Cdk1-mediated Phosphorylation of Receptor Associated Protein 80 (RAP80) Serine 677 Residues Modulate DNA Damage-induced G2/M Checkpoint and Cell Survival.

Hyun Jung Cho, Yun Jung Oh, Seung Hun Han, Hee Jin Chung, Chang Hee Kim, Nam Soo Lee, Won-Ju Kim, Je-Min Choi, and Hongtae Kim

1Department of Biological Sciences, Sungkyunkwan University; 2Department of Life Science, Research Institute for Natural Sciences, Hanyang University,

Running title: Post-translational phosphorylation of RAP80

*Address correspondence to: Hongtae Kim, 300, Cheoncheon-dong, Jangan-gu, Suwon 440-746, Republic of Korea; Tel: 82-31-299-4497; Fax: 82-31-290-7015; E-mail: khtcat@skku.edu

Or Je-Min Choi, 55, Wangsimni-ro, Seongdong-gu, Seoul 133-791, Republic of Korea; Tel: 82-2-2220-4765; Fax: 82-2-2299-3495; E-mail: jeminchoi@hanyang.ac.kr

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Background: RAP80, a member of the BRCA1-A complex, is crucial in cell cycle checkpoint and DNA damage repair.

Results: RAP80 phosphorylation by Cdk1 is important for sensitivity to ionizing radiation and G2/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.

SUMMARY

Post-translational phosphorylation plays critical roles in the assembly of signaling and repair proteins in the DNA damage response pathway. Receptor associated protein 80 (RAP80), a member of the BRCA1-A complex, is crucial in cell cycle checkpoint activation and DNA damage repair. However, its molecular mechanism is unclear. This study identifies cyclin dependent kinase 1 (Cdk1) as a new RAP80 binding protein and demonstrates that the Cdk1-cyclin B1 complex phosphorylates RAP80 at the Ser677 residue using an in vitro kinase assay and a phosphopeptide-specific antibody against the phosho-Ser677 residue of RAP80. RAP80 Ser677 phosphorylation occurred in the M phase of the cell cycle when Cdk1 was in an active state. In addition, ionizing radiation induced RAP80 phosphorylation at the Ser677 residue. Mutation of the Ser677 residue to alanine sensitized cells to ionizing radiation and functioned in G2/M checkpoint control. These results suggest that post-translational phosphorylation of RAP80 by the Cdk1-cyclin B1 complex is important for RAP80 functional sensitivity to ionizing radiation and G2/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), and many chemicals. DNA lesions such as double strand breaks (DSBs) 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 comprised of a series of events tightly regulated by cyclin and cyclin dependent kinase (Cdk) 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 G1 to S phases (G1/S transition). The Cdk1-cyclin B...
complex initiates the G2/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 G1/S checkpoint, intra-S checkpoint, and G2/M checkpoint (5, 7). The G2/M cell cycle checkpoint blocks cell cycle progression into mitosis and the Cdk1-cyclin B complex is the main target molecule of the G2/M cell cycle checkpoint (6, 7).

RAP80 is a component of the BRCA1-A complex, which contains a coiled-coil domain-containing protein98 (CCDC98/ABRAXAS), the BRCA1/BRCA2-containing complex subunit 45/brain and reproductive organ-expressed protein (BRCC45/BRE), the BRCA1/BRCA2-containing complex subunit 36 (BRCC36), mediator of RAP80 interactions and targeting subunit of 40/new component of the BRCA1-A complex (MERIT40/NBA1), and breast cancer 1 (BRCA1) (8-16). RAP80 targets BRCA1-A complex to DNA damage sites by interacting with lysine 63-mediated polyubiquitinated histones through its two tandem ubiquitin interacting motifs (UIMs) (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 phospho-protein 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 identify Cdk1 as a new RAP80 binding protein and demonstrate that the Cdk1-cyclin B1 complex directly phosphorylates RAP80 at the Ser677 residue, one of three phosphorylated SP sites, by using an in vitro kinase assay and phosphopeptide-specific antibody against the phospho-Ser677 residue in RAP80. We also demonstrate that post-translational phosphorylation of RAP80 by Cdk1-cyclin B1 complex is important for RAP80 functions in sensitivity to ionizing radiation and G2/M checkpoint control.

**Experimental procedures**

**Cell culture**

HeLa and human embryonic kidney (HEK) 293T cell lines were purchased from American Type Culture Collection (Manassas, VA). The cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO2 (v/v).

**Small interfering RNAs (siRNAs)**

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

**In vitro kinase assays**

The assays were performed with the recombinant Cdk1-cyclin B complex (Millipore, Billerica, MA). Glutathione S-transferase (GST)-RAP80 protein was incubated with two units of Cdk1-cyclin B complex, 200 μM ATP, and 10 μCi of [γ-32P]-ATP for 1 hr at 30 °C. The reaction products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and autoradiography.

**Antibodies, transfection, and immunoprecipitation**

Rabbit anti-phospho Ser677 antibody was raised by immunizing rabbits with phospho-peptide (672RDLNEpSPVKSF681). The resulting rabbit polyclonal antibodies were affinity-purified using the Sulfolink Plus Immobilization and Purification Kit (Pierce, Rockford, IL). Anti-RAP80 antibody was previously described (8). Anti-Flag, -HA, -Myc, and -β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Human cyclin E1, cyclin A2 and cyclin B1, pH3, and Cdk1 antibodies were purchased from Cell Signaling Technology (Danvers, MA). The dilutions of the various antibodies for Western blot analysis were: anti-RAP80 (1 : 1000), anti-phospho Ser677 (1 : 250), anti-Flag (1 : 1000), anti-HA (1 : 1000), anti-Myc (1 : 1000), anti-β-actin (1 : 4000).
anti-Myc (1 : 1000), anti-β-actin (1 : 5000), anti-cyclin E1 antibody (1 : 1000), anti-cyclin A2 antibody (1 : 1000), anti-cyclin B1 antibody (1 : 1000), anti-ph3 antibody (1 : 200), and anti-Cdk1 antibody (1 : 500). The dilutions of the various antibodies for immunofluorescence (IF) were: anti-phospho Ser677 (1 : 200), anti-Flag (1 : 500), anti-cyclin B1 (1 : 200), anti-PCNA (1 : 200), and anti-γ-H2AX (1 : 200). Transient transfection was performed using the Fugene 6 reagent (Roche Applied Science, Basel, Switzerland). For immunoprecipitation, cells were washed with ice-cold phosphate buffered saline (PBS) and then lysed in NETN buffer (0.5% Nonidet P-40, 20 mM Tris [pH 8.0], 50 mM NaCl, 50 mM NaF, 100 µM Na3VO4, 1 mM dithiothreitol, and 50 µg/ml phenylmethanesulfonylfluoride) 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 NETN buffer and subjected to SDS-PAGE. Western blotting was performed using the antibodies indicated in the figure legend.

Establishment of stable cell lines and affinity purification of S-Flag-Streptavidin binding peptide (SBP) [SFB]-tagged RAP80-containing complexes
The establishment of stable cell lines was previously described (8). To establish cell lines stably expressing epitope-tagged proteins, HeLa cells were transfected with plasmids encoding RAP80 wild type (WT), RAP80S677A or RAP80S677D, 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 previously described (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 G1 phase using the thymidine double-blocking 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 hrs. After removal of the thymidine and incubation for 10 hrs in fresh medium, thymidine was added to a final concentration of 2 mM for an additional 16 hrs. After removal of 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 hrs 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 G1/S boundary. After washing twice with PBS, cells were fixed with chilled 70% alcohol at 20 °C for 24 hrs. The fixed cells were collected by centrifugation (2000 rpm, 5 min), washed twice with PBS, incubated with RNaseA (30 mg/ml) for 30 min at 37 °C, and stained with 50 µg/ml propidium iodide (Sigma-Aldrich) for 30 min at room temperature, and then analyzed by flow cytometry.

G2/M cell-cycle checkpoint assay
G2/M cell-cycle checkpoint assay was performed as previously described (8). HeLa cells in a 100 mm-diameter plate were transfected twice with control or RAP80 siRNA at 24-hr intervals. Forty-eight hours after the second transfection, transfected cells were mock-treated or irradiated at the indicated doses using a radiation source. One hr after irradiation, cells were fixed with 70% (v/v) ethanol at ~20 °C for 24 hrs, then stained with rabbit antibody to pH3 (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 then analyzed by flow cytometry.

Cell survival assay
Cell survival assay was done as previously described (8). HeLa cells in a 60 mm-diameter plate were transfected twice with control or RAP80 siRNA at 24-hr 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-RAP80 N and C constructs were previously described (8, 11). RAP80 point mutants were generated by site-directed mutagenesis. The HA-tagged Cdk1 expression vector was generated by polymerase chain reaction (PCR). The small interfering RNA (siRNA) resistant RAP80 expression plasmid was previously described (20).

Statistical analysis
Student’s t-test was performed. Error bars represent standard deviation (SD) of several independent experiments. A value of \( P<0.05 \) (2-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-agarose beads. We detected several specific bands indicative of eluted from the SFB-RAP80 cell line, but not from the SFB control cell line (Fig. 1A). Mass spectrometric analysis indicated that the first band was SFB-RAP80 protein and the third band contained numerous proteins including BRCA1/2-containing complex 36 (BRCC36), a known component of RAP80-binding protein complexes. Cdk1 was detected in the eluted RAP80-binding complex (Fig. 1A). 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 an anti-Cdk1 antibody showed that Cdk1 specifically bound to RAP80 at endogenous levels (Fig. 1B). The immunoprecipitation and purification analysis suggested the possibility that RAP80 may be a substrate of Cdk1-cyclin B1 complex. This was confirmed by an in vitro kinase assay using GST-RAP80 wild-type fusion proteins and purified recombinant Cdk1-cyclin B1 complex, which phosphorylates the fusion proteins. In the assay RAP80 proved to be a substrate for the Cdk1-cyclin B1 complex (Fig. 1C). Next, we analyzed the RAP80 phosphorylation sites in an in vitro kinase assay that used N-terminus (GST-RAP80N), or C-terminus (GST-RAP80C) fusion proteins and purified recombinant Cdk1-cyclin B1 complex. The Cdk1-cyclin B1 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. 1D). The phosphorylation sites in the RAP80 C-terminal region are summarized in Fig. 1E (one SD, three SQs, and three SPs). Since SP site is putative Cdk1 phosphorylation site, point-mutated GST-fusion proteins of each serine residue (GST-RAP80S473A, GST-RAP80S653A, or GST-RAP80S677A) were used to examine the phosphorylated sites. GST-RAP80 WT, GST-RAP80S473A, or GST-RAP80S653A were phosphorylated by the Cdk1-cyclin B1 complex, but GST-RAP80S677A was not phosphorylated (Fig. 1F), suggesting that the Ser677 residue of RAP80 is a target phosphorylation site by the Cdk1-cyclin B1 complex.

Detection of RAP80 phosphorylation by phospho-Ser677 specific antibody
To further confirm in vivo RAP80 phosphorylation at the Ser677 residue, we generated a phosphopeptide-specific antibody against the phosphopeptide sequence surrounding the Ser677 residue. Specificity of the phospho-antibody was validated using in vitro phosphorylation of GST-RAP80C by the Cdk1-cyclin B1 complex in the presence of cold ATP. The phospho-specific antibody against the RAP80 phospho-Ser677 residue detected RAP80 protein in the presence of both the Cdk1-cyclin B1 complex and cold ATP (Figs. 2A and
but could not detect the GST-RAP80S677A mutant protein (Fig. 2B). Next, in vivo detection of RAP80 phosphorylation at the Ser677 residue was tested using cell lysates of HEK293T cells transfected with plasmids expressing Flag and HA-tagged wild-type RAP80 (FH-RAP80WT), the Flag and HA-tagged RAP80S667A mutant (FH-RAP80S677A) incapable of being phosphorylated at Ser677, or the Flag and HA-tagged RAP80S677D mutant (FH-RAP80S677D) that mimics phosphorylated Ser677. The phospho-specific antibody recognized wild-type RAP80 but not the RAP80S667A or RAP80S677D mutants (Fig. 2C). Endogenous RAP80 proteins were also recognized by the phospho-specific antibody but phosphatase treated endogenous RAP80 proteins were not (Fig. 2D). This phosphorylation site is conserved in many vertebrate species including human, mouse, and rat, and the surrounding sequence matches the consensus target site (S/TPxK/R) of Cdk1 (Fig. 2E). Taken together, these data suggest that the generated antibody is specific for the phosphorylated Ser677 residue of RAP80 proteins and that the Ser677 residue of RAP80 is a target phosphorylation site of the Cdk1-cyclin B1 complex. Next, we explored the phosphorylation of RAP80 at the Ser677 residue by IR. RAP80 phosphorylation was detected within 30 min and disappeared at 1 hr after IR treatment (Fig. 2F) indicating that phosphorylation of RAP80 at Ser677 residue is also dependent on DNA damage.

**Cdk1-cyclin B1 complex phosphorylates RAP80 at Ser677 residue during mitosis**

Since activity of the Cdk1-cyclin B1 complex is dependent on cell cycle progression, we checked whether RAP80 phosphorylation at the Ser677 residue was regulated by cell cycle progression. Cells were synchronized with a double thymidine block, released from the block, and harvested at designated times. RAP80 phosphorylation levels at the Ser677 residue were highest 8 hrs after release of the double thymidine block. Cyclin B1 expression levels and flow cytometric analysis confirmed cell cycle progression (Fig. 3A). Consistent with previous data, Fig. 3A also showed that the RAP80 stability was dependent on the cell cycle (20). Additionally, the RAP80 phosphorylation levels at the Ser677 residue were detected in the mitotic phase (Fig. 3B). Elevated cyclin A2 expression or Ser10 phosphorylation of histone H3 (a marker of chromosome condensation) was observed for each respective stage. Mitotic RAP80 proteins were also recognized by the phospho-specific antibody but phosphatase treated mitotic RAP80 proteins were not (Fig. 3C). To further confirm the RAP80 phosphorylation at Ser677 residue, we used a cell-free system derived from asynchronous or mitotic HeLa cells. GST-RAP80 protein was phosphorylated at the Ser677 site (detected by the specific antibodies) in nocodazole-treated mitotic cell extracts (M) and in asynchronous cell extract (As) containing active Cdk1-cyclin B1 complex (Fig. 3D). Mitotic RAP80 phosphorylation at Ser677 site was inhibited by the cyclin dependent kinase family inhibitor roscovitine (Ros) (Fig. 3E), but not by the phosphoinositol-3-kinase family inhibitor wortmannin (Wor). Next, we evaluated the phosphorylation at Ser 677 residue in cells after knockdown of Cdk1. RAP80 phosphorylation at the Ser677 residue was reduced in Cdk1 siRNA-transfected cells compared with the control siRNA-transfected cells (Fig. 3F). These results suggested that Cdk1-cyclin B1 complex phosphorylates RAP80 at Ser677 residue during the mitotic phase in HeLa cells. Finally, we checked the intracellular localization of the phosphor-Ser677 RAP80 during the various phases of the cell cycle. Phosphor-Ser677 RAP80 was detected in nucleoplasm during S and G2 phase but detected within cell except for chromosome during mitosis (Fig. 3G). Additionally, consistent with Figs. 3A and 3B, elevated RAP80 phosphorylation at the Ser677 residue was evident in the mitotic phase (Fig. 3G). We confirmed various phases of cell cycle by 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-pull down 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. 4A).
Immunoprecipitation analysis using an anti-RAP80 antibody also showed that RAP80 specifically bound to Cdk1 at endogenous levels (Fig. 4B). The data supported the suggestion that Cdk1 is a RAP80 binding protein.

**Phosphorylation at Ser677 residue sensitizes cells to IR and functions in G2/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 Ser677 residue could affect its localization to sites of DNA damage after IR treatment. No differences were observed in the localization of RAP80WT, RAP80S677A, or RAP80S677D proteins to DNA damage sites (Fig. 5A). In addition, the Ser677 mutants did not exhibit defects in CCDC98 association (Fig. 5B). 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 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, RAP80S677A, and RAP80S677D, which are resistant to RAP80 siRNA, were generated (Fig. 5C). Using a previously established cell survival assay (8), we showed that Mock or RAP80S677A expressing cell lines were more sensitive to radiation than RAP80WT or RAP80S677D expressing cells (Supplemental Fig. 1). We repeated this experiment using endogenous RAP80 knockdowned RAP80WT- or RAP80S677A-overexpressing cell lines with more IR doses (0, 2, 4, 6, or 8 Gy) to show the maximized effect. Mock or RAP80S677A expressing cell lines were more sensitive to ionizing radiation than RAP80WT or RAP80S677D expressing cells at high dose of ionizing irradiation (Fig. 5D). These data suggested that phosphorylation at Ser677 residue render the cell more resistant to ionizing irradiation. In addition, endogenous RAP80 knockdowned RAP80S677A expressing cells showed defective G2/M checkpoint control after DNA damage (Figs. 5E).

**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 phospho-specific antibody also demonstrate that Cdk1 mediated RAP80 phosphorylation at an evolutionarily conserved Ser677 residue. RAP80 phosphorylation at Ser677 residue was induced by IR treatment. In addition, Cdk1-cyclin B1 complex-mediated RAP80 phosphorylation at the Ser677 residue affected the sensitivity to IR and G2/M checkpoint control. From these results, it is reasonable to suggest that the post-translational modification of RAP80 Ser677 could be a novel mechanism that functions in sensitivity to IR and defective G2/M checkpoint control. Furthermore, RAP80 phosphorylation at the Ser677 residue is highest during the mitotic phases and it correlates with cyclin B1 expression levels. This indicates that RAP80 phosphorylation at the Ser677 residue is regulated in a cell cycle dependent manner.

Previous studies have shown that RAP80 localizes to DNA DSB regions called DNA foci. RAP80 regulates the DNA damage checkpoint to delay the G2/M transition, although the exact mechanism is unclear. In this study, we conducted experiments focused on an unknown function of RAP80 related to phosphorylation at Ser677 residue. The findings that phosphorylation at the Ser677 residue of RAP80 was not critical in translocating into the DNA damage sites or in binding to CCDC98 (Figs 5A and 5B) suggest that RAP80 phosphorylation at the Ser677 residue 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 Ser677 residue. Surprisingly, compared to other cell lines, cell survival of the S677A mutant cell line was significantly reduced after treatment with IR. Therefore, RAP80 phosphorylation at Ser677 must be important for the DNA damage response. Furthermore, S677 phosphorylation was altered within 30 min when cells were treated with IR. Various proteins involved in 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 G2/M checkpoint in response to DNA damage. In addition, a significant percentage of RAP80S677A expressing stable cells were arrested in the G2/M phase compared RAP80WT-expressing cells. This may result from the attenuation of DNA repair because of a deficient G2/M checkpoint in RAP80S677A expressing stable cells.

However, there remains an unsolved question. If Cdk1 is a major molecular target of the G2/M checkpoint, how would RAP80 phosphorylation at the Ser677 residue be mediated by Cdk1 when the G2/M checkpoint is on? Presently, RAP80 phosphorylation at Ser677 residue 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 may suggest that Cdk1-mediated RAP80 phosphorylation at Ser677 residue is probably used as rapid means of RAP80 activation to function on 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 G2/M checkpoint control by Cdk1-mediated RAP80 phosphorylation will be further studied.

In addition to the Ser677 residue, two other phospho-SP sites were previously identified by mass spectrometry analysis (8). However, these two sites were not phosphorylated by Cdk1 in an in vitro kinase assay, suggesting that those sites may be phosphorylated by an unknown kinase(s) and that the Ser677 residue might be a specific substrate to Cdk1. We speculate that mutations at RAP80 Ser677 residue that blocks 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.

References
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Footnote
Abbreviations used are: RAP80, receptor associated protein 80; siRNA, small interfering RNA; Cdk1, cyclin dependent kinase 1; IR, ionizing radiation; CCDC98, coiled-coil domain-containing protein 98; BRCC45/BRE, BRCA1/BRC2-containing complex subunit 45/brain and reproductive organ-expressed protein; BRCC36, BRCA1/BRC2-containing complex subunit 36; MERIT40/NBA1, mediator of RAP80 interactions and targeting subunit of 40/new component of the BRCA1-A complex; BRCA1, breast cancer 1; SFB, S-Flag-Streptavidin binding peptide; DAPI, 4’,6-diamidino-2-phenylindole

Figure legends
Fig. 1. RAP80 is a new substrate of Cdk1. (A) Tandem repeat affinity purified SFB-RAP80 complexes were sequentially subjected to polyacrylamide gel electrophoresis 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 through 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 reaction was performed using rabbit IgG or anti-Cdk1 antibodies and subjected to Western blot analysis using the indicated antibodies. (C) In vitro kinase assays were performed by incubating recombinant Cdk1-cyclin B1 and [γ-32P]ATP with purified wild-type GST-RAP80. After incubation for 1 hr at 30 °C, the reaction mixture was separated by SDS-PAGE and detected by autoradiography (upper panel). The Coomassie stained bottom panel shows the amount of GST fusion proteins added. In vitro kinase assays were performed by incubating recombinant Cdk1-
cyclin B1 and [γ-32P]ATP with purified GST-RAP80N or C terminus (D), or GST-RAP80, GST-RAP80S473A, GST-RAP80S653A, or GST-RAP80S677A fusion proteins (F). After incubation for 1 hr at 30 °C, the reaction mixture was separated by SDS-PAGE and detected by autoradiography (upper panel). The Coomassie stained bottom panel shows the amount of GST fusion proteins added. (E) Summary of the RAP80 phosphorylation sites. Abbreviations are: UIM, ubiquitin interacting motif; ZFD, zinc finger domain; aas, amino acids.

**Fig. 2. Detection of RAP80 phosphorylation by phos-Ser677 specific antibody.** (A and B) In vitro kinase assays were performed by incubating Cdk1-cyclin B1 and cold ATP with purified GST-RAP80C (A), or GST-RAP80C, GST-RAP80CS473A, GST-RAP80CS653A, and GST-RAP80CS677A fusion proteins (B). After incubation for 1 hr at 30 °C, the reaction mixture was separated by SDS-PAGE and RAP80 phosphorylation was detected using the RAP80 phospho-Ser677 antibody. The Coomassie stained bottom panel shows the amount of GST fusion proteins added. (C) FH-RAP80WT, -RAP80S677A, or -RAP80S677D expression plasmids were transfected into HEK293T cells. Transfected cell lysates were subjected to Western blot analysis using the indicated antibodies. (D) Phosphatase-treated endogenous RAP80 protein is not recognized by the phospho-Ser677 specific antibody. 293T cell lysates were immunoprecipitated 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 the Ser677 residue 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.

**Fig. 3. Cdk1-cyclin B1 phosphorylates RAP80 at the Ser677 residue 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 (left panel). Error bars represent the standard deviation from three independent experiments. *P<0.05 and **P<0.01 two-tailed Student’s t-test (right panel). (B and C) Regulation of RAP80 phosphorylation at the Ser677 residue. (B) HeLa cells were synchronized at the G1/S boundary by a double thymidine block. Cells were subsequently washed and allowed to progress through the cell cycle for 12 hrs in the presence of 1 μg/ml of nocodazole. Mitotic (M) round cells were collected by shake-off, and the remaining attached cells (G2 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-Ser677 specific antibody. Mitotic 293T cell lysates were immunoprecipitated with anti-RAP80 antibody. Immunoprecipitated RAP80 proteins were incubated with or without γ-phosphatase and subjected to Western blot analysis using the indicated antibodies. (D) Mitotic cell extracts and recombinant Cdk1-cyclinB1 added asynchronous cell extracts directly phosphorylated GST-RAP80 at Ser677 residue. GST-RAP80C was incubated using the RAP80 phospho-Ser677 antibody. The Coomassie stained bottom panel shows the amount of GST fusion proteins added. (E) The added cyclin-dependent kinase family inhibitor, roscovitine (Ros), inhibited mitotic cell extracts (M)-mediated RAP80 phosphorylation. GST-RAP80C was incubated for 1 hr at 30 °C with HeLa cell extracts derived from asynchronous cells (As) or mitotic cells (M). Mitotic arrested cell extracts containing Ros, or the phosphoinositol-3-kinase family inhibitor, wortmannin (Wor), were also incubated with indicated for 1 hr at 30 °C, analysed by SDS–PAGE, and immunoblotted with the specified antibodies. (F) Knock-down of Cdk1 reduced RAP80 phosphorylation at the Ser677 residue. Control, Cdk1 siRNA1, or 2 transfected cell lysates were analyzed by SDS–PAGE and immunoblotted with the specified antibody. (G) HeLa cells were synchronized at the G1/S, and G2/M phases by double thymidine
block and released. Immunofluorescence assays were performed using anti-phospho-Ser677 RAP80 and anti-PCNA, or -cyclinB1. 4',6-Diamidino-2-phenylindole (DAPI) was used as an indicator for the nucleus (upper panel). Cell cycle distributions were analyzed by flow cytometry (bottom panel).

Fig. 4. Cdk1 is a RAP80-binding protein. (A) An 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 hr at 4 °C. The bound complexes were separated by SDS-PAGE and subjected to Western blot analysis using an anti-HA antibody (upper panel). The Coomassie stained bottom panel shows the GST fusion proteins. (B) Endogenous binding between RAP80 and Cdk1. Immunoprecipitation was performed using rabbit IgG or anti-RAP80 antibody and subjected to Western blot analysis using the indicated antibodies. The bottom panel shows equal volumes of cell lysates probed with anti-Cdk1 antibody.

Fig. 5. Phosphorylation of Ser677 causes severe G2/M arrest in cell cycle. (A) Translocation of FH-RAP80WT, -RAP80S677A, or -RAP80S677D to DNA damage sites. FH-RAP80WT, -RAP80S677A, or -RAP80S677D expression plasmid transfected cells were treated with 0 or 10 Gy of IR. After 6 hrs, cells were subjected to immunofluorescence assays using anti-Flag and 7-H2AX antibodies. 4',6-Diamidino-2-phenylindole (DAPI) was used as an indicator for the nucleus. (B) Ser677 phosphorylation does not affect RAP80 association with CCDC98. A Myc-tagged CCDC98 expression plasmid (Myc-CCDC98) was co-transfected with indicated expression plasmids into HEK293T cells. Transfected cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with the indicated antibodies. Expression levels of the overexpressed proteins were analyzed by immunoblottting with the indicated antibodies. (C) HeLa cell lines stably expressing the FH-RAP80WT, -RAP80S677A, or -RAP80S677D which are resistant to siRNA were established. Each stable cell line was immunoblotted using the indicated antibodies. (D) Phosphorylation of the RAP80 Ser677 residue affects cell survival after DNA damage. HeLa cell lines stably expressing Mock, FH-RAP80WT, -RAP80S677A or -RAP80S677D (Fig. 5C) were transfected twice with control or RAP80 siRNA at 24-hr 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 the standard deviation for each irradiated dose. *P<0.01 two-tailed Student’s t-test. (E) G2/M checkpoint in the stable cell lines expressing Mock, FH-RAP80WT, -RAP80S677A or -RAP80S677D. The stable cells were transfected twice with control or RAP80 siRNA at 24-hr intervals. Forty-eight hours after the second transfection, transfected cells were irradiated with 0 or 2 Gy. Cells were incubated for 1 hr before fixation and subjected to staining with antibody to phosphorylated histone H3 (pH3) and propidium iodide. The percentages of mitotic cells were determined by fluorescence-activated cell sorting (FACS) analysis (left panel). The percentage of the M population was determined. The results represent the average of two independent experiments. Error bars indicate the standard deviation (SD) *P<0.05 two-tailed Student’s t-test (right panel). (F) Phosphorylation of the RAP80 Ser677 residue affects G2/M arrest in the cell cycle. The stable cell lines expressing RAP80WT, or RAP80S677A were transfected twice with control or RAP80 siRNA at 24-hr intervals. Forty-eight hours after the second transfection, transfected cells were synchronized by a double thymidine block and released. Cells were harvested for FACS analysis a certain time after release of the cell cycle block. The arrow indicates the mitotic delay in the HeLa cell line stably expressing RAP80S677A. The number indicates the percentage of the G2/M population (left panel). The percentage of G2/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 the standard deviation (SD). *P<0.05 and **P<0.0005 two-tailed Student’s t-test (right panel).
Fig. 1

A

- Complement component 1 Q subcomponent-binding protein, mitochondrial (C1QBP) (8)
- Heat-shock protein 70 (HSP70) (8)
- Receptor-associated protein 80 (RAP80) (7)
- BRCA1/2-containing complex 36 (BRCC36) (6)
- Cyclin-dependent kinase (Cdk1) (5)
- Ribosomal protein 36 (RPS6) (2)

SFB-RAP80

B

10% input Rabat-MG Anti-Cdk1

W: Anti-RAP80
W: Anti-Cdk1

C

$^{32}$P-ATP:

Cdk1-cyclin B1:

GST-RAP80

$^{32}$P-ATP: + - - +

GST-RAP80 (17-311 a.a)

GST-RAP80C (143-719 a.a)

D

$^{32}$P-ATP (GST-RAP80 mutants)

Coomassie

GST-RAP80N

E

1 UIM

ZFD 719

46 101 140 400 473 653 677

GST-RAP80N (1-431 a.a)

GST-RAP80C (432-719 a.a)

$^{32}$P-ATP (GST-RAP80)

Coomassie

GST-RAP80C variants

F

GST-RAP80C: GST-RAP80 GST-RAP80S97A GST-RAP80S97A

$^{32}$P-ATP (GST-RAP80)

Coomassie (GST-RAP80C variants)
Fig. 3

G

S phase

G2 phase

Mitotic phase

Cell number

DNA content

G1/S  S  G2/M
Fig. 5

E

[Cell cycle distribution plots for Mock, RAP80 WT, RAP80 S677A, RAP80 S677D under 0 Gy and 2 Gy conditions.

Bar graph showing Mitotic cells relative to untreated (%) for RAP80: Mock, WT, S677A, S677D.

* Significant difference compared to Mock.
Cdk1-mediated Phosphorylation of Receptor Associated Protein 80 (RAP80) Serine 677 Residues Modulate DNA Damage-induced G2/M Checkpoint and Cell Survival

Hyun Jung Cho, Yun Jung Oh, Seung Hun Han, Hee Jin Chung, Chang Hee Kim, Nam Soo Lee, Won-Ju Kim, Je-Min Choi and Hongtae Kim

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