Cdc25A is a dual specificity protein phosphatase that activates cyclin/cyclin-dependent protein kinase (Cdk) complexes by removing inhibitory phosphates from conserved threonine and tyrosine in Cdks. To address how Cdc25A promotes apoptosis, Jurkat cells were treated with staurosporine, an apoptosis inducer. Upon staurosporine treatment, a Cdc25A C-terminal 37-kDa fragment, designated C37, was generated by caspase cleavage at Asp-223. Thr-507 in C37 became dephosphorylated, which prevented 14-3-3 binding, as shown previously. C37 exhibited higher phosphatase activity than full-length Cdc25A. C37 with alanine substitution for Thr-507 (C37/T507A) that imitated the cleavage product during staurosporine treatment interacted with Cdc2, Cdk2, cyclin A, and cyclin B1 and markedly activated cyclin B1/Cdc2. The dephosphorylation of Thr-507 might expose the Cdc2/Cdk2-docking site in C37. C37/T507A also induced apoptosis in Jurkat and K562 cells, resulting from activating cyclin B1/Cdc2 but not Cdk2. Thus, this study reveals that Cdc25A is a pro-apoptotic protein that amplifies staurosporine-induced apoptosis through the activation of cyclin B1/Cdc2 by its C-terminal domain.

Cdc25A is one of the three members of the mammalian Cdc25 protein phosphatase family. It is a dual specificity protein phosphatase and a positive cell cycle regulator. Cdc25A activates cyclin/cyclin-dependent protein kinase (Cdk) complexes, which are central cell cycle regulators, by removing inhibitory phosphates from the conserved threonine and tyrosine in Cdks. Cdc25A directly associates with cyclin A, cyclin B1, cyclin D1, and cyclin E (1). Cyclin A and cyclin E bind to Cdc25A at the same site located between residues 10 and 14 (1). The cyclin B1-docking site is situated at the C terminus between amino acids 498 and 524 (2), and the binding site of cyclin D1 in Cdc25A is currently unknown. Cdc25A regulates the progression and transition of multiple cell cycle phases. In addition to it required for S phase entry (3, 4), Cdc25A promotes the S phase progression and initiation of mitosis (5–8). Cdc25A with oncogenic potential works together with either H-RasG12V or deprivation of RB1 in tumorigenic transformation of primary mouse embryonic fibroblasts (9). Hemizygous loss of Cdc25A in mice suppresses mammary tumorigenesis induced by H-RasG12V or Her2 (10), indicating that Cdc25A is a rate determiner in the mammary transformation. Furthermore, the overexpression of Cdc25A is correlated with poor clinical outcomes in a variety of human cancers.

The stability of Cdc25A is regulated by the ubiquitin-proteasome pathway. During mitotic exit and the early G1 phase, Cdc25A is degraded through Cdh1-directed anaphase-promoting complex, which also requires a KEN box motif between residues 141 and 143 (11). Subsequently, the phosphorylation of Ser-76 and Thr-80 by the Plk-3/GSK-3β pathway promotes Cdc25A ubiquitination and turnover during the middle and late G1 phase (12). During the S phase, Scfβ-TrCP regulates Cdc25A degradation in an unperturbed cell division cycle and in response to DNA damage, requiring phosphorylation of serines 76, 79, 82, and 85 (13–15). The Cdc25A destruction induced by ionizing and UV irradiation requires the phosphorylation of Ser-123 mediated by the ATM/Chk2 or ATR/Chk1 pathway (7, 16, 17) and the phosphorylation of Ser-76, respectively (18, 19). Rapid Cdc25A degradation in response to DNA damage is a part of the DNA damage checkpoint mechanism that protects genomic integrity by arresting the cell cycle progression and allowing cells to repair damaged DNA.

Although Cdc25A plays a clear role in both cell cycle regulation and cell cycle checkpoint control, it is unknown how Cdc25A promotes apoptosis. It has been reported that the ectopic expression of Cdc25A induces apoptosis in 3T3 L1 cells depleted of growth factors (20), but this mechanism remains unclear. Cdc25A activates Cdc2 or Cdk2 by removing inhibitory phosphates from Cdks. Cdc2 and Cdk2 are activated in a caspase-dependent manner during Fas- or staurosporine-induced apoptosis (21). Moreover, the activation of Cdc2 is required for apoptosis induced by fragmentin-2 or staurosporine (26). The activation mechanism for Cdc2 or Cdk2 during apoptosis remains unclear. After examining the amino acid sequence of Cdc25A, we discovered several possible caspase consensus motifs. Based on this information, we hypothesize that Cdc25A is a substrate for caspase, and the Cdc25A fragment generated by caspase cleavage activates Cdc2 and/or Cdk2, resulting in apoptosis. Thus, we used staurosporine, a typical inducer for apoptosis, to elucidate the possible role of Cdc25A in promoting apoptosis in Jurkat cells. This study reports a novel molecular mechanism in which Cdc25A, a pro-

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3 The abbreviations used are: Cdk, cyclin-dependent protein kinase; copGFP, copepod green fluorescence protein; siRNA, small interfering RNA.
Apoptosis Induced by Cdc25A

apoptotic protein, promotes apoptosis. Upon staurosporine treatment, a Cdc25A C-terminal 37-kDa fragment, designated C37, was generated by caspase cleavage at Asp-223, and its Thr-507 became dephosphorylated. C37 with alanine substitution for Thr-507 (C37/T507A) interacted with Cdc2, Cdk2, cyclin A, and cyclin B1 and significantly activated cyclin B1 and Cdc2-associated kinase activities. The dephosphorylation of Thr-507 might expose the Cdc2/Cdk2-docking site in C37. In the absence of staurosporine, C37/T507A induced apoptosis, resulting from activating cyclin B1/Cdc2 but not Cdk2. In summary, staurosporine causes caspase to cleave Cdc25A and promotes the dephosphorylation of Thr-507. As a result, the cyclin B1-Cdc2 complex is activated but not through the activation of Cdk2, which leads to apoptosis. Therefore, this study presents a novel pathway of staurosporine induced-apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Antibodies—Jurkat and K562 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM GlutaMAX (Invitrogen). HeLa and 293T cells were maintained in Dulbecco’s modified Eagle’s medium with the same supplements. Staurosporine and caspase inhibitor I were purchased from Calbiochem, and [γ-32P]ATP (3000 Ci/mmol) was acquired from PerkinElmer Life Sciences. Cdc25A (F-6), cyclin A, cyclin B1, Cdc2, and Cdk2 antibodies were obtained from Santa Cruz Biotechnology. Poly(ADP-ribose) polymerase, α-tubulin, and M2 monoclonal antibodies were purchased from Sigma. Cleaved caspase 3 (Asp-175) (5A1) rabbit monoclonal antibody (only against activated caspase3) and phospho-Cdc2 (Tyr-15) antibody were acquired from Cell Signaling. Anti-Fas (7C11), anti-His (RGS-His), CD20, anti-TurboGFP(d) (against copepod green fluorescence protein (copGFP)), and phospho-Cdc25A-Thr-507 antibodies were purchased from Beckman Coulter, Qiagen, NeoMarkers, Evrogen, and Abgent, respectively. Anti-rabbit IgG antibody conjugated with Alexa Fluor 546 or 647 was obtained from Invitrogen.

Generation of Expression Vectors and Site-directed Mutagenesis—Plasmids, pcDNA3-Myc-Cdc25A, pGEX2T'-Cdc25A, pGEX2T'-Cdc25A/C431S, and pCD20, were gifts from Dr. Helen Piwnica-Worms (Washington University). pcDNA3-His was constructed by subcloning the adaptor (supplemental Table S1) to pcDNA3 through HindIII and BamHI. The Cdc25A BamHI/EcoRI fragment from pcDNA3-Myc-Cdc25A and the first 223 amino acids amplified by PCR using two primers (supplemental Table S2) were subcloned to pcDNA3-His, labeled as pcDNA3-His-Cdc25A and pcDNA3-His-Cdc25A/1–223.

The DNA fragment encoding three copies of FLAG epitope was amplified by PCR using p3×FLAG CMV10 (Sigma) as a template and two primers (supplemental Table S2) and subsequently subcloned to pcDNA3 through HindIII and BamHI, designated as pcDNA3–3×FLAG. pcDNA3–3×FLAG Cdc25A/224–524 was made by subcloning the Cdc25A/224–524 BamHI/EcoRI fragment amplified by PCR using two primers (supplemental Table S2) into pcDNA3–3×FLAG. Using the primers listed in supplemental Table S2, Cdc25A-His and Cdc25A/224–524-His BamHI/EcoRI fragments were amplified by PCR and subsequently subcloned to pcDNA3, called pcDNA3-Cdc25A-His and pcDNA3-Cdc25A/224–524-His. To generate pGEX2T'-Cdc25A/1–223 or Cdc25A/224–524, the Cdc25A fragment in pGEX2T'-Cdc25A was replaced with the Cdc25A/1–223 or Cdc25A/224–524 BamHI/EcoRI fragment from pcDNA3-His-Cdc25A/1–223 or pcDNA3–3×FLAG Cdc25A/224–524.

To transiently co-express copGFP and any of Cdc25A and its mutants, pCDF1-MCS2-EF1-copGFP (System Biosciences), only with a poly(A) signal downstream of the copGFP-coding sequence, was modified by adding a NotI/PvuII fragment containing a poly(A) signal from pcDNA3 between the cytomegalovirus and EF1 promoters through a NotI site, labeled as pCDF1-MCS2-poly(A)-EF1-copGFP. The His-Cdc25A HindIII/EcoRI fragment from pcDNA3-His-Cdc25A was subcloned to pCDF1-MCS2-poly(A)-EF1-copGFP through BamHI and EcoRI, named pCDF1-MCS2-poly(A)-EF1-copGFP-His-Cdc25A. A Cdc25A/1–223 or Cdc25A/224–524 BamHI/EcoRI fragment replaced the Cdc25A fragment in pCDF1-MCS2-poly(A)-EF1-copGFP-His-Cdc25A to create pCDF1-MCS2-poly(A)-EF1-copGFP-His-Cdc25A/1–223 or His-Cdc25A/224–524, respectively.

Plasmid pcDNA3-CD20 was used to construct serial plasmids expressing fusion proteins with a presumed caspase consensus motif from Cdc25A between N-terminally tagged His6 and CD20. CD20 cDNA was amplified by PCR using pCD20 as a template and two primers (supplemental Table S2) and subsequently subcloned to pcDNA3 through Xhol. pcDNA3-CD20 was prepared by subcloning a C20 BamHI/XbaI fragment from pcDNA3-CD20 to pcDNA3-His. CD20 in pcDNA3-His-CD20 is not in the reading frame until the adapter (supplemental Table S3) encoding a presumed caspase consensus motif containing eight amino acids from Cdc25A was subcloned to pcDNA3-His-CD20 through BamHI and NotI.

All of the mutation sites were mutated by site-directed mutagenesis using the QuikChange XL site-directed mutagenesis kit (Stratagene) and primers listed in supplemental Table S4. Each mutation site was verified by sequencing.

Transfection and Small Interfering RNA Knockdown Experiments—To transfec
different time periods after electroporation. Green fluorescent protein and luciferase siRNAs (supplemental Table S5) were used as controls. Small interfering RNAs were synthesized by Ambion.

**Phospho-specific Flow Cytometric and Flow Cytometric Analyses**—0.5–1 × 10^6 cells were fixed with 1.5% paraformaldehyde in phosphate-buffered saline (pH 8.0) at room temperature for 10 min, immunostained for Cdc2/Cdk2-Tyr-15 phosphorylation with a phospho-Cdc2-Tyr-15 antibody, and analyzed with a FACSCalibur cytometer (BD Biosciences). This method was described in detail by Krutzik and Nolan (35). Because of low transfection efficiency, copGFP-positive cells were gated with a FACSCalibur cytometer after electroporation with various pCDFS1-MCS2-poly(A)-EF1-copGFP constructs.

**Apoptotic Assays**—3-(4,5-Dimethylthiazolyl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay was performed using the CellTiter96 Aqueous One Solution cell proliferation assay kit (Promega). Apoptotic cells were detected by the annexin V-Cy3 apoptosis detection kit (BioVision), the phycoerythrin-conjugated monoclonal active caspase-3 antibody apoptosis kit (Pharmergen), or the terminal deoxynucleotidyltransferase dUTP nick end-labeling assay using the apo-BrdUrde Red in situ DNA fragmentation assay kit (BioVision).

To detect sub-G1 cells, cells were fixed with 0.5% paraformaldehyde in phosphate-buffered saline (pH 8.0) at room temperature for 10 min, washed with phosphate-buffered saline, permeabilized with ice-cold 70% ethanol, stained by 30 μg/ml propidium iodide, and analyzed with a FACSCalibur cytometer.

**Immunoblot Analysis, Immunoprecipitation, and Co-immunoprecipitation**—Cell lysates for immunoblotting were made in MCLB buffer (2) supplemented with 30 mM NaCl three times (for immunoprecipitation of the Cdc25A C-terminal fragment).

Cyclin A or Cdk2 immunoprecipitates, and 30 min for Cdc2 immunoprecipitates.

To examine phosphorylation status of Thr-507 in Cdc25A, Jurkat cells were electroporated with pCDA3–3×FLAG Cdc25A/224–524, treated with vehicle or 300 nM staurosporine at a 37 °C incubator for 2 h, and lysed with MCLB buffer with the described supplements. Each cell lysate was incubated overnight with M2 antibody-agarose beads at 4 °C, and each bead pellet was washed with MCLB buffer six times. Proteins immobilized in the beads were resolved by SDS-PAGE and analyzed by immunoblotting using antibodies as indicated in the figures.

To determine whether there is a Cdc2/Cdk2-docking site in Cdc25A/224–524/T507A, 293T cells were transfected with either pCDA3–3×FLAG Cdc25A/224–524/T507A or Cdc25A/224–524/R446L/R450L/Y455A/T507A, suspended in buffer A (2) with the supplements, and lysed by passage through a 27-gauge needle 30 times. Each cell lysate was precleared by centrifugation at 100,000 × g for 1 h followed by incubation with 10 μl of protein A-agarose beads at 4 °C for 2 h. Each precleared cell lysate was incubated with 10 μl of M2 antibody-agarose beads at 4 °C for 2 h. The resulting immunoprecipitates were washed with buffer A six times or buffer A three times plus buffer A with 30 mM NaCl three times (for immunoprecipitation using Cdc2 antibody) and analyzed by SDS-PAGE and immunoblotting using antibodies as indicated in figures.

Expression and Purification of Bacterial GST Fusion Proteins and Phosphatase Activity Assay—DH5α cells were transformed with various pGEX2T constructs encoding GST-tagged Cdc25A or its mutants. GST-tagged Cdc25A and mutants were purified following the method described previously (2). Proteins immobilized in glutathione beads (GE Healthcare) were eluted with 40 mM glutathione reduced form in 50 mM Tris-HCl (pH 8.0) and dialyzed against 20 mM Tris-HCl (pH 8.0) containing 1 mM diethiothreitol. Purified proteins were concentrated with a YM-30 Centricon (Amicon) and stored at −80 °C.

The phosphatase activity of 5 or 10 μg of GST-tagged Cdc25A or its mutants was assayed using 3-O-methylfluorescein phosphate (Sigma) as substrates. The detailed steps of this process have been described previously (2). The absorbance values at 477 nm were normalized with the number of moles of GST-tagged Cdc25A or its mutants. The absorbance value at 477 nm of 59 pmol of GST-Cdc25A was set at 1.

**Kinase Assay**—293T cells were transfected with empty vector (pcDNA3–3×FLAG) or vector expressing 3×FLAG-tagged Cdc25A/224–524/T507A, and lysed with MCLB with the supplements 24 h after transfection. Cell lysates were precleared by centrifugation at 100,000 × g for 1 h and then incubated with protein A-agarose beads at 4 °C for 2 h. Each cell lysate containing 150 μg of protein was immunoprecipitated with 2 μg of cyclin A or Cdk2 antibody immobilized on 10 μl of protein A-agarose beads or 5 μl of anti-cyclin B1-agarose beads (Santa Cruz Biotechnology). Alternatively, 300 μg of protein was immunoprecipitated overnight with 10 μl of anti-Cdc2-agarose beads (Santa Cruz Biotechnology) at 4 °C. Each bead pellet was washed with MCLB buffer four times and then subjected to kinase assay using a Cdk1/Cdc2 kinase assay kit (Upstate). The kinase reaction incubation time was 10 min for cyclin A, cyclin B1, or Cdk2 immunoprecipitates, and 30 min for Cdc2 immunoprecipitates.

**RESULTS**

**Cdc25A Involvement in Staurosporine-induced Apoptosis Is through Its 37-kDa C-terminal Fragment Generated by Caspase Cleavage**—To understand how Cdc25A promotes apoptosis, Jurkat cells were treated with staurosporine or anti-Fas antibody, functioning as a ligand for Fas, to induce apoptosis. At 2 h after staurosporine treatment, a 37-kDa Cdc25A C-terminal fragment was generated (Fig. 1A). This Cdc25A C-terminal fragment also appeared during 500 or 750 ng/ml anti-Fas antibody treatment (Fig. 1B). Poly(ADP-ribose) polymerase, a known caspase substrate, was proteolyzed concomitantly with the generation of the Cdc25A C-terminal fragment (Fig. 1A and B). These results suggest that caspase mediates the generation of the Cdc25A C-terminal fragment.

Cdc25A is an activator for Cdc2 and Cdk2. Cdc2 and Cdk2 were activated in a caspase-dependent manner during staurosporine-induced apoptosis, as reported by Zhou et al. (21). Tyr-15 in Cdc2 and Cdk2 was gradually dephosphorylated 1 h after staurosporine treatment, as shown in immunoblot analysis (Fig. 1C) and phospho-specific flow cytometric analysis (Fig. 1D). The extent of Cdc2 and Cdk2 dephosphorylation was consistent with an increase in caspase 3 activity and apoptotic cells during staurosporine treatment (Fig. 1, C–E). These results...
agree with the report of Zhou et al. (21) and show that staurosporine treatment induces apoptosis in Jurkat cells, activates caspases and Cdc2/Cdk2, and promotes the generation of a 37-kDa Cdc25A C-terminal fragment.

To determine whether the Cdc25A C-terminal fragment is generated by caspase cleavage and plays a role in staurosporine-induced apoptosis, Jurkat cells were pretreated with vehicle or caspase inhibitor I in the presence or absence of staurosporine. Caspase inhibitor I pretreatment blocked the generation of the Cdc25A C-terminal fragment (Fig. 2A) and inhibited staurosporine-induced apoptosis (Fig. 2B). Furthermore, the knockdown of endogenous Cdc25A using siRNA against Cdc25A suppressed staurosporine-induced apoptosis, as indicated by immunoblotting (Fig. 2C, left panel) and a decrease in annexin V-positive cells after staurosporine treatment (Fig. 2C, right panel). These results suggest that Cdc25A is a pro-apoptotic protein and is involved in staurosporine-induced apoptosis through the Cdc25A C-terminal fragment generated by caspase cleavage.

In Cdc25A, Aspartate 223 Is the Caspase Cleavage Site to Generate the 37-kDa C-terminal Fragment, and There Are Eight Other Caspase Cleavage Sites N-terminal of Aspartate 223—C-terminal His-tagged Cdc25A was similarly cleaved as endogenous Cdc25A to generate the Cdc25A C-terminal fragment during staurosporine treatment (Fig. 3A). The Cdc25A C-terminal fragments accumulated abundantly at 1 h after staurosporine treatment (Fig. 3A, lane 3) and decreased thereafter (lane 4). The accumulation and decline of the ectopic Cdc25A C-terminal fragment levels were faster than that of endogenous Cdc25A (Fig. 1A). This phenomenon may be due to a slight activation of caspase by the ectopic expression of Cdc25A and/or electroporation.

To identify the caspase cleavage site to generate the Cdc25A C-terminal fragment, site-directed mutagenesis was performed. Mutation at Asp-223 to alanine blocked the generation of the Cdc25A C-terminal fragment after staurosporine treatment (Fig. 3B, lane 5) as compared with the C-terminal His-tagged Cdc25A (lane 3). Moreover, the C-terminal His-tagged Cdc25A/224–524 migrated in the gel at the same position as that of the Cdc25A C-terminal fragment generated after staurosporine treatment (Fig. 3B, lanes 3 and 7). The migration of the C-terminal His-tagged Cdc25A/224–524 without staurosporine treatment was slightly slower than that with treatment (Fig. 3B, lanes 6 and 7), which was due to the protein kinase inhibition effect of staurosporine (22). The levels of the C-ter-
minal His-tag and C-terminal CD20 (Fig. 3 consisting of eight amino acids from Cdc25A between the N-ter-
fusion proteins with a presumed caspase consensus motif con-
223, serial constructs were made. These constructs encoded 
caspase cleavage site(s) in Cdc25A/1–223.
results show that Asp-223 is the cleavage site to generate the 
level of the N-terminal His-tagged Cdc25A/1–223 and 
9 means 
porine-induced Cdc25A cleavage and apoptosis, respectively.
FIGURE 2. Caspase inhibitor I and knockdown of Cdc25A block stauro-
porine-induced Cdc25A cleavage and apoptosis, respectively. A, 1 × 
10^6/ml Jurkat cells were preincubated with 100 μM caspase inhibitor I or vehi-
cel and then treated with or without 300 nM staurosporine in the presence or absence of caspase inhibitor I for 2 h. Cell lysates were analyzed by 
immunoblotting using Cdc25A or α-tubulin antibody. B, Jurkat cells were 
preincubated with caspase inhibitor Iαs in A and then treated with or without 
300 nM staurosporine in the presence or absence of caspase inhibitor I for 4 h. 
Cell viability was measured by a 3-(4,5-dimethylthiazolyl)-5-(3-carboxyme-
thsxoxyphenyl)-2-(4-sulfophenyl)-2'-H-tetrazolium assay. Data are displayed as 
means ± S.D. of three independent experiments, each in triplicate. C, Jurkat 
cells were electroporated with Cdc25A or green fluorescent protein (GFP) siRNA. 
At 48 h post-electroporation, Cdc25A and α-tubulin levels were analyzed by 
immunoblotting (left), and a portion of cells was treated with 300 nM stauros-
porine for another 4 h. The percentage of apoptotic cells before (black bars) or after 
(gray bars) staurosporine treatment was determined by annexin V-Cy3 staining 
and flow cytometry (right). Data are displayed as mean ± S.D., n = 3.

minal His-tagged Cdc25A/224–524 with and without stauro-
porine treatment were very similar (Fig. 3B, lanes 6 and 7), 
whereas the level of the N-terminal His-tagged Cdc25A/1–223 
after staurosporine treatment decreased dramatically (lanes 8 
and 9). Furthermore, the Cdc25A C-terminal fragment gener-
ated during Fas-dependent apoptosis was the same as that 
generated during staurosporine-induced apoptosis (Fig. 3C). 
These results show that Asp-223 is the cleavage site to generate the 
Cdc25A C-terminal domain, and there are other possible 
caspase cleavage site(s) in Cdc25A/1–223.

To further identify the caspase cleavage site(s) in Cdc25A/1– 
223, serial constructs were made. These constructs encoded 
fusion proteins with a presumed caspase consensus motif con-
sisting of eight amino acids from Cdc25A between the N-ter-
mal His-tag, tag and C-terminal CD20 (Fig. 3D, top). The mobi-
ity of these fusion proteins in gel with or without staurosporine 
treatment was examined using CD20 antibody. In addition to 
using the Asp-223 caspase cleavage motif as a positive control, 
16 possible caspase cleavage sites in the first 223 amino acids of 
Cdc25A were tested (Fig. 3D). Results show that Asp-223 is the 
most frequent cleavage site, followed by aspartates 128, 130, 
and 215, while aspartates 81, 87, 136, 190, and 216 are less 
cleaved. Furthermore, compared with wild type Cdc25A, the 
substitution of aspartates 81, 87, 128, 130, 136, 190, 215, 216, 
and 223 with alanines inhibited the Cdc25A cleavage induced 
by staurosporine treatment (Fig. 3E). Thus, in addition to aspartate 
223, there are eight caspase cleavage sites in Cdc25A/1–223.

Phosphatase Activity of Cdc25A/224–524 Is 3-Fold Higher than That of Full-length Cdc25A, and Threonine 507 Is Dephosphory-
lated after Staurosporine Treatment, Which Cannot Recruit 14-3-3 
Binding—The Cdc25A C-terminal fragment generated by 
caspase cleavage at Asp-223 contains a phosphatase catalytic 
domain from residues 336 to 497. To compare the phosphatase 
activity of wild type Cdc25A with its mutants, bacterially pro-
duced GST-tagged Cdc25A, Cdc25A/C431S (phosphatase 
dead mutant), Cdc25A/1–223, and Cdc25A/224–524 were 
purified by pull down using glutathione beads. These proteins 
were purified to 95% homogeneous, as indicated by Coomassie 
Blue staining (Fig. 4A). Phosphatase activity was assayed using 
3-O-methylfluorescence phosphate, an artificial substrate. The 
phosphatase activity of GST-Cdc25A/224–524 was 3-fold higher than that of GST-Cdc25A, whereas that of GST-tagged 
Cdc25A/C431S and Cdc25A/1–223 was nondetectable (Fig. 4B). 
These results suggest that the conformation of the active 
site in Cdc25A/224–524 was quite unlike that in the full-length 
Cdc25A, resulting in an increase of Cdc25A/224–524 phospha-
tase activity.

In an unperturbed cell division cycle, Thr-507 is phosphory-
lated by Chk1 at the G_{1}/S boundary until the G_{2} phase. The 
phosphorylation of Thr-507 recruits 14-3-3 binding, which 
shields the cyclin B1-docking site. In the G_{2} phase, Thr-507 is 
dephosphorylated, and Cdc25A can then access and activate 
cyclin B1/Cdc2, promoting the G_{2}/M transition (2). Because 
staurosporine inhibits Chk1 activity (22), the phosphorylation 
status of Thr-507 was examined using phospho-Cdc25A-Thr-
507 antibody. Thr-507 was indeed dephosphorylated at 2 h 
after staurosporine treatment (Fig. 4C), and in turn 14-3-3 
binding could not be recruited.

In Addition to Amplification of Staurosporine-induced Apo-
ptosis, the Ectopic Expression of Cdc25A/224–525/T507A 
Induces the Activation of Cdc2/Cdk2 and Caspase 3 and 
Apoptosis—The phosphatase activity of Cdc25A/224–524 was 
higher than that of full-length Cdc25A, and staurosporine 
treatment induced dephosphorylation of Thr-507. To deter-
mine the function of Cdc25A/224–524 with the dephospho-
lated Thr-507 generated during staurosporine treatment, Jur-
kat cells were electroporated with the expression plasmid with 
two promoters to drive any of the His-tagged Cdc25A/1–223, 
Cdc25A/224–524, and Cdc25A/224–524/T507A and copedeg 
green fluorescence protein (copGFP) expression. At 10 h after 
electroporation, the protein levels of these Cdc25A truncated 
mutants and copGFP were determined by immunoblotting 
(Fig. 5A, top panel), and some cells were treated with stauro-
sporine for another 12 h. The percentage of apoptotic cells in the 
cells expressing copGFP alone, the ectopic expression of 
either His-tagged Cdc25A/224–524 or Cdc25A/224–524/ 
T507A amplified staurosporine-induced apoptosis but not His-
tagged Cdc25A/1–223. The ectopic expression of either His-
tagged Cdc25A/224–524 or Cdc25A/224–524/T507A for 24 h 
also promoted the dephosphorylation of Tyr-15 in Cdc2/Cdk2.
Apoptosis Induced by Cdc25A

(Fig. 5B) and apoptosis, as indicated by the activation of caspase-3 (Fig. 5C, top panel) or an increase in terminal deoxynucleotidyltransferase dUTP nick end labeling-positive cells (Fig. 5C, bottom panel). Moreover, compared with His-tagged Cdc25A/224–524, the ectopic expression of His-tagged Cdc25A/224–524/T507A had a greater effect on the amplification of staurosporine-induced apoptosis, activation of Cdc2/ Dck2 and caspase 3, and induction of apoptosis. copGFP was cleaved by an unknown protease or caspase during the ectopic expression of Cdc25A/224 with or without T507A mutation (Fig. 5, A, top panel, and B, top panel). However, the electroporation efficiency did not decrease, indicating that the

![Diagram](image-url)
cleaved copGFP still had fluorescent activity. Interestingly, the level of cleaved copGFP is correlated with the apoptotic effect induced by the ectopic expression of Cdc25A/224–524 with or without T507A (Fig. 5, B, top panel, and C).

We identified nine caspase cleavage sites in Cdc25A. Amino acids around Asp-81 and 87 are involved in the regulation of Cdc25A protein stability. Substituting these two aspartates with alanines dramatically increased Cdc25A protein levels (data not shown). Moreover, Asp-81 and 87 are minor caspase cleavage sites. Thus, we used the caspase-resistant mutant that contained mutations at aspartates 128, 130, 136, 190, 215, 216, and 223 to alanines to further confirm that the generation of Cdc25A/224–524 with dephosphorylated Thr-507 during staurosporine treatment amplified staurosporine-induced apoptosis. The overexpression of wild type Cdc25A, the ectopic expression of the caspase-resistant mutant amplified staurosporine-induced apoptosis to as less extent, even though the protein level of the caspase-resistant mutant was much higher than that of wild type. These results further confirm that Cdc25A is a pro-apoptotic protein that amplifies staurosporine-induced apoptosis through its C-terminal domain.

The ectopic expression of Cdc25A/224–524/T507A induced apoptosis in Jurkat cells. To determine whether Cdc25A/224–524/T507A also induced apoptosis in the other cell types, His-tagged Cdc25A/224–524/T507A was expressed in K562 cells for 12 h. The protein levels of His-tagged Cdc25A/224–524/T507A and copGFP were examined by immunoblotting (Fig. 5E, top panel), and the percentage of apoptotic cells in the cells expressing copGFP was determined by annexin V staining (Fig. 5E, bottom panel). Results show that the ectopic expression of Cdc25A/224–524/T507A also induced apoptosis in K562 cells.

Arginine 446 and 450 and Tyrosine 455 in Cdc25A Are Involved in the Activation of Cdc2/Cdk2 and Induction of Apoptosis—Cdc25A and Cdc25B are highly homologous in their C-terminal halves. Arg-488 and 492 and Tyr-497 in Cdc25B mediate Cdk2 docking, which requires Asp-206 and
FIGURE 5. Ectopic expression of Cdc25A/224–524 or Cdc25A/224–524/T507A amplifies staurosporine-induced apoptosis and promotes apoptosis.

A, Jurkat cells were electroporated with plasmids expressing copGFP alone or co-expressing copGFP and either His-tagged Cdc25A/1–223, Cdc25A/224–524, or Cdc25A/224–524/T507A. At 10 h post-electroporation, SDS-PAGE analysis was followed by immunoblotting using antibodies as indicated (top). The protein level of copGFP was as an electroporation efficiency control. Furthermore, a portion of cells was treated with 300 nM staurosporine for another 12 h. The percentage of apoptotic cells in copGFP-positive cells before (black bars) or after (gray bars) staurosporine treatment was determined by propidium iodide staining and flow cytometry and represented by the sub-G1 cell population (bottom).

B, Jurkat cells were electroporated with plasmids expressing copGFP alone or co-expressing copGFP and either His-tagged Cdc25A/224–524 or Cdc25A/224–524/T507A and incubated at 37 °C for 24 h. Immunoblotting was analyzed using antibodies as indicated (top). The activity of Cdc2/Cdk2 in copGFP-positive cells was examined by phospho-flow cytometric analysis after immunostaining with phospho-Tyr-15-Cdc2 antibody and anti-rabbit IgG antibody conjugated with Alexa Fluor 647 (bottom).

C, caspase 3 activation in copGFP-positive cells was detected by flow cytometry after immunostaining with anti-active caspase 3 antibody conjugated with phycoerythrin (top). The percentage of apoptotic cells in copGFP-positive cells was also measured by terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) assay and flow cytometry (bottom). Column data are displayed as mean ± S.D., n = 3.

D, Jurkat cells were electroporated with plasmids expressing copGFP alone or co-expressing copGFP and either His-tagged Cdc25A/224–524 or Cdc25A/224–524/T507A and incubated at 37 °C for 24 h. Immunoblotting was analyzed using antibodies as indicated (top). The activity of Cdc2/Cdk2 in copGFP-positive cells was examined by phospho-flow cytometric analysis after immunostaining with phospho-Tyr-15-Cdc2 antibody and anti-rabbit IgG antibody conjugated with Alexa Fluor 647 (bottom). The percentage of apoptosis in copGFP-positive cells was also measured by terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) assay and flow cytometry (bottom). Column data are displayed as mean ± S.D., n = 3.

E, K562 cells were electroporated with plasmids expressing copGFP alone or co-expressing copGFP and His-Cdc25A/224–524/T507A. At 12 h post-electroporation, immunoblotting using antibodies as indicated (top) and annexin V-Cy3 staining followed by flow cytometry were performed. The percentage of annexin V-positive cells in copGFP-positive cells is displayed as mean ± S.D., n = 3 (bottom).
Substitutions of leucine for both arginine 446 and 450 and plus alanine for tyrosine 455 inhibit apoptosis induced by Cdc25A/224–524/T507A and the amplification of apoptosis promoted by Cdc25A after staurosporine treatment, respectively. A, Jurkat cells were electroporated with plasmids expressing copGFP alone or co-expressing copGFP and either His-tagged Cdc25A/224–524/T507A or Cdc25A/224–524/R446L/R450L/T507A. At 24 h post-electroporation, SDS-PAGE analysis was followed by immunoblotting using antibodies as indicated (top), and the activity of Cdc2/Cdk2 in copGFP-positive cells was detected by phospho-flow cytometry after immunostaining with phospho-Tyr-15-Cdc2 antibody and anti-rabbit IgG antibody conjugated with Alexa Fluor 647 (bottom). B, at 24 h post-electroporation, some cells were also subjected to propidium iodide staining and flow cytometry. The percentage of apoptotic cells in copGFP-positive cells was represented by the sub-G₁ cell population. C, Jurkat cells were electroporated with plasmids expressing copGFP alone or co-expressing copGFP and either His-tagged Cdc25A or Cdc25A/R446L/R450L/Y455A. At 8 h post-electroporation, immunoblotting using the indicated antibodies was analyzed (top), and a portion of cells was treated with 300 nm staurosporine for another 12 h. The percentage of apoptotic cells in copGFP-positive cells before (black bars) or after (gray bars) staurosporine treatment was examined by propidium iodide staining followed by flow cytometry and represented by the sub-G₁ cells population (bottom). Column data are displayed as mean ± S.D., n = 3.
nine 446 and 450 and alanine for Tyr-455 did not completely block the interaction between Cdc25A/224–524/T507A and either Cdc2, Cdk2, cyclin B1, or cyclin A, suggesting that other residues may be involved in Cdc2/Cdk2 docking. Because Cdc25A/224–524/T507A interacted with Cdc2, Cdk2, cyclin B1, and cyclin A, we further examined if the ectopic expression of Cdc25A/224–524/T507A significantly increases cyclin B1 and Cdc2-associated kinase activities. The ectopic expression of 3×FLAG-tagged Cdc25A/224–524/T507A dramatically increased cyclin B1- and Cdc2-associated kinase activities but only slightly increased cyclin A- and Cdc2-associated kinase activities (Fig. 7D). These results suggest that the apoptosis induced by the ectopic expression of Cdc25A/224–524/T507A was due to the activation of cyclin B1/Cdc2.

**Apoptosis Induced by the Ectopic Expression of Cdc25A/224–524/T507A Resulted from the Activation of Cyclin B1/Cdc2, although Knockdown of Cdc2, but Not Cdk2, Suppresses Staurosporine-induced Apoptosis—Cdc25A/224–524/T507A, mimicking the Cdc25A C-terminal fragment generated during staurosporine treatment, interacts with Cdc2, Cdk2, cyclin A, and cyclin B1 and activates their associated kinase activities in different degrees. To clarify which cyclin-Cdk complex is involved in Cdc25A/224–524/T507A-induced apoptosis, we performed knockdown experiments using siRNA against Cdc2, Cdk2, cyclin A, and cyclin B1 and analyzed the associated kinase activities in different degrees. To clarify which cyclin-Cdk complex is involved in Cdc25A/224–524/T507A-induced apoptosis, we performed knockdown experiments using siRNA against Cdc2, Cdk2, cyclin B1, or cyclin A. Cdc2, Cdk2, cyclin B1, or cyclin A in Jurkat cells was knocked down for 36, 24, 44, and 18 h, respectively, and His-tagged Cdc25A/224–524/T507A was then expressed for 6 h. The protein levels and percentage of apoptotic cells in the cells expressing copGFP were determined.
by immunoblotting using the indicated antibodies and annexin V staining, respectively. These results show that knockdowns of Cdc2 and cyclin B1 (Fig. 8, A and B), but not Cdk2 and cyclin A (data not shown), dramatically inhibit the apoptosis induced by the ectopic expression of His-tagged Cdc25A/224–524/T507A. Thus, the apoptosis induced by Cdc25A/224–524 with dephosphorylated Thr-507 generated by staurosporine treatment is due to the activation of cyclin B1/Cdc2.

This study shows that Cdc25A is involved in staurosporine-induced apoptosis through the knockdown of Cdc2 (Fig. 2C). Cdc25A/224–524/T507A interacts with and activates Cdc2 and Cdk2 (Fig. 7, A, B, and D), whereas the apoptosis induced by the ectopic expression of Cdc25A/224–524/T507A is due to the activation of cyclin B1/Cdc2 (Fig. 8, A and B). To further confirm that the function of Cdc25A/224–524 with dephosphorylated Thr-507 in staurosporine induced apoptosis, we performed knockdown experiments using siRNA against Cdc2 or Cdk2. Knockdowns of Cdc2 and Cdk2 in Jurkat cells were carried out for 36 h, and then some cells were treated with staurosporine for another 4 h. The protein levels before staurosporine treatment and percentage of apoptotic cells before or after staurosporine treatment were detected by immunoblotting using the indicated antibodies and annexin V staining, respectively (Fig. 8C). These results indicate that knockdown of Cdc2, but not Cdk2, attenuates staurosporine-induced apoptosis. Thus, Cdc25A is a pro-apoptotic protein that amplifies staurosporine-induced apoptosis by activating cyclin B1/Cdc2 through its C-terminal fragment.

DISCUSSION

This study proposes a novel function of Cdc25A, a pro-apoptotic protein, in staurosporine-induced apoptosis (Fig. 9). Staurosporine treatment activates caspase and induces apopto-
Apoptosis Induced by Cdc25A

sis in Jurkat cells (Fig. 1, C and E). Cdc25A is a caspase substrate. Staurosporine induces the cleavage of Cdc25A, which can be inhibited by caspase inhibitor I (Fig. 2A). Caspase mainly cleaves Cdc25A at Asp-223, and it slightly cleaves Cdc25A at several residues in the N-terminal 223 amino acids during staurosporine treatment (Fig. 3, B and D). The Cdc25A C-terminal fragment containing the last 301 residues, C37, has a higher phosphatase activity than full-length Cdc25A (Fig. 4B). Staurosporine, a broad spectrum protein kinase inhibitor, also induces the dephosphorylation of Thr-507 (Fig. 4C). C37 with or without the substitution of alanine for Thr-507 amplifies staurosporine-induced apoptosis, promotes the activation of Cdc2/Cdk2 and caspase 3, and induces apoptosis (Fig. 5, A–C). C37 with the substitution of alanine for threonine 507 (C37/T507A), mimicking the Cdc25A cleavage product generated during staurosporine treatment, has a greater effect than C37 (Fig. 5, A–C). Arg-446 and 450 and Tyr-455 in C37/T507A mediate Cdc2/Cdk2 activation and binding. It is shown by that mutation at both Arg-446 and 450 to leucine or both of these mutation sites plus Tyr-455 to alanine weakens the activation of Cdc2/Cdk2 and apoptosis and also reduces the interaction with Cdc2, Cdk2, cyclin B1, or cyclin A (Fig. 6, A and B, and Fig. 7, A–C). The ectopic expression of C37/T507A dramatically activates cyclin B1- and Cdk2-associated kinase activities but only slightly activates cyclin A- and Cdk2-associated kinase activities (Fig. 7D). Moreover, the apoptosis induced by the ectopic expression of C37/T507A is due to the activation of cyclin B1/Cdc2, as indicated by our Cdc2 or cyclin B1 siRNA knockdown experiment (Fig. 8, A and B). Because the ectopic expression of either C37/T507A or Cdc25A amplifies staurosporine-induced apoptosis, and knockdown of Cdc2 attenuates staurosporine-induced apoptosis (Fig. 5A, Fig. 6C, and Fig. 8C), Cdc25A is a pro-apoptotic protein that amplifies staurosporine-induced apoptosis through the activation of cyclin B1/Cdc2 by C37 with dephosphorylated Thr-507. The phosphorylation of Thr-507 is required for recruiting 14-3-3 binding (2). C37/T507A has a greater effect on the activation of Cdc2/Cdk2 and the induction of apoptosis than C37. This suggests that the dephosphorylation of Thr-507 exposes the Cdc2/Cdk2-docking site, which prevents C37 from interacting with and activating Cdc2/Cdk2. The ectopic expression of C37/T507A markedly activates Cdc2/Cdk2-associated kinase activity but only slightly activates Cdk2-associated kinase activity. This means that Cdc2 and Cdk2 can be activated to a different extent by the ectopic expression of C37/T507A. Although premature activation of Cdc2 is required for apoptosis induced by a lymphocyte granule protease or staurosporine (26), the mechanism is not clear. Our results provide strong evidence explaining why the premature activation of Cdc2 is involved in staurosporine-induced apoptosis and even lymphocyte granule protease-induced apoptosis. The mechanisms for staurosporine-induced apoptosis are quite complicated. In addition to caspases, staurosporine-induced apoptosis can proceed through cathepsin D (27). Staurosporine also triggers DNA double strand breaks and inhibits transcription during early treatment (28). Our results show that C37 with the dephosphorylation at Thr-507 generated during early staurosporine treatment is involved in the early stages of apoptosis. Previous research shows that Cdc2 induces neuronal apoptosis in the absence of electrical activity or growth factors through the phosphorylation of Bad at Ser-128 (29), whereas the phosphorylation of Bad at Ser-128 does not promote apoptosis in non-neural cells (30). The identification of the downstream substrate of cyclin B1/Cdc2 will further elucidate the mechanism of apoptosis induced by C37/T507A. Cdc25A activates different types of cyclin-Cdk complexes by directly associating with different cyclins. Interestingly, our study shows that there is a Cdc2/Cdk2-docking site at C37. Thus, C37 can activate Cdc2 and Cdk2. The property of C37 is quite different from that of full-length Cdc25A. Using small artificial molecules as substrates, the phosphatase activity of C37 is 3-fold higher than that of full-length Cdc25A, suggesting the active site conformation of C37 is unlike that of Cdc25A, and the catalytic activity of C37 is much higher than that of full-length Cdc25A. Compared with C37/T507A, the substitution of leucine for both Arg-446 and 450 and alanine for Tyr-455 only decreases the interaction between C37/T507A and either Cdc2, Cdc2, cyclin A, or cyclin B1. This suggests that other residues are involved in Cdc2/Cdk2 binding. We only showed that Cdc2, Cdc2, cyclin A, or cyclin B1 were co-immunoprecipitated with C37/T507A. It is unknown if C37 interacts with other cyclins and Cdk2.

The ubiquitin-mediated proteasome pathway regulates Cdc25A protein stability in unperturbed and perturbed cell division cycles. The protein stability of C37 is likely more stable than that of Cdc25A, because almost all of the residues and the motifs required for the ubiquitin-mediated proteasome pathways are located at N-terminal 223 residues. The phosphorylation status of Thr-507 may regulate the interaction of Cdc2 and Cdk2 with C37. Thr-507 is phosphorylated by Chk1 from the G1/S boundary until the G2 phase (2). The ectopic expression of C37/T507A or Cdc25A amplifies staurosporine-induced apoptosis, and knockdown of Cdc2 attenuates staurosporine-induced apoptosis (Fig. 5A, Fig. 6C, and Fig. 8C), indicating that C37/T507A also functions as a pro-apoptotic protein in the Fas-dependent apoptotic pathway. A recent study showed that Cdc25A was cleaved by caspase 3 at Asp-223 to generate an active C-terminal fragment that activates Cdk2 and induced apoptosis (25). However, our present findings show that the same C-terminal fragment generated during staurosporine treatment, but with dephosphorylated Thr-507, induces apoptosis through the activation of cyclin B1/Cdc2 but not Cdk2. Furthermore, the ectopic expression of C37/T507A markedly activates Cdc2/Cdk2-associated kinase activity but only slightly activates Cdk2-associated kinase activity. This means that Cdc2 and Cdk2 can be activated to a different extent by the ectopic expression of C37/T507A. Although premature activation of Cdc2 is required for apoptosis induced by a lymphocyte granule protease or staurosporine (26), the mechanism is not clear. Our results provide strong evidence explaining why the premature activation of Cdc2 is involved in staurosporine-induced apoptosis and even lymphocyte granule protease-induced apoptosis. The mechanisms for staurosporine-induced apoptosis are quite complicated. In addition to caspases, staurosporine-induced apoptosis can proceed through cathepsin D (27). Staurosporine also triggers DNA double strand breaks and inhibits transcription during early treatment (28). Our results show that C37 with the dephosphorylation at Thr-507 generated during early staurosporine treatment is involved in the early stages of apoptosis. Previous research shows that Cdc2 induces neuronal apoptosis in the absence of electrical activity or growth factors through the phosphorylation of Bad at Ser-128 (29), whereas the phosphorylation of Bad at Ser-128 does not promote apoptosis in non-neural cells (30). The identification of the downstream substrate of cyclin B1/Cdc2 will further elucidate the mechanism of apoptosis induced by C37/T507A. Cdc25A activates different types of cyclin-Cdk complexes by directly associating with different cyclins. Interestingly, our study shows that there is a Cdc2/Cdk2-docking site at C37. Thus, C37 can activate Cdc2 and Cdk2. The property of C37 is quite different from that of full-length Cdc25A. Using small artificial molecules as substrates, the phosphatase activity of C37 is 3-fold higher than that of full-length Cdc25A, suggesting the active site conformation of C37 is unlike that of Cdc25A, and the catalytic activity of C37 is much higher than that of full-length Cdc25A. Compared with C37/T507A, the substitution of leucine for both Arg-446 and 450 and alanine for Tyr-455 only decreases the interaction between C37/T507A and either Cdc2, Cdc2, cyclin A, or cyclin B1. This suggests that other residues are involved in Cdc2/Cdk2 binding. We only showed that Cdc2, Cdc2, cyclin A, or cyclin B1 were co-immunoprecipitated with C37/T507A. It is unknown if C37 interacts with other cyclins and Cdk2.

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