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Cdk1 Protein-mediated Phosphorylation of Receptor-associated Protein 80 (RAP80) Serine 677 Modulates DNA Damage-induced G_2/M Checkpoint and Cell Survival*[S]

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Background: RAP80, a component of the BRCA1-A complex, is crucial in the cell cycle checkpoint and DNA damage repair.
Results: RAP80 phosphorylation by Cdk1 is important for sensitivity to ionizing radiation and G_2/M checkpoint control.
Conclusion: Cdk1-mediated RAP80 phosphorylation is important for the DNA damage response.
Significance: The findings provide new implications for the interplay of the DNA damage signaling pathway and RAP80 phosphorylation.

Post-translational phosphorylation plays critical roles in the assembly of signaling and repair proteins in the DNA damage response pathway. RAP80, a component of the BRCA1-A complex, is crucial in cell cycle checkpoint activation and DNA damage repair. However, its molecular mechanism is unclear. In this study, we identified Cdk1 as a new RAP80-binding protein and demonstrated that the Cdk1-cyclin B_1 complex phosphorylates RAP80 at Ser-677 using an *in vitro* kinase assay and a phosphopeptide-specific antibody against phospho-Ser-677 of RAP80. RAP80 Ser-677 phosphorylation occurred in the M phase of the cell cycle when Cdk1 was in an active state. In addition, ionizing radiation (IR) induced RAP80 phosphorylation at Ser-677. Mutation of Ser-677 to alanine sensitized cells to IR and functioned in G_2/M checkpoint control. These results suggest that post-translational phosphorylation of RAP80 by the Cdk1-cyclin B_1 complex is important for RAP80 functional sensitivity to IR and G_2/M checkpoint control.

The main cause of genomic instability is DNA lesions induced by DNA-damaging agents such as reactive oxygen species, ionizing radiation (IR),[3] and many chemicals. DNA lesions such as double-strand breaks or stalled replication forks activate various cell cycle checkpoints. DNA-damaging agent-induced cell cycle checkpoints in eukaryotic cells are generally connected with cell cycle progression (1–3). The eukaryotic cell cycle is composed of a series of events tightly regulated by cyclin and Cdk (cyclin-dependent kinase) complexes, leading to the division and duplication of cells. The Cdk4-cyclin D complex promotes initiation of the cell cycle, and Cdk2-cyclin E promotes cell cycle progression from the G_1 to S phase (G_1/S transition). The Cdk1-cyclin B complex initiates the G_2/M transition (4). Cell cycle progression is also regulated by cell cycle checkpoints. DNA damage-mediated cell cycle checkpoints can induce apoptosis when damaged DNA cannot be repaired or cell cycle progression cannot be coordinated with DNA repair (5–7). The main purpose of DNA damage-induced cell cycle checkpoints is to assess DNA damage and allow sufficient time to repair the damaged DNA, leading to maintenance of the human genome in a stable state. The cell cycle checkpoints include the G_1/S, intra-S, and G_2/M checkpoints (5, 7). The G_2/M cell cycle checkpoint blocks cell cycle progression into mitosis, and the Cdk1-cyclin B complex is the main target molecule of the G_2/M cell cycle checkpoint (6, 7).

RAP80 (receptor-associated protein 80) is a component of the BRCA1-A complex, which contains CCDC98 (coiled-coil domain-containing protein 98)/ABRAXAS, BRCC45 (BRCA1/BRCA2-containing complex subunit 45/BRE (brain and reproductive organ-expressed protein), BRCC36, MERIT40 (mediator of RAP80 interactions and targeting subunit 40)/NBA1 (new component of the BRCA1-A complex 1), and BRCA1 (breast cancer 1) (8–16). RAP80 targets the BRCA1-A complex to DNA damage sites by interacting with lysine 63-mediated polyubiquitinated histones through its two tandem ubiquitin-interacting motifs (8–11). Recruitment of BRCA1 to the DNA damage sites plays an important role in cell cycle checkpoint activation and DNA damage repair. RAP80 is a nuclear phosphoprotein, and phosphorylated SQ and SP sites in the RAP80 protein have been identified by mass spectrometry (8). The SQ sites in RAP80 are phosphorylated by ATM kinase in response to DNA damage, but the kinase(s) that phosphorylate SP sites remain unclear (8).

In this study, we identified Cdk1 as a new RAP80-binding protein and demonstrated that the Cdk1-cyclin B_1 complex directly phosphorylates RAP80 at Ser-677, one of three phos-

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[3] The abbreviations used are: IR, ionizing radiation; SFB, S-protein/FLAG/streptavidin-binding peptide; FH, FLAG- and HA-tagged; Gy, grays.

phorylated SP sites, by using an *in vitro* kinase assay and phosphopeptide-specific antibody against phospho-Ser-677 in RAP80. We also demonstrate that post-translational phosphorylation of RAP80 by the Cdk1-cyclin B_1 complex is important for RAP80 functions in sensitivity to IR and G_2/M checkpoint control.

EXPERIMENTAL PROCEDURES

Cell Culture—The HeLa and HEK293T cell lines were purchased from American Type Culture Collection (Manassas, VA). The cell lines were maintained in DMEM supplemented with 10% FBS at 37 °C in 5% (v/v) CO_2.

siRNAs—Control, RAP80 (8), and Cdk1 siRNAs were described previously (17, 18). siRNAs were transfected into cells using Oligofectamine (Invitrogen).

In Vitro Kinase Assays—The assays were performed with the recombinant Cdk1-cyclin B complex (Millipore). GST-RAP80 protein was incubated with 2 units of Cdk1-cyclin B complex, 200 μM ATP, and 10 μCi of [γ-^{32}P]ATP for 1 h at 30 °C. The reaction products were analyzed by SDS-PAGE and autoradiography.

Antibodies, Transfection, and Immunoprecipitation—Rabbit anti-phospho-Ser-677 antibody was raised by immunizing rabbits with phosphopeptide ^{672}RDLNEpSPVKSF^{682}. The resulting rabbit polyclonal antibodies were affinity-purified using a SulfoLink Plus immobilization and purification kit (Pierce). Anti-RAP80 antibody was described previously (8). Anti-FLAG, anti-HA, anti-Myc, and anti-β-actin antibodies were purchased from Sigma-Aldrich. Antibodies against human cyclins E_1, A_2, and B_1; phosphorylated histone H3; and Cdk1 were purchased from Cell Signaling Technology (Danvers, MA). The dilutions of the various antibodies for Western blot analysis were as follows: anti-RAP80, 1:1000; anti-phospho-Ser-677, 1:250; anti-FLAG, 1:1000; anti-HA, 1:1000; anti-Myc, 1:1000; anti-β-actin, 1:5000; anti-cyclin E_1, 1:1000; anti-cyclin A_2, 1:1000; anti-cyclin B_1, 1:1000; anti-phosphorylated histone H3, 1:200; and anti-Cdk1, 1:500. The dilutions of the various antibodies for immunofluorescence were as follows: anti-phospho-Ser-677, 1:200; anti-FLAG, 1:500; anti-cyclin B_1, 1:200; anti-proliferating cell nuclear antigen, 1:200; and anti-γ-H2AX, 1:200. Transient transfection was performed using FuGENE 6 reagent (Roche Applied Science). For immunoprecipitation, cells were washed with ice-cold PBS and then lysed in buffer A (0.5% Nonidet P-40, 20 mM Tris (pH 8.0), 50 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM dithiothreitol, and 50 μg/ml PMSF) at 4 °C for 10 min. Crude lysates were cleared by centrifugation at 14,000 rpm at 4 °C for 5 min, and supernatants were incubated with protein A-agarose-conjugated primary antibodies. The immunocomplexes were washed three times with buffer A and subjected to SDS-PAGE. Western blotting was performed using the antibodies indicated in the figure legends.

Establishment of Stable Cell Lines and Affinity Purification of S-protein/FLAG/Streptavidin-binding Peptide (SFB)-tagged RAP80-containing Complexes—The establishment of stable cell lines was described previously (8). To establish cell lines stably expressing epitope-tagged proteins, HeLa cells were transfected with plasmids encoding RAP80WT, RAP80(S677A), or RAP80(S677D) and puromycin-resistant protein. Forty-eight hours after transfection, the cells were split at a 10:1 ratio and cultured in medium containing puromycin (10 μg/ml) for 3 weeks. Individual puromycin-resistant colonies were isolated and screened by Western blotting for expression of the RAP80 protein. Affinity purification using 293T cells stably expressing SFB-RAP80 has been described previously (8).

Purification of GST Fusion Proteins—The GST fusion protein was expressed in *Escherichia coli* and purified as described previously (19).

Cell Synchronization—Cells were synchronized at late G_1 phase using the double thymidine block method (20). Briefly, the cells were plated in 100-mm diameter Petri dishes, and thymidine was added to a final concentration of 2 mM after cell adherence. The cells were cultured for 16 h. After removal of the thymidine and incubation for 10 h in fresh medium, thymidine was added to a final concentration of 2 mM for an additional 16 h. After removal of the thymidine, synchronized cells were cultured in fresh medium and collected at different times for cell cycle analysis and Western blotting. Cells were synchronized in prometaphase with 17 h of nocodazole treatment and then released into fresh medium for further incubation.

Cell Cycle Analysis by Flow Cytometry—The double thymidine- or nocodazole-synchronized cells were collected at different times after release from a G_1/S boundary. After washing twice with PBS, cells were fixed with chilled 70% alcohol at 20 °C for 24 h. The fixed cells were collected by centrifugation at 2000 rpm for 5 min, washed twice with PBS, incubated with 30 mg/ml RNase A for 30 min at 37 °C, stained with 50 μg/ml propidium iodide (Sigma-Aldrich) for 30 min at room temperature, and then analyzed by flow cytometry.

G_2/M Cell Cycle Checkpoint Assay—G_2/M cell cycle checkpoint assay was performed as described previously (8). HeLa cells in a 100-mm diameter plate were transfected twice with control or RAP80 siRNA at 24-h intervals. Forty-eight hours after the second transfection, transfected cells were mock-treated or irradiated at the indicated doses using a radiation source. One hour after irradiation, cells were fixed with 70% (v/v) ethanol at −20 °C for 24 h, stained with rabbit antibody to phosphorylated histone H3 (1:200 dilution), and incubated with fluorescein isothiocyanate-conjugated goat secondary antibody to rabbit immunoglobulin. The stained cells were treated with RNase A, incubated with propidium iodide, and analyzed by flow cytometry.

Cell Survival Assay—Cell survival assay was done as described previously (8). HeLa cells in a 60-mm diameter plate were transfected twice with control or RAP80 siRNA at 24-h intervals. Forty-eight hours after the second transfection, transfected cells were irradiated at the indicated doses using a radiation source. Eleven days after irradiation, cells were washed with PBS, fixed, and stained with 2% (w/v) methylene blue, and the colonies were counted.

Plasmids—The SFB-RAP80, GST-RAP80, and Myc-CCDC98 expression vectors and the GST-RAP80N and GST-RAP80C constructs were described previously (8, 11). RAP80 point mutants were generated by site-directed mutagenesis. The HA-tagged Cdk1 expression vector was generated by PCR. The siRNA-resistant RAP80 expression plasmid was described previously (20).

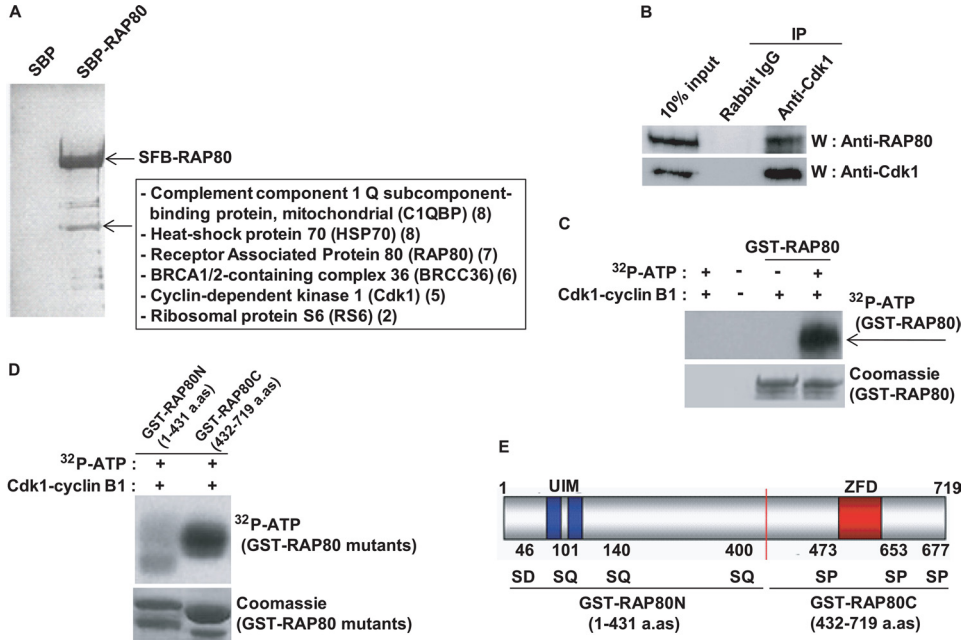


FIGURE 1. **RAP80 is a new substrate of Cdk1.** *A*, tandem repeat affinity-purified SFB-RAP80 complexes were sequentially subjected to PAGE and silver staining. The first and third bands specific to the SFB-RAP80 cells (*arrows*) were excised from the silver-stained gel, and peptides were identified by mass spectrometric analysis. The first band was identified as SFB-RAP80. A summary of the proteins identified in the third band is presented, with the *numbers* in *parentheses* indicating the number of peptides detected for each protein. *B*, endogenous Cdk1 binds to endogenous RAP80. Immunoprecipitation (*IP*) was performed using rabbit IgG or anti-Cdk1 antibody, followed by Western blot analysis (*W*) using the indicated antibodies. *C*, *in vitro* kinase assays were performed by incubating recombinant Cdk1-cyclin B_1 and [γ-^{32}P]ATP with purified GST-RAP80WT. After incubation for 1 h at 30 °C, the reaction mixture was separated by SDS-PAGE and detected by autoradiography (*upper panel*). The Coomassie Blue-stained *lower panel* shows the amount of GST fusion proteins added. *In vitro* kinase assays were performed by incubating recombinant Cdk1-cyclin B_1 and [γ-^{32}P]ATP with purified GST-RAP80N or GST-RAP80C (*D*) or GST-RAP80, GST-RAP80(S473A), GST-RAP80(S653A), or GST-RAP80(S677A) (*F*) fusion protein. After incubation for 1 h at 30 °C, the reaction mixture was separated by SDS-PAGE and detected by autoradiography (*upper panel*). The Coomassie Blue-stained *lower panel* shows the amount of GST fusion proteins added. *E*, summary of the RAP80 phosphorylation sites. *UIM*, ubiquitin-interacting motif; *ZFD*, zinc finger domain; *a.as*, amino acids.

Statistical Analysis—Student's *t* test was performed. *Error bars* represent S.D. of several independent experiments. A p value of <0.05 (two-tailed) was considered statistically significant.

RESULTS

RAP80 Is a Novel Substrate of Cdk1—To identify new RAP80-binding proteins, we performed tandem repeat affinity purification using HEK293T cells that stably express SFB-RAP80 (8). Silver staining was performed for RAP80-binding complexes using cell lysates prepared by sequential affinity chromatography with streptavidin- and S-protein-agarose beads. We detected several specific bands indicative of elution from the SFB-RAP80 cell line, but not from the SFB control cell line (Fig. 1*A*). Mass spectrometric analysis indicated that the first band was SFB-RAP80 protein, and the third band contained numerous proteins, including BRCC36, a known component of RAP80-binding protein complexes. Cdk1 was detected in the eluted RAP80-binding complex (Fig. 1*A*). To evaluate the molecular interaction between RAP80 and Cdk1, the binding between RAP80 and Cdk1 was verified using *in vivo* binding assays. Immunoprecipitation analysis using anti-Cdk1 antibody showed that Cdk1 specifically bound to RAP80 at endogenous levels (Fig. 1*B*). The immunoprecipitation and purification analysis suggested the possibility that RAP80 may be a substrate of the Cdk1-cyclin B_1 complex. This was confirmed by an *in vitro* kinase assay using GST-RAP80WT fusion proteins and purified recombinant Cdk1-cyclin B_1 complex, which phosphorylates the fusion proteins. In this assay, RAP80 proved to be a substrate for the Cdk1-cyclin B_1 complex (Fig. 1*C*). Next, we analyzed the RAP80 phosphorylation sites in an *in vitro* kinase assay using the GST-RAP80N or GST-RAP80C fusion protein and purified recombinant Cdk1-cyclin B_1 complex. The Cdk1-cyclin B_1 complex phosphorylated GST-RAP80 and GST-RAP80C, but not GST-RAP80N, suggesting the presence of a phosphorylated region at the C terminus of RAP80 (Fig. 1*D*). The phosphorylation sites in the RAP80 C-terminal region are summarized in Fig. 1*E* (one SD site and three SQ, and three SP sites). Because the SP site is a putative Cdk1 phosphorylation site, GST-fused point mutants of each serine residue (GST-RAP80(S473A), GST-RAP80(S653A), or GST-RAP80(S677A)) were used to examine the phosphorylated sites. GST-RAP80WT, GST-RAP80(S473A), or GST-RAP80(S653A) was phosphorylated by the Cdk1-cyclin B_1 complex, but GST-RAP80(S677A) was not phosphorylated (Fig. 1*F*), suggesting that Ser-677 of RAP80 is a target phosphorylation site for the Cdk1-cyclin B_1 complex.

Detection of RAP80 Phosphorylation by Phospho-Ser-677-specific Antibody—To further confirm *in vivo* RAP80 phosphorylation at Ser-677, we generated a phosphopeptide-specific antibody against the phosphopeptide sequence surrounding Ser-677. Specificity of the phosphospecific antibody was validated using *in vitro* phosphorylation of GST-RAP80C by the Cdk1-cyclin B_1 complex in the presence of unlabeled ATP. The phosphospecific antibody against RAP80 phospho-Ser-677 detected RAP80 protein in the presence of both the Cdk1-cyclin B_1 complex and unlabeled ATP (Fig. 2, *A* and *B*), but could

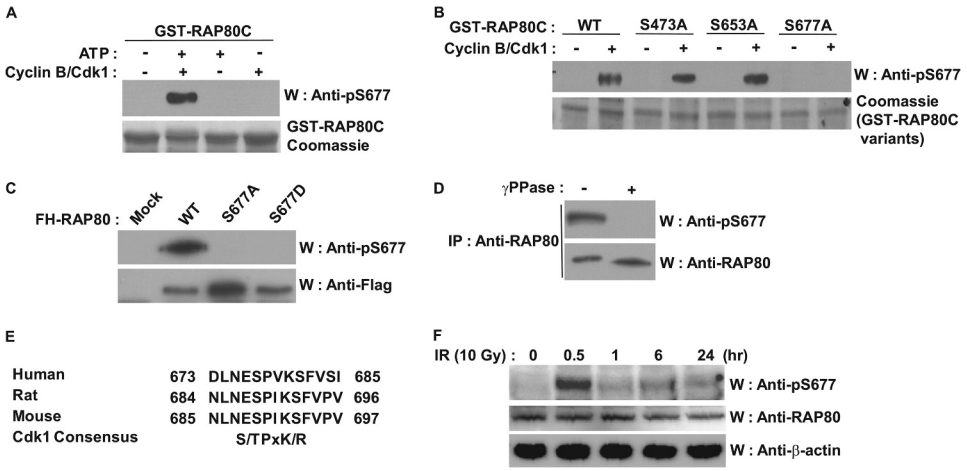
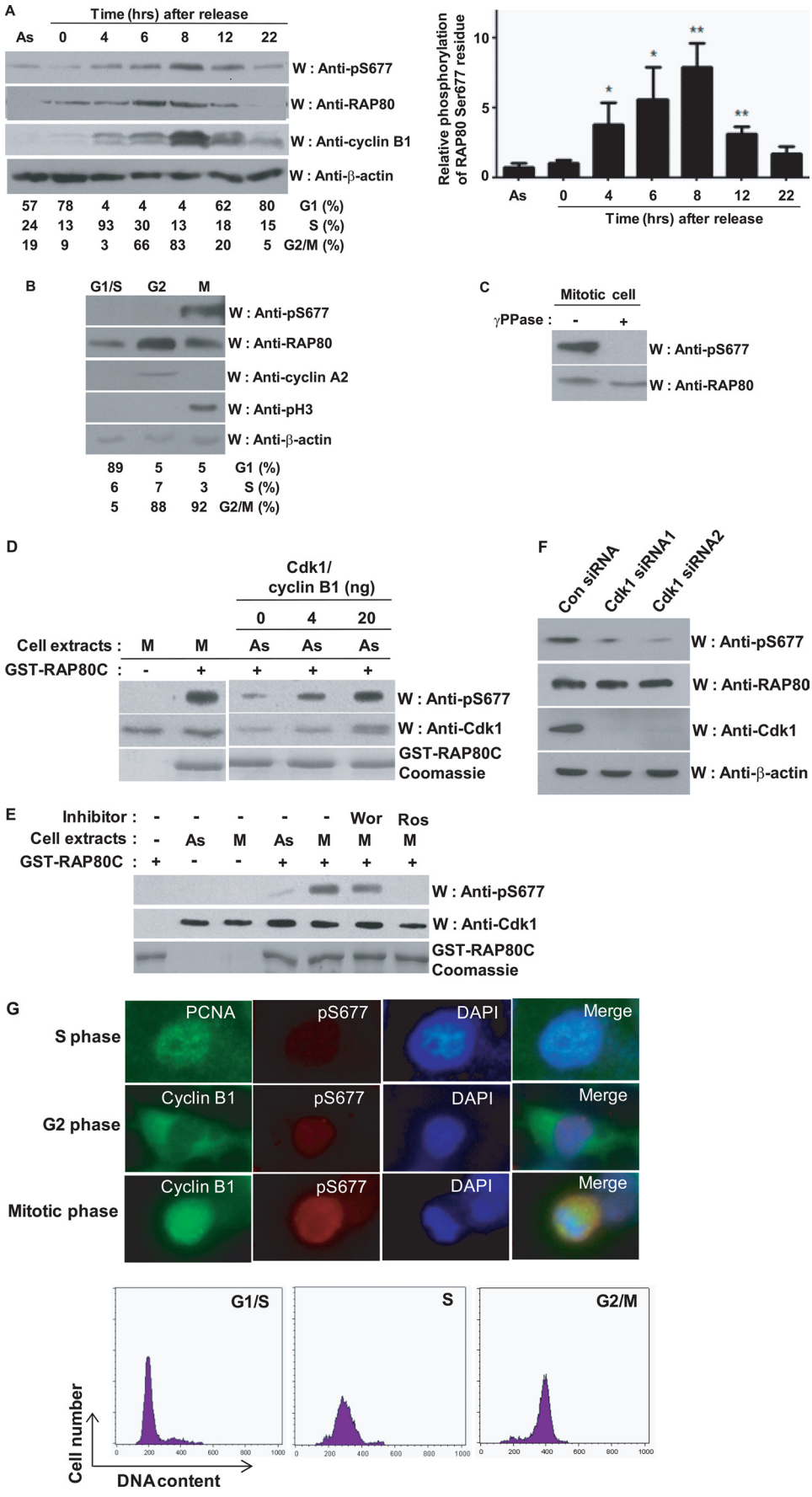


FIGURE 2. **Detection of RAP80 phosphorylation by phospho-Ser-677-specific antibody.** *In vitro* kinase assays were performed by incubating Cdk1-cyclin B_1 and unlabeled ATP with purified GST-RAP80C (*A*) or GST-RAP80C, GST-RAP80C(S473A), GST-RAP80C(S653A), or GST-RAP80C(S677A) (*B*) fusion protein. After incubation for 1 h at 30 °C, the reaction mixture was separated by SDS-PAGE, and RAP80 phosphorylation was detected using the antibody against RAP80 phospho-Ser-677. The Coomassie Blue-stained *lower panels* show the amount of GST fusion proteins added. *C*, FH-RAP80WT, FH-RAP80(S677A), or FH-RAP80(S677D) expression plasmid was transfected into HEK293T cells. Transfected cell lysates were subjected to Western blot analysis (*W*) using the indicated antibodies. *D*, phosphatase-treated endogenous RAP80 protein is not recognized by the phospho-Ser-677-specific antibody. 293T cell lysates were immunoprecipitated (*IP*) with anti-RAP80 antibody. Immunoprecipitated RAP80 proteins were incubated with or without γ-phosphatase and subjected to Western blot analysis using the indicated antibodies. *E*, sequence alignment of the RAP80 region containing Ser-677 in mammalian species. *F*, HeLa cells were irradiated with 10 Gy and then harvested at the indicated times. Cell lysates were subjected to Western blot analysis using the indicated antibodies.

not detect the GST-RAP80(S677A) mutant protein (Fig. 2*B*). Next, *in vivo* detection of RAP80 phosphorylation at Ser-677 was tested using lysates of HEK293T cells transfected with plasmids expressing FLAG- and HA-tagged (FH) RAP80WT, FH-RAP80(S677A) (which is incapable of being phosphorylated at Ser-677), or FH-RAP80(S677D) (which mimics phosphorylated Ser-677). The phosphospecific antibody recognized RAP80WT, but not the RAP80(S677A) or RAP80(S677D) mutant (Fig. 2*C*). Endogenous RAP80 proteins were also recognized by the phosphospecific antibody, but phosphatase-treated endogenous RAP80 proteins were not (Fig. 2*D*). This phosphorylation site is conserved in many vertebrate species, including human, mouse, and rat, and the surrounding sequence matches the consensus target site ((S/T)P*X*(K/R)) of Cdk1 (Fig. 2*E*). Taken together, these data suggest that the generated antibody is specific for phosphorylated Ser-677 of RAP80 and that Ser-677 of RAP80 is a target phosphorylation site for the Cdk1-cyclin B_1 complex. Next, we explored the phosphorylation of RAP80 at Ser-677 by IR. RAP80 phosphorylation was detected within 30 min and disappeared at 1 h after IR treatment (Fig. 2*F*), indicating that phosphorylation of RAP80 at Ser-677 is also dependent on DNA damage.

Cdk1-Cyclin B_1 Complex Phosphorylates RAP80 at Ser-677 during Mitosis—Because the activity of the Cdk1-cyclin B_1 complex is dependent on cell cycle progression, we checked whether RAP80 phosphorylation at Ser-677 is regulated by cell cycle progression. Cells were synchronized with a double thymidine block, released from the block, and harvested at the designated times. The levels of RAP80 phosphorylation at Ser-677 were highest 8 h after release of the double thymidine block. Cyclin B_1 expression levels and flow cytometric analysis confirmed cell cycle progression (Fig. 3*A*). Consistent with previous data (20), Fig. 3*A* also shows that the RAP80 stability was dependent on the cell cycle. Additionally, RAP80 phosphorylation at Ser-677 was detected in the mitotic phase (Fig. 3*B*). Elevated cyclin A_2 expression or Ser-10 phosphorylation of histone H3 (a marker of chromosome condensation) was observed for each respective stage. Mitotic RAP80 proteins were also recognized by the phosphospecific antibody, but phosphatase-treated mitotic RAP80 proteins were not (Fig. 3*C*). To further confirm the RAP80 phosphorylation at Ser-677, we used a cell-free system derived from asynchronous or mitotic HeLa cells. GST-RAP80 protein was phosphorylated at the Ser-677 site (detected by the specific antibodies) in nocodazole-treated mitotic cell extracts and in asynchronous cell extracts containing the active Cdk1-cyclin B_1 complex (Fig. 3*D*). Mitotic RAP80 phosphorylation at Ser-677 was inhibited by the Cdk inhibitor roscovitine (Fig. 3*E*), but not by the phosphoinositol 3-kinase family inhibitor wortmannin. Next, we evaluated the phosphorylation at Ser-677 in cells after knockdown of Cdk1. RAP80 phosphorylation at Ser-677 was reduced in Cdk1 siRNA-transfected cells compared with control siRNA-transfected cells (Fig. 3*F*). These results suggest that the Cdk1-cyclin B_1 complex phosphorylates RAP80 at Ser-677 during the mitotic phase in HeLa cells. Finally, we checked the intracellular localization of Ser-677-phosphorylated RAP80 during the various phases of the cell cycle. Ser-677-phosphorylated RAP80 was detected in the nucleoplasm during the S and G_2 phases, but was detected within cells except for chromosomes during mitosis (Fig. 3*G*). Additionally, consistent with Fig. 3 (*A* and *B*), elevated RAP80 phosphorylation at Ser-677 was evident in the mitotic phase (Fig. 3*G*). We confirmed various phases of the cell cycle using specific antibodies and flow cytometric analysis.

Cdk1 Is a RAP80-binding Protein—To evaluate the molecular interaction between RAP80 and Cdk1, the binding between RAP80 and Cdk1 was verified using *in vitro* and *in vivo* binding assays. A GST pulldown assay using a GST-RAP80 fusion protein and cell lysates of 293T cells overexpressing Cdk1 showed that GST-RAP80 specifically bound to overexpressed Cdk1, in contrast to GST only (Fig. 4*A*). Immunoprecipitation analysis


A
Time (hrs) after release
As 0 4 6 8 12 22
W : Anti-pS677
W : Anti-RAP80
W : Anti-cyclin B1
W : Anti-β-actin
57 78 4 4 4 62 80 G1 (%)
24 13 93 30 13 18 15 S (%)
19 9 3 66 83 20 5 G2/M (%)
Relative phosphorylation of RAP80 Ser677 residue
Time (hrs) after release
B
G1/S G2 M
W : Anti-pS677
W : Anti-RAP80
W : Anti-cyclin A2
W : Anti-pH3
W : Anti-β-actin
89 5 5 G1 (%)
6 7 3 S (%)
5 88 92 G2/M (%)
C
Mitotic cell
γPPase : - +
W : Anti-pS677
W : Anti-RAP80
D
Cdk1/ cyclin B1 (ng)
0 4 20
Cell extracts : M M As As As
GST-RAP80C : - + + + +
W : Anti-pS677
W : Anti-Cdk1
GST-RAP80C Coomassie
F
Con siRNA
Cdk1 siRNA1
Cdk1 siRNA2
W : Anti-pS677
W : Anti-RAP80
W : Anti-Cdk1
W : Anti-β-actin
E
Inhibitor : - - - - - Wor Ros
Cell extracts : - As M As M M M
GST-RAP80C : + - - + + + +
W : Anti-pS677
W : Anti-Cdk1
GST-RAP80C Coomassie
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S phase
G2 phase
Mitotic phase
PCNA
Cyclin B1
pS677
DAPI
Merge
G1/S
S
G2/M
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DNA content

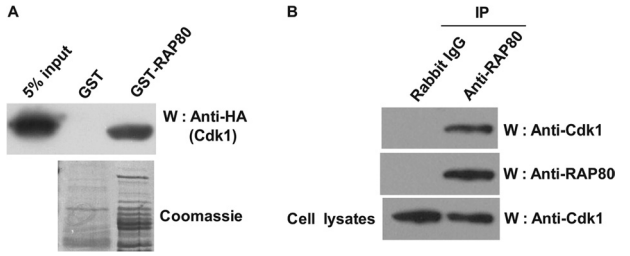

FIGURE 4. **Cdk1 is a RAP80-binding protein.** *A*, *in vitro* binding assay between GST-RAP80 and HA-Cdk1. Cell lysates of 293T cells transfected with an HA-Cdk1 expression plasmid were incubated with 2 μg of GST or GST-RAP80 fusion protein for 1 h at 4 °C. The bound complexes were separated by SDS-PAGE and subjected to Western blot analysis (*W*) using anti-HA antibody (*upper panel*). The Coomassie Blue-stained *lower panel* shows the GST fusion proteins. *B*, endogenous binding between RAP80 and Cdk1. Immunoprecipitation (*IP*) was performed using rabbit IgG or anti-RAP80 antibody, followed by Western blot analysis using the indicated antibodies (*upper* and *middle panels*). The *lower panel* shows equal volumes of cell lysates probed with anti-Cdk1 antibody.

using anti-RAP80 antibody also showed that RAP80 specifically bound to Cdk1 at endogenous levels (Fig. 4*B*). The data support the suggestion that Cdk1 is a RAP80-binding protein.

Phosphorylation at Ser-677 Sensitizes Cells to IR and Functions in the G_2/M Checkpoint—RAP80 is translocated to DNA damage sites and is required for recruitment of the BRCA1-A complex to DNA damage sites through binding with CCDC98 (8–16). We tested whether RAP80 phosphorylation at Ser-677 could affect its localization to sites of DNA damage after IR treatment. No differences were observed in the localization of the RAP80WT, RAP80(S677A), and RAP80(S677D) proteins to DNA damage sites (Fig. 5*A*). In addition, the Ser-677 mutants did not exhibit defects in CCDC98 association (Fig. 5*B*). Knockdown of RAP80 leads to defects in the DNA damage response, in particular impaired cell cycle checkpoints and increased sensitivity to DNA-damaging agents (8). In addition, *Rap80*$^{-/-}$ cells display increased chromosome anomalies (21). This may result from the defects in either the cell cycle checkpoint or DNA repair. We therefore examined whether RAP80 phosphorylation would result in similar defects in the DNA damage response. Stable HeLa cell lines expressing RAP80WT, RAP80(S677A), and RAP80(S677D), which are resistant to RAP80 siRNA, were generated (Fig. 5*C*). Using a previously established cell survival assay (8), we showed that mock or RAP80(S677A)-expressing cell lines were more sensitive to radiation than RAP80WT- or RAP80(S677D)-expressing cells (supplemental Fig. 1). We repeated this experiment using endogenous RAP80 knockdown RAP80WT- and RAP80(S677A)-overexpressing cell lines with more IR doses (0, 2, 4, 6, or 8 grays (Gy)) to show the maximized effect. Mock or RAP80(S677A)-expressing cell lines were more sensitive to IR than RAP80WT- or RAP80(S677D)-expressing cells at high doses of IR (Fig. 5*D*). These data suggest that phosphorylation at Ser-677 renders the cell more resistant to IR. In addition, endogenous RAP80 knockdown RAP80(S677A)-expressing cells showed defective G_2/M checkpoint control after DNA damage (Fig. 5*E*). Furthermore, we analyzed the cell cycle index of mock, RAP80WT-overexpressing, or RAP80(S677A)-overexpressing cell lines. The cells were synchronized with a double thymidine block, released from the block, and harvested at the designated times. Flow cytometric analysis revealed that a significant percentage of stable RAP80(S677A)-expressing cells were arrested in the G_2/M phase compared with mock or RAP80WT-expressing cells (supplemental Fig. 2). The effects of G_2/M arrest in RAP80(S677A)-expressing cell lines were more significant when we transfected with RAP80 siRNA (Fig. 5*F*).

DISCUSSION

The present data obtained using the tandem repeat affinity purification technique indicate that Cdk1 is a novel RAP80-binding protein. *In vitro* kinase assays and use of a phosphospecific antibody also demonstrated that Cdk1 mediates RAP80 phosphorylation at an evolutionarily conserved Ser-677 residue. RAP80 phosphorylation at Ser-677 was induced by IR treatment. In addition, Cdk1-cyclin B_1 complex-mediated RAP80 phosphorylation at Ser-677 affected the sensitivity to IR and G_2/M checkpoint control. Based on these results, it is reasonable to suggest that the post-translational modification of RAP80 Ser-677 could be a novel mechanism that functions in sensitivity to IR and defective G_2/M checkpoint control. Furthermore, RAP80 phosphorylation at Ser-677 is highest during the mitotic phases, and it correlates with cyclin B_1 expression levels. This indicates that RAP80 phosphorylation at Ser-677 is regulated in a cell cycle-dependent manner.

Previous studies have shown that RAP80 localizes to DNA double-strand break regions called DNA foci. RAP80 regulates the DNA damage checkpoint to delay the G_2/M transition, although the exact mechanism is unclear. In this study, we con-

FIGURE 3. **Cdk1-cyclin B_1 phosphorylates RAP80 at Ser-677 in mitosis.** *A*, HeLa cells were synchronized by a double thymidine block and released. After a certain amount of time after release of the cell cycle block, cells were harvested for analysis. Cell lysates were immunoblotted using the indicated antibodies. Cell cycle distributions were analyzed by flow cytometry, and the results are summarized at the bottom of the *left panel*. In the *right panel*, *error bars* represent S.D. from three independent experiments. *, $p < 0.05$; **, $p < 0.01$ (two-tailed Student's *t* test). *B* and *C*, regulation of RAP80 phosphorylation at Ser-677. *B*, HeLa cells were synchronized at the G_1/S boundary by a double thymidine block. Cells were subsequently washed and allowed to progress through the cell cycle for 12 h in the presence of 1 μg/ml nocodazole. Mitotic (*M*) round cells were collected by shake-off, and the remaining attached cells (G_2 phase) were also harvested. Cell lysates were immunoblotted using the indicated antibodies. Cell cycle distributions were analyzed by flow cytometry, and the results are summarized at the bottom. *C*, phosphatase-treated mitotic RAP80 protein is not recognized by the phospho-Ser-677-specific antibody. Mitotic 293T cell lysates were immunoprecipitated with anti-RAP80 antibody. Immunoprecipitated RAP80 proteins were incubated with or without γ-phosphatase (*γPPase*) and subjected to Western blot analysis using the indicated antibodies. *D*, mitotic cell extracts and asynchronous cell extracts (*As*) containing recombinant Cdk1-cyclin B_1 directly phosphorylated GST-RAP80 at Ser-677. GST-RAP80C was incubated in asynchronous cell extracts containing Cdk1-cyclin B_1 or mitotic extracts made from nocodazole-treated cells. After incubation for 1 h at 30 °C, the reaction mixture was separated by SDS-PAGE, and RAP80 phosphorylation was detected using the antibody against RAP80 phospho-Ser-677. The Coomassie Blue-stained *lower panel* shows the amount of GST fusion proteins added. *E*, the Cdk family inhibitor roscovitine (*Ros*) inhibited mitotic cell extract-mediated RAP80 phosphorylation. GST-RAP80C was incubated for 1 h at 30 °C with HeLa cell extracts derived from asynchronous or mitotic cells. Mitotic arrested cell extracts containing roscovitine or the phosphoinositol 3-kinase family inhibitor wortmannin (*Wor*) were also incubated for 1 h at 30 °C, analyzed by SDS-PAGE, and immunoblotted with the specified antibodies. *F*, knockdown of Cdk1 reduced RAP80 phosphorylation at Ser-677. Control (*Con*) siRNA-, Cdk1 siRNA1-, or Cdk1 siRNA2-transfected cell lysates were analyzed by SDS-PAGE and immunoblotted with the specified antibodies. *G*, HeLa cells were synchronized at the G_1/S and G_2/M phases by double thymidine block and released. Immunofluorescence assays were performed using anti-RAP80 phospho-Ser-677, anti-proliferating cell nuclear antigen (*PCNA*), or anti-cyclin B_1 antibody. DAPI was used as an indicator for the nucleus (*upper panels*). Cell cycle distributions were analyzed by flow cytometry (*lower panels*).

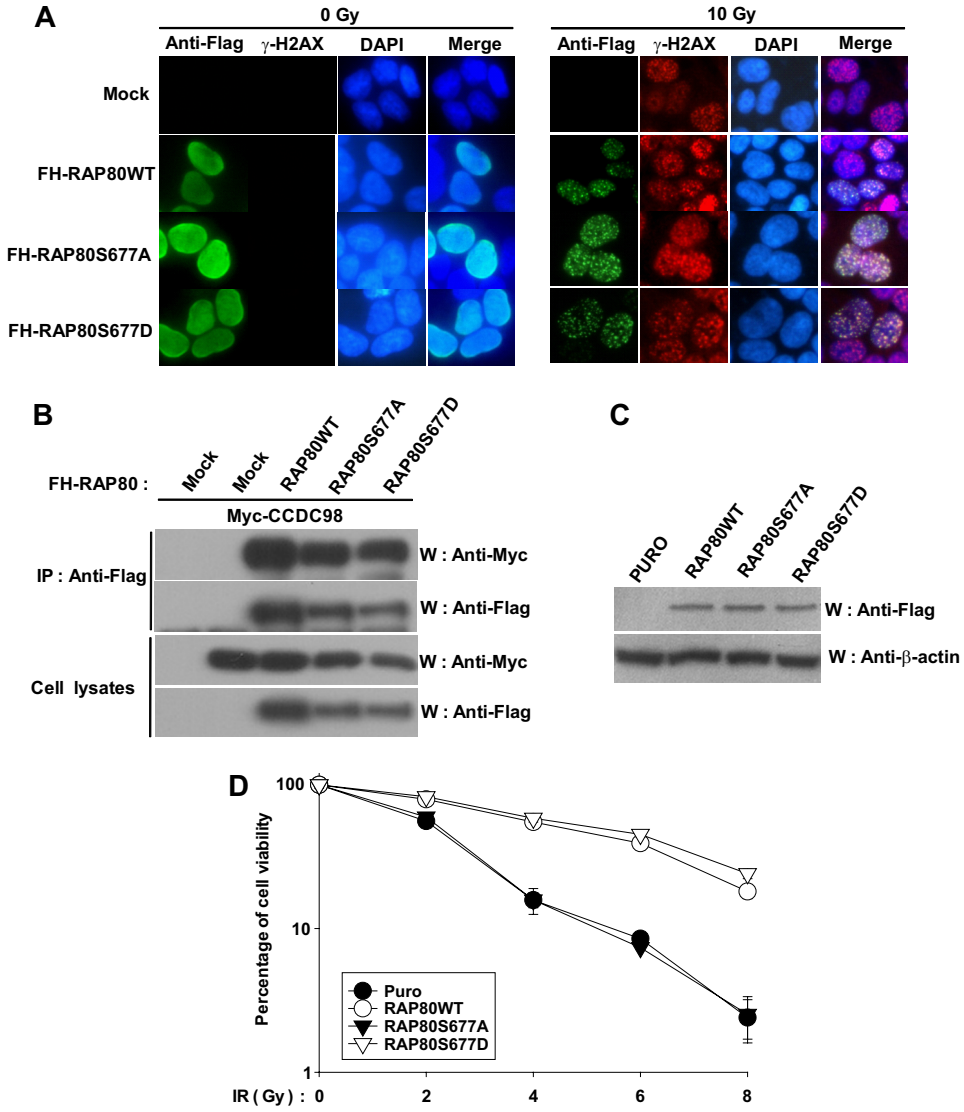

FIGURE 5. **Phosphorylation of Ser-677 causes severe G_2/M cell cycle arrest.** *A*, translocation of FH-RAP80WT, FH-RAP80(S677A), or FH-RAP80(S677D) to DNA damage sites. FH-RAP80WT, FH-RAP80(S677A), or FH-RAP80(S677D) expression plasmid-transfected cells were treated with 0 or 10 Gy of IR. After 6 h, cells were subjected to immunofluorescence assays using anti-FLAG and γ-H2AX antibodies. DAPI was used as an indicator for the nucleus. *B*, Ser-677 phosphorylation did not affect RAP80 association with CCDC98. A Myc-CCDC98 expression plasmid was cotransfected with the indicated expression plasmids into HEK293T cells. Transfected cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody and immunoblotted with the indicated antibodies. Expression levels of the overexpressed proteins were analyzed by immunoblotting with the indicated antibodies. *W*, Western blot. *C*, HeLa cell lines stably expressing FH-RAP80WT, FH-RAP80(S677A), or FH-RAP80(S677D), which are resistant to siRNA, were established. Each stable cell line was immunoblotted using the indicated antibodies. *PURO*, puromycin. *D*, phosphorylation of RAP80 at Ser-677 affected cell survival after DNA damage. Stable mock or FH-RAP80WT-, FH-RAP80(S677A)-, or FH-RAP80(S677D)-expressing HeLa cell lines (*C*) were transfected twice with control or RAP80 siRNA at 24-h intervals. Forty-eight hours after the second transfection, transfected cells were irradiated with 0, 2, 4, 6, or 8 Gy. The percentage of surviving colonies was determined 11 days later. These experiments were performed in duplicate, and the results represent the average of two independent experiments. *Error bars* indicate S.D. for each irradiated dose. *, $p < 0.01$ (two-tailed Student's *t* test). *E*, G_2/M checkpoint in the stable mock or FH-RAP80WT-, FH-RAP80(S677A)-, or FH-RAP80(S677D)-expressing cell lines. The stable cells were transfected twice with control or RAP80 siRNA at 24-h intervals. Forty-eight hours after the second transfection, transfected cells were irradiated with 0 or 2 Gy. Cells were incubated for 1 h before fixation and subjected to staining with antibody to phosphorylated histone H3 (*pH3*) and propidium iodide. The percentage of mitotic cells was determined by FACS analysis (*left panels*). The percentage of the mitotic population was determined (*right panel*). The results represent the average of two independent experiments. *Error bars* indicate S.D. *, $p < 0.05$ (two-tailed Student's *t* test). *F*, phosphorylation of RAP80 at Ser-677 affected G_2/M arrest in the cell cycle. The stable cell lines expressing RAP80WT or RAP80(S677A) were transfected twice with control or RAP80 siRNA at 24-h intervals. Forty-eight hours after the second transfection, transfected cells were synchronized by a double thymidine block (*DTB*) and released. Cells were harvested for FACS analysis at a certain time after release of the cell cycle block. The *arrow* indicates the mitotic delay in the HeLa cell line stably expressing RAP80(S677A) (*left panels*). The *numbers* indicate the percentage of the G_2/M population (*left panel*). The percentage of the G_2/M population was summarized for each time after release of the cell cycle block. The results represent the average of three independent experiments. *Error bars* indicate S.D. *As*, asynchronous. *, $p < 0.05$; **, $p < 0.0005$ (two-tailed Student's *t* test).

ducted experiments focused on an unknown function of RAP80 related to phosphorylation at Ser-677. The finding that phosphorylation of RAP80 at Ser-677 is not critical in translocating into the DNA damage sites or in binding to CCDC98 (Fig. 5, *A* and *B*) suggests that RAP80 phosphorylation at Ser-677 may have functions other than translocation into DNA damage sites and binding to CCDC98. We tested cell survival under the condition of radiation related to Cdk1-mediated RAP80 phosphorylation at Ser-677. Surprisingly, compared with other cell lines, the survival of the S677A mutant cells was significantly reduced

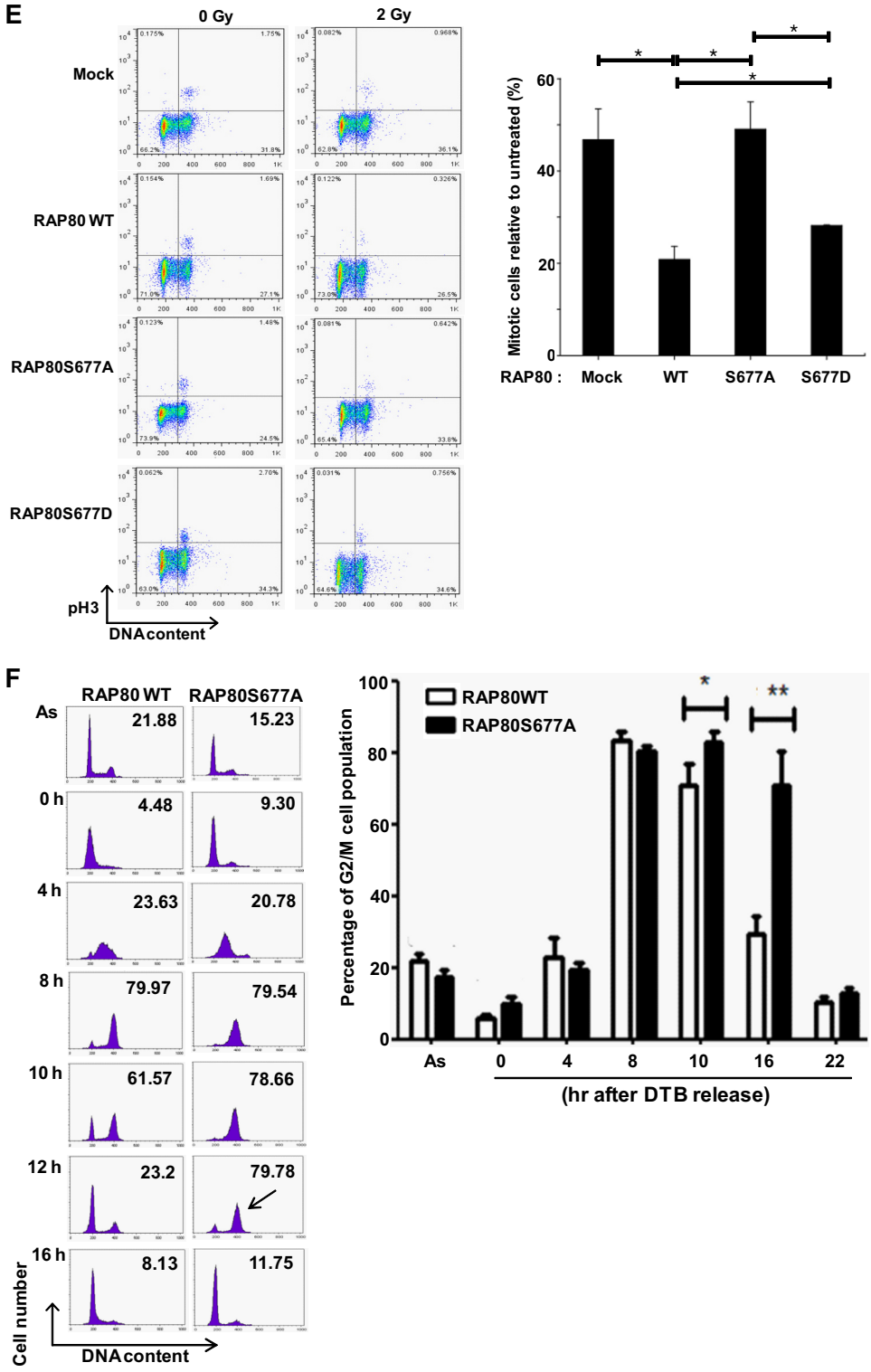

FIGURE 5—*continued*

after treatment with IR. Therefore, RAP80 phosphorylation at Ser-677 must be important for the DNA damage response. Furthermore, Ser-677 phosphorylation was altered within 30 min when cells were treated with IR. Various proteins involved in the DNA damage response and DNA repair have been identified as Cdk targets (22–24). The present study implicates RAP80 as a direct Cdk1 substrate and indicates that Cdk1-mediated phosphorylation is required for RAP80 activation at the G_2/M checkpoint in response to DNA damage. In addition, a significant percentage of stable RAP80(S677A)-expressing cells were arrested in the G_2/M phase compared RAP80WT-expressing cells. This may result from the attenuation of DNA repair because of a deficient G_2/M checkpoint in stable RAP80(S677A)-expressing cells.

However, there remains an unsolved question. If Cdk1 is a major molecular target of the G_2/M checkpoint, how would RAP80 phosphorylation at Ser-677 be mediated by Cdk1 when the G_2/M checkpoint is on? In this study, RAP80 phosphorylation at Ser-677 was induced within 30 min in response to DNA damage, so we think that Cdk1 phosphorylates RAP80 early in the response to DNA damage by an as yet unknown mechanism. These observations suggest that Cdk1-mediated RAP80 phosphorylation at Ser-677 is probably used as rapid means of RAP80 activation to function in the DNA damage response, but we cannot exclude the possibility that another kinase(s) phosphorylates RAP80 in response to DNA damage. The exact molecular mechanism of G_2/M checkpoint control by Cdk1-mediated RAP80 phosphorylation will be further studied.

In addition to Ser-677, two other phospho-SP sites were previously identified by mass spectrometric analysis (8). However, these two sites were not phosphorylated by Cdk1 in an *in vitro* kinase assay, suggesting that they may be phosphorylated by an unknown kinase(s) and that Ser-677 might be a specific substrate for Cdk1. We speculate that mutations of RAP80 Ser-677 that block phosphorylation may have a potential role in tumorigenesis. Future studies will be done to elucidate the molecular mechanisms of the RAP80 phosphorylation-dependent DNA repair pathway and regulation of mitotic progress.

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