BRCA2 is a breast tumor susceptibility gene encoding a 390-kDa protein with functions in maintaining genomic stability and cell cycle progression. Evidence has been accumulated to support the concept that BRCA2 has a critical role in homologous recombination of DNA double-stranded breaks by interacting with RAD51. In addition, BRCA2 may have chromatin modifying activity through interaction with a histone acetyltransferase protein, p300/CBP-associated factor (P/CAF). To explore how the functions of BRCA2 may be regulated, the post-translational modifications of BRCA2 throughout the cell cycle were examined. We found that BRCA2 is hyperphosphorylated specifically in the M phase and becomes dephosphorylated as cells exit the M phase and enter interphase. This specific phosphorylation of BRCA2 was not observed in cells treated with DNA-damaging agents. Systematic mapping of the potential mitosis specific phosphorylation sites revealed the N-terminal 284 amino acids of BRCA2 (BR-N1) as the major region of phosphorylation and mass spectrometric analysis identified two phosphopeptides that contain “phosphorylation consensus motifs” for Polo-like kinase 1 (Plk1). Phosphorylation of BR-N1 with Plk1 recapitulated the electrophoretic mobility change as seen in BR-N1 isolated from M phase cells. Plk1 interacts with BRCA2 in vitro, and mutation of Ser1096, Ser1098, and Thr203/207 to Ala in BR-N1 abolished Plk1 phosphorylation, suggesting that BRCA2 is the substrate of Plk1. Furthermore, both the hyperphosphorylated and hypophosphorylated forms of BRCA2 bind to RAD51, whereas the M phase hyperphosphorylated form of BRCA2 no longer associates with the P/CAF, suggesting that the dissociation of P/CAF-BRCA2 complex is regulated by phosphorylation. Taken together, these results implicate a potential role of BRCA2 in modulating M phase progression.

Mutations in the breast cancer susceptibility gene BRCA2 are responsible for about 45% of hereditary early-onset breast cancer (1, 2). Inheritance of one defective allele confers cancer predisposition and tumor cells from predisposed individuals exhibit loss of heterozygosity (3, 4). Expression of the wild type BRCA2 in a human pancreatic cell line, Capan 1, which contains a truncated BRCA2, suppresses its neoplastic phenotypes (5). These results indicate that BRCA2 is a bona fide tumor suppressor protein (6, 7). In addition, biallelic homoplastic mutations in BRCA2 have recently been found in cell lines derived from patients in the B and D1 subgroups of Fanconi anemia, a recessive cancer susceptibility disorder (8).

Inactivation of Brca2, a murine homolog, leads to embryonic lethality at an early stage of embryo development (9–12). Brca2-null mouse embryonic fibroblasts show increased sensitivity to genotoxic agents (12, 13), spontaneous accumulation of chromosomal abnormalities, including aberrant chromatid exchanges, triradial and quadriradial chromosome structures, and gross chromosomal rearrangements (13, 14), and they frequently develop micronuclei, chromosome missegregation, and centrosome amplification (15). These observations implicate a requirement for BRCA2 in maintaining chromosome stability. Similar aberrant chromosome configurations are observed in cells mutant for proteins involved in homologous recombination (16, 17), suggesting that BRCA2 is needed for this error-free repair process. Homologous recombination (HR) involves the repair of DNA strand breaks by using an adjacent sister chromatid as a template (18). Loss of BRCA2 reduces the efficiency of HR-mediated double-stranded break repair, resulting in the repair of these lesions through error-prone mechanisms such as nonhomologous end joining (19–21).

The role of BRCA2 in HR is mediated through an interaction with RAD51, the evolutionarily conserved homologue of bacteriophage RecA, which plays an essential role in DNA double strand repair and exchange during HR (22). RAD51 interacts with the BRCA2 repeats, a stretch of unique amino acid sequences that is repeated eight times within the central region of BRCA2 (23–25). Disruption of the endogenous interaction between RAD51 and BRCA2 by ectopic expression of the BRCA2 repeats leads to radiation sensitivity and G2/M checkpoint failure (26). Recently, two crystallographic studies of the highly conserved C-terminal domain of murine Brca2 (Brca2CTD) indicated that this domain binds to single-stranded and double-stranded DNA hybrid structures. Moreover, purified Brca2CTD is able to stimulate RAD51-mediated DNA strand pairing and exchange in vitro (27). These studies suggest that BRCA2 may have a direct role in facilitating crucial steps of RAD51-mediated HR.
Besides roles in HR, BRCA2 has been linked to transcription regulation. The region encoded by exon 3 in the N terminus of BRCA2 when fused to a DNA-binding domain has the ability to activate transcription in yeast and mammalian cells; this transactivation activity is severely compromised by a cancer-predisposing mutation, tyrosine 42 to cysteine (28). In addition, it has been shown that the N-terminal region of BRCA2 interacts with P/CAF (29), a transcriptional co-activator with intrinsic histone acetyltransferase activity (30). Based on these observations, the ability of BRCA2 to activate transcription may be explained via the recruitment of chromatin modifying activity in the form of P/CAF. The exact mechanism of how P/CAF or other histone acetyltransferases act as co-activators remains unclear, but it is believed that the transfer of acetyl groups to histone tails may act either as a “flag” for recruitment or to “loosen” chromatin structure to create an “activated state” by making nucleosomal DNA more accessible to transcription factors and polymers (31, 32). To date, BRCA2 has not been shown to directly activate the transcription of a specific gene. Therefore, it is possible that the association of P/CAF may be required for other chromosomal metabolic functions.

The genetic instability seen in BRCA2-deficient cells cannot be attributed solely to an absence of BRCA2-mediated HR. We therefore postulate that BRCA2 must be involved in chromosome metabolism throughout the cell cycle and predict that this must be a regulated function. A common mechanism for regulation is by post-translational modifications such as phosphorylation, acetylation, and ubiquitination. Thus, we explored whether BRCA2 may be regulated through some form of post-translational modifications during the cell cycle. Here, we report that endogenous human BRCA2 protein is hyperphosphorylated specifically in M phase and show that the mitotic Pole-like kinase 1 (Plk1) interacts with BRCA2 in vitro and phosphorylates at least Ser193 and one or two residues in a Ser/Thr cluster (amino acids 203–207) of BRCA2 in vivo. Moreover, we found that RAD51 associates with both hyperphosphorylated (M phase form) and hypophosphorylated (interphase form) BRCA2 in vivo. On the other hand, P/CAF associates only with the hypophosphorylated form rather than the hyperphosphorylated form of BRCA2 in vivo. These results suggest that the phosphorylation of BRCA2 in M phase, at least by Plk1, regulates the dissociation of the BRCA2-P/CAF complex, implying a potential role of BRCA2 in modulating M phase progression.

EXPERIMENTAL PROCEDURES

Cell Culture, Synchronization, and Transfection—Human bladder carcinoma T24 and osteosarcoma U2OS cell line (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 units of penicillin/streptomycin at 37 °C with 10% CO2.

T24 cells, synchronized at G0/G1 by contact inhibition (33), were seeded at low density to release them back into the cell cycle and thereafter were harvested at 0, 12, 18, and 24 h after seeding. M phase cells were enriched by treatment with 0.3 μg/ml nocodazole (Sigma) for another 16–18 h before being harvested. To further isolate a distinct M phase cell population, the slightly detached nocodazole-treated cells were shaken off (shake-off method). In order to enrich for M phase cells without nocodazole treatment, cells first were synchronized at S phase by double-thymidine block and then were released back into the cell cycle. Briefly, the cells were treated with 2 mM thymidine for 18–24 h, released for 8–10 h, and then treated with the same concentration of thymidine for another 16–18 h; the cells were released for another 8–10 h before being harvested. Finally, to release cells from nocodazole block (prometaphase or metaphase), arrested cells were collected using the mitotic shake-off method and washed with phosphate-buffered saline before being plated to allow re-entry into the cell cycle.

Transfections were performed with Lipofectin reagents (Invitrogen) according to the manufacturer’s instructions. U2OS cells (1 × 106 cells/10-cm dish) were seeded 1 day before transfection, and fresh medium was added 18 h after transfection. Cells were then harvested for 18 h and then harvested for analyses.

Cell Treatment with DNA-damaging Agents— Cycling T24 cells were exposed to 4000 rads of ionizing radiation (IR), 10 μM of ultraviolet light (UV), and then harvested 1 h later. The time of treatment of 20 μg/ml mitomycin C (Sigma), 10 μg hydroxyurea (Sigma), or 0.3 μg/ml nocodazole was 1.2, or 18 h, respectively. IR was delivered using a 137Cs source. UV was delivered using a Stratalinker (Stratagene, La Jolla, CA) without culture medium.

Construction of Plasmids—Full-length human BRCA2 cDNA was flanked with NotI and Xhol linkers at its 5’ and 3’ ends, respectively, and subcloned in pDONR207 (Invitrogen). The 5’- and 3’-end sequences of BRCA2 cDNA obtained using polymerase chain reactions were subcloned in pUHG1, a pUHD10-3-based vector (Stratagene), or pCHPL-GFP2 (24). Both of the vectors were engineered for gene fragments as N-terminal fusion with Myc-green fluorescent protein (Myc-GFP).

To generate FLAG-mGST-BR-N1, the BR-N1 fragment was subcloned in FLAG-mGST, an engineered plasmid created by assembling mammalian GST cDNA in p3×FLAG-CMV-10 (Sigma).

GST-BR-N1 wild type (WT) was generated by subcloning the BR-N1 fragment in pGEX-4T-1 (Amersham Biosciences). GST-BR-N1 mutants S183A, S193A, S239A, A4, S193A-a4, and Δ193–207 were created using a QuickChange site-directed mutagenesis kit (Stratagene), and the results were verified by sequencing.

The murine HA-Plk1(WT) and HA-Plk1(K82M) (kinase-inactive) DNAs were kindly provided by Dr. Eisuke Nishida (Kyoto University, Japan) and Dr. Kyung S. Lee (National Institutes of Health, Bethesda, MD). The FLAG-P/CAF construct was kindly provided by Dr. Yoshihiro Nakatani (Dana Farber Cancer Institute, Boston, MA).

Preparation of Whole-cell Extracts—Whole-cell extracts were prepared as described previously (34). Briefly, cells were resuspended in ice-cold NETN buffer (250 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 5 μg/ml antipain, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 2 mM Na3VO4, and 10 mM glycerophosphate), and then subjected to three freeze/thaw cycles. Insoluble debris was removed by centrifugation at 14,000 × g for 10 min at 4 °C. Total protein concentrations were determined by the Bradford assay (Bio-Rad).

Immunoprecipitation, Phosphatase Treatment of Immunoprecipitated Western Blot—Immunoprecipitations were carried out with a modification from Chen et al. (24). Briefly, whole-cell extracts (about 3 mg) were pre-cleared by incubation with 40 μl of protein A-Sepharose beads (1.1 slurry) (Amersham Biosciences) at 4 °C for 30 min. The supernatants were then incubated with 3 μg of murine IgG or anti-BRCA2 (BBA) monoclonal antibody (24) at 4 °C for 1.5 h and then incubated with 40 μl of a mixture of protein A- and G-Sepharose beads at 4 °C for 1.5 h. Finally, beads were washed in ice-cold NETN buffer.

Immunoprecipitation, Phosphatase Treatment of Immunoprecipitated Western Blot—Immunoprecipitations were carried out using anti-HA (12CA5), anti-BRCA2 (BBA), anti-RAD51 (14B4) (35), or anti-FLAG (M2) antibody (Sigma), respectively.

For phosphatase treatment, BRCA2 immune complexes were washed in NETN buffer in the absence of phosphatase inhibitors and resuspended in phosphate buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM MgCl2, and 1 mM dithiothreitol). Parallel samples were incubated with or without calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA) at 30 °C for 30 min.

Western blots were developed by standard colorimetric procedures using the appropriate alkaline phosphataseoma specific secondary antibodies (Promega, Madison, WI) or ECL (Amersham Biosciences). The BRCA2 immunoprecipitates were analyzed by 6.5% SDS-PAGE and Western with anti-BRCA2 (Ab2) polyclonal antibody (Oncogene Research Products, Boston, MA). Ectopically expressed GFP fusion proteins in U2OS cells were probed with anti-GFP monoclonal antibody (Roche Applied Science).

In Vitro Kinase Assays—Plk1 kinase assay was adapted from Lee et al. (36). Briefly, nocodazole-treated U2OS whole-cell extracts (500 μg) in NETN buffer were immunoprecipitated with 1 μg of murine IgG, anti-HA (12CA5), or anti-Plk1 monoclonal antibody (Zymed Laboratories Inc., South San Francisco, CA) at 4 °C for 1.5 h and then incubated with 1 μg of a mixture of protein A- and G-Sepharose beads at 4 °C for another 1.5 h. Following extensive washing of the immune complexes with NETN buffer, kinase reactions were initiated by adding 50 μM cold ATP and 5 μCi of [γ-32P]ATP (Amersham Biosciences) in the presence of 12 μl of Plk1 kinase buffer (50 mM Tris-HCl, pH 7.4, 2 mM
EDTA, pH 8.0, 2 mM dithiothreitol, 10 mM MgCl₂, 50 mM NaF, 2 mM Na₃VO₄, and 10 mM glycerophosphate) using 6 μg of dephosphorylated casein (Sigma), GST, or GST-BRN1 (wild type and mutants) as substrates.

Cdk1 kinase assay was adapted from Park et al. (37). Briefly, cycling U2OS whole-cell extracts (500 μg) in NETN buffer were immunoprecipitated with 1 μg of anti-Cdk1 (p34) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and washed as described above. Kinase reactions were initiated by adding 50 μl cold ATP and 5 μCi of [γ-32P]ATP in the presence of 12 μl of Cdk1 kinase buffer (50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, and 10 mM MgCl₂) using 3 μg of histone H1 (Roche Applied Science) and 6 μg of GST or GST-BRN1 as substrates.

The kinase reactions were carried out at 30 °C for 30 min and stopped by the addition of SDS protein sample buffer. One-third of the total volume was fractionated on SDS-PAGE and analyzed by Coomassie Blue staining, autoradiography, or Western blot.

Recombinant Proteins—Recombinant BRCA2 protein was expressed using the baculovirus expression system according to the manufacturer’s guide (Pharmingen, San Diego, CA). Briefly, ~1 x 10⁵ yeast insect cells were infected with the baculovirus expressing full-length His-tagged BRCA2 at the appropriate multiplicity of infection. Infected cells were then harvested 48 h later and lysed in NETN buffer.

For partial purification of recombinant FLAG-mGST-BR-N1 proteins, nocodazole-treated whole-cell extracts (200-10 cm dishes of U2OS) prepared in NETN buffer 48 h after transient transfection with FLAG-mGST-BR-N1 were subjected to immunosaffinity purification using anti-FLAG (M2) resins. The FLAG-mGST-BR-N1 immune complexes were eluted using SDS protein sample buffer, fractioned on 8.5% SDS-PAGE, and analyzed by Coomassie Blue staining and Western blot with anti-BRCA2 (1-17) polyclonal antibody (Santa Cruz Biotechnology).

To purify GST fusion proteins, all of the recombinant GST-BR-N1 (wild type and mutants) proteins expressed in BL21 Star (DE3) (Invitrogen) were purified using glutathione-agarose resin according to the manufacturer’s instructions (Amersham Biosciences).

Mass Spectrometry for the Identification of Phosphorylation Sites—Identification of phosphorylation sites using mass spectrometry was performed as described previously (38).

RESULTS

BRCA2 Is Hyperphosphorylated Specifically in M Phase—BRCA2 mRNA and protein levels have been shown to be present at high levels at late G₁/early S phase in rapidly proliferating cells (39, 40). However, the precise expression profile of BRCA2 throughout the cell cycle remains unclear. As shown in Fig. 1A, BRCA2 was expressed once cells entered S phase (lane 3, top panel). Conspicuously, we observed the appearance of several slower migrating forms of BRCA2 in extracts from cells enriched for M phase by nocodazole treatment (Fig. 1A, lane 6, top panel). This unique migration pattern was not observed in cell extracts from interphase cells or s9 insect cells expressing recombinant BRCA2 (Fig. 1A, top panel, compare lane 6 with lanes 1–5). The phosphorylation pattern of retinoblastoma protein (Fig. 1A, middle panel, RB) was used as an indicator for the cell cycle phase as described previously (33).

To rule out the possibility that the slower migrating forms of BRCA2 resulted from the treatment of cells with nocodazole, M phase enriched cell extracts were prepared from T24 cells released from double-thymidine block without nocodazole (Fig. 1B; M-SN, M phase-synchronized extracts; M-EN, M phase-enriched extracts by nocodazole). Consistently, BRCA2 displayed similar slower migrating forms (Fig. 1B, top panel, compare lane 4 with lane 3). Furthermore, the slower migrating forms of BRCA2 were more distinct and prominent, using highly purified M phase cells, which were collected by shaking off slightly detached mitotic cells (Fig. 1B, top panel, lane 2; M-SO, “mitotic shake-off” extracts). Based on these observations, we conclude that the slower migrating pattern seen with BRCA2 is M phase-specific. Similar results were seen with another microtubule depolymerizing drug, vinblastine (41), which is known to arrest cells in M phase (data not shown).

To determine whether the alteration in electrophoretic mobility of BRCA2 was attributed to phosphorylation, immune complexes of BRCA2 from M phase and interphase cells were treated with phosphatase. As shown in Fig. 1C, the slower migrating forms of BRCA2 from M phase cells converted to a faster migrating form, which co-migrated with the form of BRCA2 from interphase cells (Fig. 1C, compare lanes 3–6), suggesting that BRCA2 becomes hyperphosphorylated as cells enter M phase.

DNA Damage Does Not Induce an M Phase Phosphorylation Pattern Similar to BRCA2—DNA damage-induced phosphorylation of BRCA1 by ATM (ataxia telangiectasia-mutated), ATR (ataxia telangiectasia and Rad3-related), and CHK2 kinases
has been shown (42–45). However, it is currently unknown whether DNA damage also induces phosphorylation of BRCA2. To test this possibility, asynchronous T24 cells were exposed to various DNA-damaging agents such as ionizing radiation, ultraviolet light, mitomycin C, and hydroxyurea. All of these genotoxic agents induced dramatic alterations in electrophoretic mobility of BRCA1 (Fig. 2, middle panel, compare lane 2 with lanes 3–6). In contrast, the genotoxic agents failed to induce the conspicuously slower migrating forms of BRCA2 as observed in M phase (Fig. 2, top panel, lanes 3–6). We noted, however, that these genotoxic agents elicited slight but not distinctive changes in the mobility of BRCA2.

BRCA2 Becomes Dephosphorylated upon Exit from M Phase—The fate of hyperphosphorylated BRCA2 in M phase was examined further in T24 cells released from nocodazole arrest. After the release from nocodazole block for 0.6–1 h, the slower migrating forms of BRCA2 were gradually converted to faster migrating forms (Fig. 3, top panel). At this time point, the fastest migrating form of RB had emerged, signifying the beginning of G1 phase (Fig. 3, middle panel, compare lane 2 with lanes 3–6). In contrast, the genotoxic agents failed to induce the conspicuously slower migrating forms of BRCA2 observed in M phase (Fig. 3, top panel, lanes 3–6). We noted, however, that these genotoxic agents elicited slight but not distinctive changes in the mobility of BRCA2.

Mapping the M Phase-specific Phosphorylation Regions of BRCA2—As an initial step toward defining a role for M phase phosphorylation in the function of BRCA2, we sought to map the regions of BRCA2 that become hyperphosphorylated. Because the hyperphosphorylated form of full-length BRCA2 showed such a dramatic alteration of gel mobility, we predicted that smaller fragments of BRCA2 that harbored the M phase-specific phosphorylation sites would have similar alteration in gel mobility and used this as a basis for a strategy to identify these sites. We initially generated four mammalian expression constructs that express fusion proteins containing Myc-GFP with overlapping fragments of BRCA2 (Myc-GFP-BR-CR, -BRC4-CR, -BRC1–5, and -BR-N, Fig. 4A) and transiently transfected them into U2OS cells. The transfected cells were then treated with nocodazole and harvested for Western blot analyses with anti-GFP (or anti-Myc) antibody. Of the four fusion proteins, only Myc-GFP-BR-N (which constituted the first 923 amino acids of BRCA2) exhibited a clear electrophoretic mobility shift following nocodazole treatment (Fig. 4B, compare lane 4 with lanes 6, 8, and 10). This finding directed us to focus on the N-terminal region of BRCA2 (designated as BR-N).

Three other derivatives of the BR-N construct were generated: Myc-GFP-BR-NS, -BR-NC, and -BR-NL (Fig. 4A). All of the fusion proteins exhibited different migration patterns after nocodazole treatment (Fig. 4B, lanes 12, 14, and 16). BR-NL showed a more distinct change in mobility shift compared with BR-NC, which showed only a slight change (Fig. 4B, compare lane 12 with lane 14). Interestingly, BR-NS showed two distinctive slower migrating forms, but the slowest migrating form appeared only after nocodazole treatment (Fig. 4B, lanes 15 and 16). These findings suggest that BR-NC contains at least one M phase-specific phosphorylation site, whereas BR-NS contains at least two phosphorylation sites (one that is phosphorylated in a nocodazole-independent manner and the other in a nocodazole-dependent manner). Furthermore, the dramatic conformational change of BR-NL that leads to electrophoretic mobility shift in the presence of nocodazole may require the extreme N-terminal region of BRCA2 (amino acids 1–190).

To further define the region of BR-NL (amino acids 1–672) that harbors the potential M phase-specific phosphorylation sites, we generated four other constructs that were serial deletions from the C terminus of BR-NL and designated them as Myc-GFP-BR-N4, -BR-N3, -BR-N2, and -BR-N1 (Fig. 4A). All four fragments of BR-NL displayed prominent changes in mobility after nocodazole treatment (Fig. 4B, lanes 18, 20, 22, and 24). Phosphatase treatment of immunoprecipitates with anti-GFP antibody confirmed that the BR-NL and BR-N1 fragments were phosphorylated (data not shown). Taken together, these studies suggest that the major M phase-specific phosphorylation sites contributing to the electrophoretic mobility shift of full-length BRCA2 reside within BR-N1 (amino acids 1–284), although we cannot exclude the possibility that other M phase-specific phosphorylation sites exist in other portions of BRCA2.

Identification of M Phase-specific Phosphorylation Sites within the BR-N1 Fragment—To identify the M phase-specific
phosphorylation sites within BR-N1, we generated a construct expressing BR-N1 translationally fused with mammalian glutathione S-transferase (mGST) and FLAG epitope and transfected the construct into U2OS cells. The ectopically expressed FLAG-mGST-BR-N1 fusion protein was then purified using anti-FLAG affinity resin from nontreated and nocodazole-treated cells (Fig. 5A, left panel). Western blot with anti-BRCA2 antibody specific for the N-terminal region confirmed the identity of the purified polypeptides (Fig. 5A, lanes 1–4), which contain various fragments of BRCA2 as shown in A. Equal amounts of whole-cell extracts from untransfected asynchronous parental (UN, lane 0), transfected asynchronous (all of the odd-numbered lanes), or nocodazole-treated (all of the even-numbered lanes except lane 0) U2OS cells were analyzed by immunoblotting with anti-GFP antibody.

analyses were able to confirm Ser\(^{239}\) of phosphopeptide B (PP-B) was phosphorylated, but only predicted 3 sites in phosphopeptide A (PP-A) were phosphorylated (Fig. 5, B and C). Nevertheless, alignment of the phosphopeptide sequences revealed that PP-A is highly conserved among mouse, chicken, and human, whereas PP-B is only conserved between mouse and human (Fig. 5C) (46), suggesting that the M phase-specific phosphorylation within this region may be vital for the overall function of BRCA2.

**Plk1 and Cdk1 Phosphorylate the BR-N1 Fragment of BRCA2 In Vitro**—The amino acid sequence surrounding the serine/threonine residues in the identified phosphopeptides (Fig. 5C) appears to show some resemblance to the predicted “consensus phosphorylation motif” for the highly conserved mitotic kinase, Plk1. This consensus motif, which is one or two negatively charged/uncharged amino acid residues preceding the Ser or Thr, followed by a hydrophobic/uncharged residue (for example, DE/S/T/L), is deduced from the sites identified in Plk1 phosphorylation of cyclin B1 (47, 48), Cdc25C (49), and

Phosphorylation sites within BR-N1, we generated a construct expressing BR-N1 translationally fused with mammalian glutathione S-transferase (mGST) and FLAG epitope and transfected the construct into U2OS cells. The ectopically expressed FLAG-mGST-BR-N1 fusion protein was then purified using anti-FLAG affinity resin from nontreated and nocodazole-treated cells (Fig. 5A, left panel). Western blot with anti-BRCA2 antibody specific for the N-terminal region confirmed the identity of the purified polypeptides (Fig. 5A, lanes 1–4), which contain various fragments of BRCA2 as shown in A. Equal amounts of whole-cell extracts from untransfected asynchronous parental (UN, lane 0), transfected asynchronous (all of the odd-numbered lanes), or nocodazole-treated (all of the even-numbered lanes except lane 0) U2OS cells were analyzed by immunoblotting with anti-GFP antibody.
TCTP (50). In addition, four potential phosphorylation sites for Cdk kinases were also found within BR-N1 (Fig. 5B), although they were not identified by mass spectrometric analysis. Cdk kinases display substrate specificity toward proteins containing the motif (S/T)P(X/R) (X stands for any amino acid) or (S/T)P(K/R) (51–53). Furthermore, it has been shown that during the cell cycle, the kinase activity of Plk1 and Cdk1 reaches maximum levels at the G2/M transition and gradually decreases as M phase proceeds and that both proteins play crucial roles in the entry into and exit from mitosis (36, 54–56). In the light of this information, we performed in vitro kinase assays using Plk1 and Cdk1 immunoprecipitated from U2OS cells with GST-BR-N1 as the substrate. As shown in Fig. 6A, phosphorylation of BR-N1 by Plk1, but not by Cdk1, resulted in a dramatic mobility change in GST-BR-N1, which was comparable with FLAG-mGST-BR-N1 and Myc-GFP-BR-N1 isolated from M phase cells (Figs. 6A, 5A, and 4B).

To confirm that Plk1 directly phosphorylates BR-N1, kinase active or inactive forms of HA-tagged Plk1 were transfected into U2OS, and nocodazole-treated extracts were subsequently...
prepared for immunoprecipitation with anti-HA antibody for in vitro kinase assays. Significantly, only the active HA-Plk1 kinase was able to phosphorylate and induce a mobility change in GST-BR-N1 (Fig. 6E, lane 3, indicated by PP). The presence of HA-Plk1 in the assays was confirmed by Western blots (Fig. 6B, lanes 7–9). The phosphoproteins with lower molecular weights, indicated by arrows, likely represent Plk1-associated substrates (Fig. 6A, lane 3).

To determine which residues on BR-N1 are targeted by Plk1, various GST-BR-N1 mutants were generated (Fig. 6C), based on the mass spectrometric results, to serve as substrates in Plk1 kinase assay. As shown in Fig. 6D, phosphorylation of the GST-BR-N1-S193A mutant was reduced, whereas phosphorylation of GST-BR-N1-Δ199–207 was completely abolished (top panel, lanes 5 and 7). Interestingly, GST-BR-N1 with combined mutations of Thr203, Ser205, Ser206, and Thr207 to Ala (GST-BR-N1–4A) showed a further reduction in Plk1-dependent phosphorylation when compared with the single S193A mutant (Fig. 6E, top panel, compare lane 4 with lane 3). Significantly, similar to GST-BR-N1-Δ193–207, phosphorylation of GST-BR-N1 harboring both S193A and 4A mutations were completely abolished (Fig. 6E, lanes 5 and 6). Because the mass spectrometric analysis predicted three phosphorylation residues within PP-A of BRCA2 (Fig. 5C), these data suggest that Plk1 targets Ser193 and at least one or two of the Ser/Thr sites of amino acids 203–207 in an M phase-specific manner.

M Phase-specific Phosphorylation of BRCA2 Does Not Affect Its Association with RAD51—Several BRCA2-interacting proteins, including RAD51 (11, 23, 24) and P/CAF (29), and their minimally interacting motifs on BRCA2 have been identified (Fig. 7A). The interaction with RAD51 is central to the function of BRCA2 in HR. To investigate whether this M phase-specific phosphorylation influences the interaction between BRCA2 and RAD51, co-immunoprecipitation experiments using anti-human RAD51 antibody and extracts from interphase and M phase T24 cells were performed. As shown in Fig. 7B (lanes 5 and 6), both the hyperphosphorylated and hypophosphorylated form of BRCA2 were co-immunoprecipitated with RAD51, suggesting that the association between BRCA2 and RAD51 is independent of the M phase-specific phosphorylation event of BRCA2.

The Hyperphosphorylated M Phase Form of BRCA2 Dissociates from P/CAF—It has been shown that the N terminus of BRCA2 associates with acetyltransferase activity when bound to P/CAF (29). By means of an in vitro GST pull-down assay, the amino acid residues 290–453 of BRCA2 were determined to be sufficient for interaction with P/CAF. This interaction was also confirmed in vivo by co-immunoprecipitation of FLAG-tagged P/CAF with HA-tagged N-terminal region of BRCA2 (amino acids 1–1963). Because the major M phase-specific phosphorylation region of BRCA2 resides in its N-terminal region, we examined whether the association of P/CAF with BRCA2 is influenced by the M phase hyperphosphorylation of BRCA2. A co-immunoprecipitation experiment was performed using cell extracts prepared from asynchronous, Adriamycin-treated (G2 arrest), nocodazole-treated (M phase-enriched), or mitotic shake-off (highly purified M phase cells) U2OS cells transiently transfected with a GFP-tagged BRCA2 N-terminal fragment (GFP-BRNL-1–672) as well as a FLAG-tagged P/CAF (FLAG-P/CAF). Fig. 7C showed that only the hypophosphorylated but not the hyperphosphorylated form of GFP-BRNL-1–672 could be co-immunoprecipitated with FLAG-P/CAF using anti-FLAG (M2) antibody (lanes 8–11). As previously shown in Fig. 1B, the predominant form of BRCA2 in M phase is the hyperphosphorylated form. Collectively, these observations suggest that the interaction between BRCA2 and P/CAF is regulated by phosphorylation in a cell cycle-dependent manner.

**DISCUSSION**

In the present study, we show that (i) BRCA2 is hyperphosphorylated specifically in M phase and becomes dephosphorylated as cells exit mitosis or enter interphase in vivo; and (ii) the N terminus of BRCA2 is the major M phase-specific phospho-
M Phase-specific Phosphorylation of BRCA2

**Fig. 8.** Model for the possible roles of the M phase-specific phosphorylation event of BRCA2. The intact BRCA2-P/CAF complex in G2 may have roles related to preventing premature entry of cells into mitosis. Because Plk1 is important for cellular mitotic entry, phosphorylation of BRCA2 by Plk1 and possibly by Cdk1 and other M phase kinases may regulate this potential function of the BRCA2-P/CAF complex. Furthermore, one of the consequences of the hyperphosphorylation event of BRCA2 is the dissociation of the BRCA2-P/CAF complex, which may allow BRCA2 to participate in modulating the chromatin structure for proper transition of cells through mitosis. Once cells exit mitosis and re-enter G1, hyperphosphorylated BRCA2 becomes dephosphorylated, perhaps to re-establish association with P/CAF for interphase functions.

The phenotypes of mice harboring various truncation mutations of 5′ of the BR repeat in BRCA2 range from embryonic lethality between E6 and E9 to survival until birth (9–12). Mouse embryonic cells derived from these mice show radiation sensitivity, defects in HR, and pronounced chromosomal aberrations (57, 58). These phenotypes can be attributed, in part, to the loss of the Rad51 interacting function within the BRCA2 repeats and the conserved CTD and possibly also to the absence of nuclear localization sequences in the extreme C terminus of BRCA2 (Fig. 7A) (57). Moreover, many cancer-predisposing point mutations have also been identified within the BRCA2 repeats (for example, D1420Y, G1529R) or clustered within the highly conserved C-terminal domain (for example, A2466V, A2951T) (Breast Cancer Information Core). Besides the potential involvement in transcription regulation, the function of the N terminus of BRCA2 remains relatively unknown (28). Cancer-predisposing point mutations have also been found within exon 3 (amino acids 23–105, Y42C), and an in-frame deletion of BRCA2 exon 3 has been identified in a Swedish family with a history of breast and ovarian cancer (59), suggesting that the N terminus of BRCA2 must provide an important contribution to its overall functions.

Interestingly, it has been shown that the N-terminal region of BRCA2 (amino acids 18–141) associates with and is phosphorylated by a yet to be identified cellular kinase, and removal of the exon 3 region (amino acids 23–105) disrupted this interaction (60). Consistently, we found the extreme N terminus of BRCA2 (amino acids 1–284, BR-N1), which includes the region encoded by exon 3, is phosphorylated specifically in M phase. More precisely, at least 4 M phase-specific phosphorylation sites within two stretches of amino acids (Fig 5C, PP-A and PP-B) within this region were identified as being targeted in vivo. Mutation of highly conserved Ser193 and one or two of the Ser/Thr residues between amino acids 203–207 leads to a reduction in the phosphorylation of BR-N1 by Plk1, whereas elimination of all these sites (Ser193, Thr204, Ser198, Thr201) abolishes BR-N1 phosphorylation by Plk1, suggesting that Plk1 targets at least Ser193 and one or two of the other four sites (the S/T cluster) within this region of BRCA2 in vivo. Plk1, the mammalian ortholog of Drosophila Polo, has been implicated in the regulation of a number of mitotic events including entry into mitosis, centrosome separation and maturation, metaphase to anaphase transition, and cytokinesis (55, 56, 61–63). These roles coincide with the potential roles of BRCA2 during the transition of G2 to M phase (to be discussed elsewhere). Although Cdk1 is able to phosphorylate BR-N1 in vitro, it does not induce the mobility change as seen with Plk1. However, we cannot eliminate the possibility that Cdk1 may also target BRCA2 in vivo. Moreover, because Ser239 has been identified by mass spectrometric analysis as another in vivo phosphorylation site, there are likely many kinases that target this region of BRCA2, implicating the potential for a very complex and intricate network of regulation for the N terminus of BRCA2.

What is the consequence of M phase-specific phosphorylation of BRCA2? Our results suggest that RAD51 associates with both the hyperphosphorylated and hypophosphorylated forms of BRCA2. Therefore, the M phase-specific phosphorylation of BRCA2 may have little influence on the function of the BRCA2-RAD51 complex. Our data, however, do suggest that P/CAF associates with the N-terminal region of BRCA2 and that this association becomes disrupted following phosphorylation of BRCA2 during M phase. One potential function of the intact BRCA2-P/CAF complex in G2 is to prevent premature entry into mitosis. This is consistent with our finding that BRCA2 associates with and is phosphorylated by Plk1, a kinase intimately linked to regulating entry into mitosis (54). It has been shown that phosphorylation of Ser138 and Ser147 on the cyclin B1 and Ser366 on the Cdc25C phosphatase by Plk1 is required for their nuclear localization and cell mitotic entry (47–49). It is possible that Plk1 may also regulate BRCA2 in a similar manner. Furthermore, the dissociation of P/CAF from BRCA2 at prometaphase may be necessary for proper chromosome segregation events, which are monitored by mitotic checkpoints. Supporting this notion, BRCA2 has been reported to associate with Bub1, a spindle checkpoint kinase (64). Similarly, inactivation of another spindle protein, Bub1, is sufficient to overcome growth arrest and promote transformation of BRCA2-deficient cells (65). Moreover, BRCA2 has been found to associate with a cruciform DNA-binding protein, BRF35, in a supramolecular protein complex that associates with chromatin during mitosis. Microinjection of BRCA2 or BRF35 antibodies results in a delayed entry into M phase (66). Deregulation of the potential BRCA2 function during mitosis may lead to major chromosomal alterations as seen in BRCA2-deficient cells. However, the precise role of the BRCA2-P/CAF complex during mitosis remains to be elucidated.

The observation that BRCA2 becomes hyperphosphorylated during M phase is significant in that it reveals a novel regulatory mechanism for BRCA2 functions. Further identification of other kinases responsible for phosphorylating BRCA2 in M phase will enhance the understanding of the role of BRCA2 during mitosis.

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**Addendum**—We have determined that BRCA2 interacts with Plk1 in vivo by transfected HA-tagged Plk1 into U2OS cells and followed by co-immunoprecipitation assays. The endogenous BRCA2 and Plk1 were reciprocally co-immunoprecipitated. Furthermore, HA-Plk1 was preferentially co-immunoprecipitated with the hyperphosphorylated form of GFP-BR-N1, suggesting that Plk1 associates with and phosphorylates this region of BRCA2.
M Phase-specific Phosphorylation of BRCA2 by Polo-like Kinase 1 Correlates with the Dissociation of the BRCA2-P/CAF Complex
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