Sgt1 Dimerization Is Negatively Regulated by Protein Kinase CK2-mediated Phosphorylation at Ser<sup>361</sup>*

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The kinetochore, which consists of centromere DNA and structural proteins, is essential for proper chromosome segregation in eukaryotes. In budding yeast, Sgt1 and Hsp90 are required for the binding of Skp1 to Ctf13 (a component of the core kinetochore complex CBF3) and therefore for the assembly of CBF3. We have previously shown that Sgt1 dimerization is important for this kinetochore assembly mechanism. In this study, we report that protein kinase CK2 phosphorylation is important for this kinetochore assembly mechanism.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

Yeast Strains and Medium—Table 1 lists the genotypes of yeast strains used in this study. The medium for yeast growth and sporulation was prepared using previously described methods (31). Yeast transformation was done according to the method of Ito <em>et al.</em> (32). Strains that expressed tagged proteins were generated according to the procedure of Longtine <em>et al.</em> (33). Regions that encoded Myc tags were inserted at the 3′-end of the endogenous locus.

Plasmid Construction and Primers—Table 2 lists the plasmids used in this study. Details about their construction (34) and primer sequences are available upon request.

Antibodies—Anti-Skp1, anti-Sgt1, and anti-Hsp82 antibodies were used as previously described (21, 24, 35). Anti-hemagglutinin (HA; Roche Applied Science), anti-Myc (Roche Applied Science), anti-GST (Abcam), and anti-His<sub>6</sub> (Qiagen) antibodies were purchased.

Protein Expression and Immunoprecipitation—Immunoprecipitation using yeast lysates was performed as described previously (24). His<sub>6</sub>-Sgt1 and GST-Sgt1 proteins were expressed and purified according to the manufacturer’s instructions, as previously described (24).

Two-dimensional Gel Electrophoresis—Myc-tagged Sgt1 was immunoprecipitated from yeast cell lysates using an anti-Myc antibody. Isoelectric focusing was performed with a 17-cm immobilized pH 3–10 gradient strips (Bio-Rad), following the manufacturer’s instructions. Gel electrophoresis was performed in a Bio-Rad PROTEAN Plus Dodeca cell.

* This work was supported, in whole or in part, by National Institutes of Health, NCI, Cancer Center Support Grant CA21765 and National Institutes of Health Grant GM68418. This work was also supported by the American Lebanese Syrian Association Charities.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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2 The abbreviations used are: HA, hemagglutinin; GST, glutathione S-transferase.
TABLE 1

Yeast strains used in this study

| Strain    | Genotype                  | Reference |
|-----------|---------------------------|-----------|
| YPH499    | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 | 49        |
| Y1662     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 prsA14-3myc-SGT1, prsA16-3HA-SGT1 |           |
| Y1684     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 prsA14-3myc-SGT1, prsA16-3HA-SGT1, sgt1-S361A, sgt1-S361D |           |
| Y1686     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 sgt1-S361A, LEU2 CFIIICEN3L.YPH983 TRP1 SUP11 |           |
| Y1761     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 sgt1-S361A, LEU2, CFIIICEN3L.YPH983 TRP1 SUP11 |           |
| Y1734     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 CKA1-ymc |           |
| Y1736     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 cka1::His3MX6 prsA14-3HA-Sgt1 pRS415-3myc-Sgt1 |           |
| Y26       | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 Sgt1-LEU2 CFIIICEN3L.YPH983 TRP1 SUP11 |           |
| YKK54     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 prsA14-3HA-Sgt1, prsA16-3HA-Sgt1, sgt1-S361A |           |
| Y1773     | mata ure3-52 topd1 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 sgt1::His3MX6 CFIIICEN3L.YPH983 TRP1 SUP11 |           |
| Y1775     | mata ure3-52 topd1 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 cka1::His3MX6 sgt1::3LEU2 |           |
| Y1870     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 sgt1::His3MX6 prsA14-3myc-Sgt1 |           |
| Y1871     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 sgt1::His3MX6 prsA14-3myc-Sgt1, sgt1-S361A |           |
| Y1872     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 prsA14-3myc-Sgt1, sgt1-S361A |           |
| Y1873     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 cka1::His3MX6 prsA14-3myc-Sgt1 |           |
| Y1874     | mata ure3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δ1 cka1::His3MX6 prsA14-3HA-sgt1-S361A, prsA14-3myc-sgt1-S361A |           |

* Strains for which no reference is given were generated during this study.

TABLE 2

Plasmids used in this study

| Plasmid       | Relevant characteristics | Reference |
|---------------|--------------------------|-----------|
| B1354         | pRS416-3HA-SGT1 URA CEN  | 30        |
| B1380         | pRS415-3myc-SGT1 LEU CEN |           |
| B1367         | pDEST17-HIS-SGT1         | 30        |
| B940          | pDESt5-GST-SGT1          |           |
| B1657         | pDESt5-GST-sgt1-S361A    |           |
| B824          | pDESt5-GST1-KG71         |           |
| B942          | pDESt5-GST-hsgt1-A-S299A | 30        |
| B191          | pRS414-3myc-SGT1 TRP CEN |           |
| B1374         | pRS414-3myc-sgt1-S361A TRP CEN |           |
| B1375         | pRS414-3HA-Sgt1-S361D TRP CEN |           |
| B1377         | pRS414-3HA-sgt1-S361A URA CEN |           |
| B1378         | pRS414-3HA-sgt1-S361D URA CEN |           |
| B1699         | pRS414-3myc-sgt1-S361A LEU CEN |           |

* Plasmids for which no reference is given were generated during this study.

Immunoprecipitated Sgt1-Myc appeared as two major spots on the two-dimensional gel (Fig. 1A, top left), and phosphatase treatment substantially diminished the low pH spot (Fig. 1A, top right), indicating that the low pH spot corresponds to phosphorylated forms. An unphosphorylated mutant sgt1-S361A protein showed a reduced low pH spot, indicating that Ser^361 is phosphorylated in vitro (Fig. 1A, bottom left). Phosphatase treatment reduced the low pH spot of sgt1-S361A protein, suggesting that there are other phosphorylation sites.

We also found that GST-Sgt1 and His^10-Sgt1 were phosphorylated by human CK2 in vitro (Fig. 1B and Figs. S2 and S3). Sgt1 was phosphorylated with 0.6 mol of phosphate/mol of substrate (Fig. 1C). The $K_a$ and $V_{max}$ of CK2 for the phosphorylation of Sgt1 were 288 $\mu$mol and 4.8 $\mu$mol/min/mg (Fig. 1D), respectively, which are within the same range as that for known CK2 substrates (37–39), indicating that Sgt1 is a suitable substrate for CK2 in vitro.

CK2 did not efficiently phosphorylate the sgt1-S361A mutant protein (Fig. 2A), indicating that the S361A on Sgt1 is a phosphorylation site for CK2 in vitro.

Serine 361 is within the consensus site that is phosphorylated by CK2 (Fig. 2B), and this residue is conserved in humans and corresponds to serine 299 in human SGT1A (Fig. 2B). Nowotny et al. (40) reported that an oligopeptide containing this serine residue, human SGT1A (263–333), is phosphorylated by CK2 in vitro. A search at PhosphoBase (41) revealed that in human SGT1A (263–333), residue Ser^299 can be phosphorylated by CK2. Human SGT1A (263–333) oligopeptide containing the E302K mutation was also efficiently phosphorylated, suggesting that the CK2 phosphorylation site might be different from that identified by computer prediction. However, whether Ser^299 is a CK2 phosphorylation site has not been directly tested. Therefore, we made the human SGT1A-S299A protein and found that CK2 did not phosphorylate the mutant protein as efficiently as the wild-type protein (Fig. 2C). This finding strongly suggests that Ser^299 is a CK2 phosphorylation site in vitro.

Inhibition of Sgt1 Dimerization by Phosphorylation of Ser^361

To examine the possible effect of phosphorylation of Ser^361, we...
generated a nonphosphorylated mutant (S361A) strain and a phosphorylation-mimic mutant (S361D) strain. The S361A mutant protein bound to itself and Skp1 in vivo (Fig. 3A). However, the S361D protein did not bind to either itself or Skp1 efficiently, suggesting that Ser361-phosphorylated Sgt1 cannot form dimers effectively. Interestingly, the sgt1-S361D but not sgt1-S361A mutation was lethal when expressed in vivo (Fig. 3B). These results suggest that the phosphorylation of Ser361 inhibits Sgt1 dimerization, Sgt1 dimerization is essential for viability, and thus that CK2 negatively regulates Sgt1 dimerization.

To confirm this hypothesis in vivo, we examined whether the CBF3 complex could be formed in sgt1-S361D mutant cells. Because the sgt1-S361D mutation is lethal, we used a conditional SGT1-null mutant by using N-degron-4HA-Sgt1 expressed under the control of a repressible promoter (25) to remove the N-degron-wild-type protein but not the Myc-tagged mutant protein from cells that were used for the CBF3 complex formation.
band shift assay (8). In this system, copper induces the expression of Ubr1 and Rox1. Ubr1 is a ubiquitin ligase that binds proteins containing an N-degron and facilitates their ubiquitination and subsequent destruction by the 26 S proteasome. Rox1 represses the transcription of the ANB1 promoter controlling the expression of N-degron-Sgt1. Thus, the addition of copper both blocks the expression of N-degron-Sgt1 and triggers its proteolysis. The levels of N-degron-Sgt1 decreased after CuSO4 was added and were substantially lower after a 4-h incubation, but the levels of Myc-tagged Sgt1 and Myc-tagged sgt1-S361D mutant protein did not change (Fig. 3C, right). The cells in which Sgt1-Myc or sgt1-S361D-Myc were expressed and N-degron-Sgt1 was depleted were used for the CBF3 band shift assay. Although the viability after an 8-h incubation was substantially reduced, that after a 4-h incubation appeared to be high enough to assess CBF3 activity (Fig. 3D). The CBF3 complex was not formed efficiently in cell lysates containing only sgt1-S361D-Myc (Fig. 3C, left), suggesting that the phosphorylation of Ser^{361} inhibits the formation of the CBF3 complex.

**CK2 Inhibits Sgt1 Dimerization**

Next, we examined whether Sgt1 is phosphorylated by CK2. We found that Sgt1 interacted with Cka1 (a subunit of yeast CK2) and that the low pH spot diminished in cka1Δ mutant cells (Fig. 4B). These results strongly suggest that CK2 phosphorylates Sgt1 in vivo.

The phosphorylation of Ser^{361} appears to negatively regulate Sgt1 dimerization; thus, if Ser^{361} is phosphorylated by CK2 in vivo, then the amount of Sgt1 dimers should be greater in yeast casein-kinase mutants than in wild-type cells. Indeed, Sgt1 dimerization increased in cka1Δ mutant cells (Fig. 4C), and Skp1 binding also increased (Fig. 4D). Furthermore, sgt1-S361A dimerization or Skp1 binding did not increase in cka1Δ mutant cells, indicating that the stimulation of dimerization by reduction of CK2 activity is dependent on Ser^{361} phosphorylation (Fig. 4C). Consistent with these results, the deletion of cka1 suppressed the benomyl sensitivity of the sgt1::3 mutant (Fig.
**CK2 Inhibits Sgt1 Dimerization**

**A** IP (anti-myc)

| Input | Pellet |
|-------|--------|
| myc-Sgt1 | myc-Sgt1 |
| HA-Sgt1 | HA-Sgt1 |
| S361S | S361S |
| S361A | S361A |
| S361D | S361D |

**B**

- LEU
- 5-FOA

**C**

![CBF3](image)

**D**

![YPD SC-TRP h in CuSO4](image)

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**FIGURE 3. Phenotypes of the sgt1-S361D mutant.** A mimic of Sgt1 phosphorylation at Ser361 inhibits Sgt1-Sgt1 and Sgt1-Skp1 binding. Sgt1-S361S (wild type), Sgt1-S361A, or sgt1-S361D was expressed as HA-tagged and Myc-tagged proteins from two different plasmids (pRS416-HA and pRS414-Myc). Sgt1-S361S was expressed in the Y1681 strain, Sgt1-S361A in the Y1682 strain, and sgt1-S361D in the Y1684 strain. (All three strains were derived from the YPH499 strain.) The YPH499 strain carrying only pRS416-HA-SGT1-S361S was used as a negative control (leftmost lane). B, mimic of Sgt1 phosphorylation at Ser361 is lethal. Yeast cells that were haploid for SGT1 (Y26), sgt1-S361A (Y1686), or sgt1-S361D carrying SGT1 on a URA/CEN plasmid (Y1687) were streaked onto plates that contained Sc+ 5-fluoroorotic acid (5-FOA) or Sc-Leu. Plates were incubated at 25 °C for 3 days. C (right), the addition of 0.5 mM CuSO4 to strain CDY26 (N-degron-Sgt1) induced the proteolysis of N-degron-4HA-Sgt1 after either a 4- or 8-h incubation with CuSO4, as determined by immunoblotting with anti-HA antibodies, but Myc-tagged Sgt1 or sgt1-S361D was not affected. Left, protein extracts of the indicated strains were collected at the indicated time points after adding CuSO4. Cell extracts (40 μg) were subjected to the band shift assay to examine the CBF3 assembly activity. Competitor CEN DNA (100 or 200 μg) or mutant CEN DNA (CCG motif at CDEIII region is mutated to CCC) was added to CuSO4-untreated extracts of each cell type. D, survival of cells after a 4- or 8-h incubation with 0.5 mM CuSO4, as determined by a dilution-spotting assay. The numbers of colonies that were spotted onto the indicated plates were as follows: 1.25 × 10^6, 2.5 × 10^6, 5 × 10^5, 1 × 10^5, and 2 × 10^4. Plates were incubated at 30 °C for 2 days. IP, immunoprecipitation.

4D) (21, 30). These results strongly suggest that CK2 phosphorylates Sgt1 at Ser361 and negatively regulates Sgt1 dimerization in vivo.

**DISCUSSION**

In this study, we have shown that Sgt1 dimerization, which is required for kinetochore assembly in budding yeast, is regulated negatively by CK2-mediated phosphorylation.

**CK2 Negatively Regulates Sgt1 Dimerization**—CK2 is a serine/threonine protein kinase that is ubiquitous in eukaryotes (42, 43). The enzyme is composed of a catalytic subunit a and a regulatory subunit b that form a native a₂b₂ holoenzyme (42, 43). CK2 of the budding yeast consists of two catalytic subunits, a and a', and two regulatory subunits, b and b', which are encoded by the CKA1, CKA2, CKB1, and CKB2 genes, respectively (44). Individual deletion of either CKA1 or CKB2 does not show any significant phenotypes, but their simultaneous deletion is lethal (45).

To demonstrate that Ser361 is phosphorylated by CK2 in vivo, we attempted to generate antibodies against phosphorylated Ser361. However, unfortunately, antibodies produced using a phosphorylated peptide were not specific for the phosphorylated form and recognized the unphosphorylated form even after affinity purification. Therefore, we used indirect approaches to answer this question. Two-dimensional gel analysis revealed that Sgt1 is phosphorylated by CK2. When CK2 activity was altered by deletion of Cka1 but not Cka2 or Ckb2, Sgt1 dimerization and Skp1 binding were stimulated (data not shown). It has been previously reported that different combinations of subunits exhibit properties typical for CK2 but differ in substrate specificity and sensitivity to inhibitors, which suggests that each CK2 isomer may regulate different processes or may have different mechanisms of regulation (46). Thus, the regulation of Sgt1 dimerization may be a specific function of Cka1.

Because the sgt1-S361D mutant is lethal, we had to perform coimmunoprecipitation experiments to test the dimerization activity of the sgt1 mutant proteins in the presence of endogenous wild-type Sgt1 (Fig. 3A). Since the sgt1-S361D protein presumably binds more efficiently to the wild-type Sgt1 than to sgt1-S361D, the dimerization activity detected by co-immunoprecipitation might be an underestimation.

The stimulation of Sgt1 dimerization by deletion of Cka1 was dependent on Ser361. The alteration of CK2 activity by deletion...
of Cka1 suppressed the benomyl sensitivity of the sgt1-3 mutant, and Sgt1 associated with Cka1 in vivo. Therefore, we conclude that CK2 negatively regulates Sgt1 dimerization by phosphorylating Ser361, which in turn represses kinetochore assembly. However, the biological significance of why Sgt1 dimerization needs to be negatively regulated is unknown and needs further analyses. A monomer form might have a different function.

Another interesting observation is that Ser361 is outside the Sgt1 dimerization domain but within the SGS domain (amino acids 307–395). Our binding analysis suggested that an inhibitory activity by affecting the structure of the SGS domain. Highly Conserved Kinetochore Function of Sgt1—The human homolog of Sgt1 can rescue the yeast sgt1-null mutant (21), suggesting that the function of Sgt1 is conserved between humans and yeast. Sgt1 interacts with Hsp90 in yeast and human cells (28, 47). CENP-I, CENP-F, and Hec1, but not CENP-C, are absent from kinetochores in human cells depleted of SGT1 or HSP90 or treated with 17-AAG (an HSP90 inhibitor) (28, 29). This result strongly suggests that the human SGT1-HSP90 complex is crucial for assembly of the human kinetochore. Therefore, the kinetochore function of Sgt1 is evolutionarily conserved between humans and yeast.

However, Nyarko et al. (48) have shown by gel filtration chromatography that human SGT1A does not form dimers efficiently. Our recent analytical ultracentrifuge experiments have also revealed that human SGT1A exists mainly as a monomer (30). Further studies are required to determine whether human SGT1 is phosphorylated by CK2 in vivo and, if so, what role phosphorylation of this protein plays.

Acknowledgments—We thank V. Measday and R. Kitagawa for helpful comments, members of the Kitagawa laboratory for stimulating conversation and advice, V. Pagala and X. Ding for two-dimensional gel electrophoresis, D. Huang and J. Easton for kinetics experiments, and Vani Shanker for editing the manuscript.

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