiently transfected into HeLa cells. Cells were treated with DMSO (control) or 150 $\mu$M $CoCl_2$ for 16 h and subjected to fluorescent-immunocytochemistry analysis. As shown in Fig. 5A and B, top panels, SAG-wildtype and SAG-T10E were localized in both cytoplasm and nucleus with nucleus localization dominant under normal growth condition. Neither hypoxia exposure (Fig. 5A and B, bottom panel), nor CK2 inhibitor treatment (not shown) was found to change this sub-cellular localization. Similar results were seen for SAG-T10A (data not shown). Thus, the failure to detect endogenous SAG phosphorylation by CK2 under hypoxia could be partially due to cellular compartmental separation between activated cytoplasmic CK2 and nuclear SAG.

### *SAG phosphorylation at Thr10 did not change its E3 ubiquitin ligase activity*

We next determined whether SAG phosphorylation by CK2 would regulate its E3 ubiquitin ligase activity, using an *in vitro* ubiquitination assay as described [13, 36]. HA-tagged wild-type SAG and two phosphor-$SAG^{Thr10}$ mutants were transiently co-transfected with cullin-1 into 293 cells. HA-SAG-Cul1 complex were immunoprecipitated by anti-HA antibody and used as the E3 source in a reaction mixture containing $^{32}$P-labeled ubiquitin, E1 and E2 (Cdc34). As shown in Fig. 6A, slow-migrating bands, representing ubiquitinated species were detected in wild-type SAG transfected cells only in the presence of E1 and E2 (lane 3, compared to lanes 1 & 2). Served as a negative control, SAG-MM10, a RING mutant previously shown to be inactive as an E3 ligase [13], again showed no such activity (lane 6), nor did the pcDNA3 vector control (lane 7). Both phosphor-$SAG^{Thr10}$ mutants, SAG-T10A and SAG-T10E were equally active as the wild type SAG in promoting poly-ubiquitination (lanes 4 and 5). Amount of each protein used was relatively equal as shown in Fig. 6B. The results strongly suggested that SAG phosphorylation by CK2 would change its protein stability, but not its enzymatic activity.

## Discussion

Although SAG E3 ligase and CK2 protein kinase belong to biochemically different enzymatic category, there are a few similarities between the two proteins with regard to their expression, stress-responsiveness, and biological functions. These include: (1) both proteins are inducible by stress stimuli; (2) both are over-expressed in a number of human cancer cells; (3) both promote cell proliferation; and (4) they both inhibit apoptosis [2, 18, 38]. Mechanistically, via its antioxidant activity, SAG protects apoptosis through

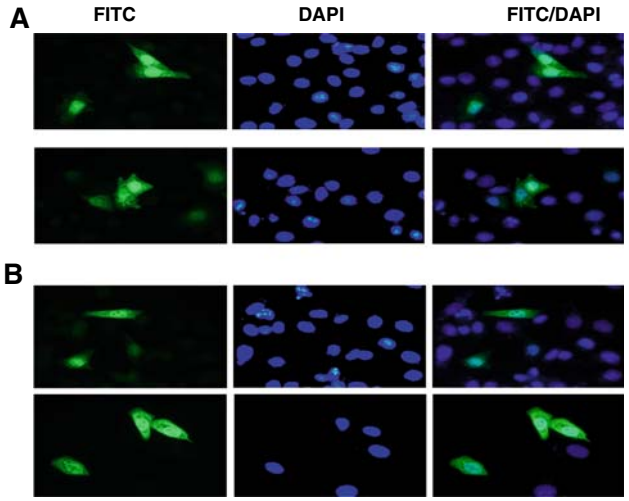


*Fig. 5.* Sub-cellular localization of SAG upon phosphorylation: HeLa cells were seeded in 8-well chamber tissue culture slide and transiently transfected next day with plasmid constructs expressing HA-tagged wild-type SAG (A) or SAG-T10E (B), respectively. After 24 h post-transfection, cells were treated with DMSO (top panels), or with 150 $\mu$M $CoCl_2$ for 16 h (bottom panels), followed by staining with HA-Ab-FITC conjugated secondary antibody (left panels) as described under the Materials and Methods. DAPI staining was used to show nucleus (middle panels). Overlay of FITC/DAPI was shown in the right panels.

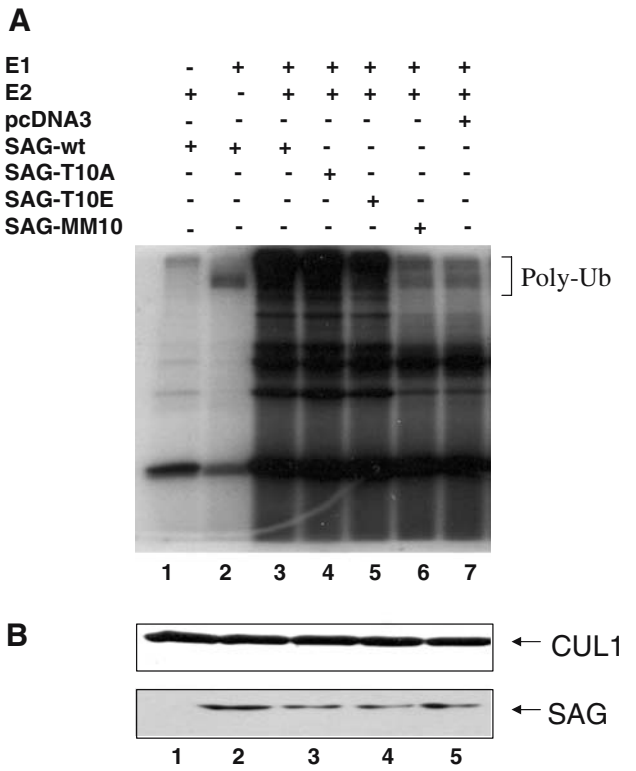


*Fig. 6.* No change in SAG E3 ubiquitin ligase activity upon SAG phosphorylation: (A) Ligase activity assay: Plasmid construct expressing cullin-1 was co-transfected into 293 cells, along with pcDNA 3 (lane 7), HA-tagged SAG wild type SAG, SAG-wt (lanes 1–3), SAG-T10A (lane 4), SAG-T10E (lane 5) or SAG RING mutant, SAG-MM10 (lane 6). HA-immunoprecipitated cullin1-SAG complex was used as E3 ligase in a reaction mixture containing $^{32}$P-labeled ubiquitin, E1, E2, and ATP. Poly-ubiquitination was resolved in a PAGE gel, followed by autoradiography. (B) Expression of cullin-1 and SAG: Cell extracts prepared from cullin1/SAG cotransfection were subjected to immunoblotting analysis using antibodies against cullin1 and SAG. The lane arrangement was following: lane 1, cullin-1/pcDNA3; lane 2, cullin1/SAG-wt; lane 3, cullin1/SAG-T10A; lane 4, cullin1/SAG-T10E; and lane 5, cullin1/SAG-MM10.

scavenging ROS and prevents cytochrome c release and caspase activation [4, 5]. CK2 also influences mitochondria and caspase activity [39]. The anti-apoptotic function of CK2 also appears to be associated with phosphorylation of several CK2 substrates. (A) CK2-mediated BID phosphorylation prevented BID cleavage by caspase 8 and BID translocation to mitochondria where it induces cytochrome c release [40]. (B) CK2 phosphorylation of ARC, an anti-apoptotic protein, targeted ARC to mitochondria where it inhibits caspase 8 [41]. And (C) CK2-induced activation of NF-$\kappa$B [29], an anti-apoptotic transcription factor [42–44]. A recent study has shown that SAG is a substrate of CK2 and SAG phosphorylation by CK2 modulated its function, although detailed mechanism remained to be determined [33, 34]. Thus, it is interesting and important to elucidate the nature of CK2-SAG cross talk and its potential functional consequences.

To this end, we showed here that indeed, under overexpression conditions, SAG is phosphorylated by CK2, and the phosphorylation was inhibited by a specific CK2 inhibitor. However, we were unable to detect such a phosphorylation under physiological or stressed conditions in the absence of MG132. Several possibilities exist to explain this phenomenon. First, CK2 phosphorylation of SAG at the Thr10 might be a rare or infrequent event that occurred in cells under unstressed or even stressed conditions, thus being beyond the detection limit of the antibody; A second possibility is that phosphor-SAG at the Thr10 is a short-lived protein and is degraded rapidly via a proteasome-dependent manner upon phosphorylation as shown in cells treated with $CoCl_2$-MG132 combination and cells transfected with SAG phosphor-mimic mutant, SAG-T10E; Third, CK2 and SAG may be partially separated in different cell compartments upon hypoxia stimulation such that hypoxia-induced cytoplasmic CK2 activity may have a limited access to a majority of SAG localized in the nucleus. Thus, although SAG, upon CK2-mediated phosphorylation, undergoes a rapid and proteasome-dependent degradation, the fact that hypoxia induces both the SAG level and CK2 activity suggested that only a small fraction of SAG was phosphorylated by CK2 for targeted degradation due to major compartmental separation of SAG and CK2.

Furthermore, our unpublished data suggest that hypoxia-induced SAG expression occurs mainly at the transcriptional level through HIF-1 transcription factor (Tan *et al.*, manuscript in preparation).

It is worth noting that intracellular CK2 shuttling has been proposed as a mechanism of CK2 functional regulation [45–47]. Intracellular shuttling of CK2 is a dynamic process in response to diverse stimuli. For example, removal of growth or survival factors promotes shuttling of CK2 from the nuclear compartment to the cytoplasmic compartment while the reverse occurs in the presence of growth or survival stimuli. Certain chemical and physical stress stimuli promote the translocation of CK2 from cytoplasm to the nucleus as a survival response [18]. In the present work, we have noted that hypoxia induced CK2 activity in the cytoplasm fraction, a phenomenon that has not been described previously. The mechanism of this induction of CK2 is unclear at present, but could represent shuttling of the kinase from other loci.

It has been previously reported that in HeLa stable cells over-expressing SAG-T10A or SAG-T10E, the levels of I$\kappa$B and p27, two known substrates of SAG-SCF E3 ligase were either accumulated or reduced upon serum starvation, respectively [34]. This would suggested that CK2 mediated SAG phosphorylation might have a positive effect on SAG's ligase activity. Our finding that SAG-T10A was indistinguishable from wild-type SAG in its protein half-life and E3 ligase activity, whereas SAG-T10E had a much shorter protein half-life, while maintaining a similar ligase activity, appeared to suggest that CK2 negatively regulated SAG activity by promoting its degradation. This discrepancy might be derived from different experimental conditions, such as cell status (stable clone vs. transient transfection) or cell growth conditions (serum starvation vs. normal serum); it may be noted that removal of serum results in translocation of CK2 from the nucleus to the cytoplasm [48]. Finally, the fact that two phosphor-SAG$^{Thr10}$ mutants still retain their SAG E3 ubiquitin ligase activity when complexed with cullin1 clearly demonstrated that CK2 mediated SAG phosphorylation did not directly change its enzymatic activity, as predicted by the presence of an intact RING structure in these mutants. We, therefore, concluded from this study that potential regulation of SAG activity by CK2-mediated phosphorylation occurs most likely at the level of the protein stability, not its ligase activity *per se*. This is analogous to other examples where CK2 mediated phosphorylation of proteins has been found to either increase (e.g., protein B23, c-myc) or decrease (e.g., I$\kappa$B, PTEN) their stability [49–52]. The *in vivo* significance of SAG phosphorylation will only be resolved by a mouse SAG-T10A knock-in study, as demonstrated by others for p27 gene [53, 54].

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