The BRCA1 COOH-terminal Region Acts as an RNA Polymerase II Carboxyl-terminal Domain Kinase Inhibitor That Modulates p21WAF1/CIP1 Expression*

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BRCA1 is involved both in positive and negative regulation of gene activity as well as in numerous other processes, such as DNA damage response and repair. We recently reported that BRCA1 inhibits RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation by TFIIH and decreases serine 5 phosphorylation by P-TEFb. Regulation of CTD phosphorylation is crucial for proper gene expression and response to cellular stresses, such as DNA damage and transcription arrest. A key player in this process, P-TEFb, phosphorylates the CTD on serine 2 of transcriptionally engaged RNA polymerase II, and its kinase activity was shown to be up-regulated when cells are exposed to transcriptional stress such as UV irradiation. Here, we investigate the effect of BRCA1 on serine 2 phosphorylation and UV-activated P-TEFb kinase activity. We now show that BRCA1 inhibits immunoprecipitated P-TEFb kinase activity from UV-irradiated cells and preferentially decreases UV-induced serine 2 phosphorylation of soluble, rather than chromatin-bound, RNAPII. We further show that BRCA1 rescues the UV-mediated inhibition of transcriptional activity from nuclear extracts and stimulates endogenous p21 gene expression upon UV irradiation, a function that is dependent of the inhibition of CTD kinase activity. Our results suggest that BRCA1 could act as a CTD kinase inhibitor and, as such, contribute to the regulation of p21 gene expression.

Mutations in BRCA1 (breast cancer susceptibility gene 1) account for ~45% of reported cases of hereditary breast cancer. The BRCA1 protein acts as a tumor suppressor that plays a crucial role in the maintenance of genomic integrity. BRCA1 achieves this function by integrating important cellular processes, such as regulation of gene expression, the DNA damage response, and cell cycle control (1). For instance, BRCA1 can positively modulate the expression of the p21 (2) and GADD45 stress-responsive genes (2, 3), the DDB2 and XPC nucleotide excision repair genes (4) and the p27 (5) and 14-3-3σ cell cycle arrest genes (5, 6). On the other hand, BRCA1 has also been shown to down-regulate the transcriptional activity elicited by the c-Myc proto-oncogene (7), as well as by ER-α (8). Importantly, BRCA1 co-purifies with a form of RNA polymerase II (RNAPII) holoenzyme by interacting with RNA helicase A (9) or by directly contacting the RBP2 and RBP10a subunits of core RNAPII (10). Interestingly, a biochemical study proposed that following replication blockage by hydroxyurea treatment, a fraction of RNAPII holoenzyme-bound BRCA1 shifts to a new complex that contains BARD1 (BRCA1-associated RING domain 1) (11). This is consistent with a previous finding that showed that BRCA1 is phosphorylated and relocated from its nuclear foci to sites of DNA damage after treatment with UV light, hydroxyurea, or γ-irradiations (12). Furthermore, a recent report revealed that the association of BRCA1 with processed RNAPII is abrogated when cells are subjected to various DNA-damaging agents (13).

The RNAPII carboxyl-terminal domain (CTD)3 constitutes an important integrator of gene expression regulation, DNA damage response, and cell cycle control. Indeed, the CTD becomes highly phosphorylated during progression through the transcription cycle, after exposure to certain genotoxic agents, or when cells undergo mitosis (14). The CTD is highly conserved in all eukaryotes and consists, in mammals, of 52 repeats bearing the consensus heptapeptide sequence YSPTSPS. RNAPII can be found in two different forms relative to its CTD phosphorylation status and electrophoretic mobility on SDS-PAGE, which are the hypophosphorylated (IIa) and the hyperphosphorylated (IIo) forms. RNAPIIa is mainly found in solution and is the polymerase form that is preferentially recruited to promoters, whereas RNAPIIo is generally associated with the coding region of transcriptionally active genes (for a review, see Ref. 15). Upon formation of a stable preinitiation complex by RNAPIIa and transcription factors on a promoter, TFIIH phosphorylates serine 5 of the CTD heptapeptide, an event that allows promoter clearance by RNAPII (16) and the recruitment of mRNA capping enzymes (17). Serine 2 is then phosphorylated by P-TEFb, which renders RNAPII more processive during elongation of the transcript (18).

Two forms of the P-TEFb complex predominate in HeLa cell extracts: first, core P-TEFb, which consists of the binary Cdk9-cyclin T complex and possesses efficient CTD kinase activity,
BRCA1 Inhibits UV-activated CTD Kinases

and second, a much less active complex composed of the 7SK small nuclear RNA, HEXIM1 (HMBA-inducible protein 1), and HEXIM2 proteins in addition to Cdk9-cyclin T (19, 20). However, once subjected to a transcription insult, caused either by UV irradiation or treatment with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) or actinomycin D, the 7SK small nuclear RNA dissociates from core P-TEFb, which results in induction of the P-TEFb CTD kinase activity in vitro (21, 22). This positive modulation of P-TEFb kinase activity has been proposed to be involved in up-regulation of stress-responsive genes. However, it has been shown that serine 2 phosphorylation by P-TEFb is not required for proper induction of the stress-responsive gene p21 following a transcriptional stress engendered by treatment with DRB (23). Furthermore, extensive CTD phosphorylation in response to UV exposure has been proposed to reduce transcription initiation on an undamaged target gene in vitro, possibly by decreasing the pool of RNAPII competent for preinitiation complex assembly (24). The later observation may reflect the proposed dual role of CTD kinases, which is to act either as a specific positive transcription factor or in some circumstances as a general negative factor (25).

We have recently discovered an inhibitory effect of the BRCA1 COOH-terminal region (BRCA1-C) on CTD phosphorylation by TFIIH in vitro and reported the decrease of serine 5 phosphorylation levels when BRCA1 is overexpressed in transfected cells (26). Here, we investigate the effect of BRCA1 on serine 2 phosphorylation and study its involvement in the P-TEFb regulation pathway upon UV irradiation. We first show that BRCA1-C can inhibit immunoprecipitated P-TEFb kinase activity and that overexpressed BRCA1 decreases serine 2 phosphorylation levels in MCF7 cells. We show that in UV-irradiated cells, BRCA1 no longer affects CTD phosphorylation of chromatin-bound RNAPII but rather decreases serine 2 phosphorylation of soluble RNAPII. Finally, we show that wild type BRCA1-C, but not a mutated version unable to inhibit CTD phosphorylation, rescues the reduced transcriptional activity of nuclear extracts prepared from UV-irradiated cells, and stimulates endogenous p21 gene expression upon UV irradiation.

MATERIALS AND METHODS

Cell Culture, Transfections, and UV Irradiation for Protein Analyses—MCF7 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. 70% confluent 100-mm dishes of MCF7 cells were transfected with 5, or 8 µg of full-length BRCA1-, BRCA1-C-, or Cdk9dn-expressing vectors (27) and 8, 3, or 0 µg of pcDNA3 using the FuGENE 6 transfection reagent (from Roche Applied Science) according to the manufacturer’s instructions. 24 h following transfection, the medium was removed, cells were exposed to 25 J/m² of UVC, and fresh medium was added. Cells were harvested 0.5–6 h later, as described in the figure legends. Details of plasmid constructions are available upon request.

Nuclear Extract Preparation and Immunoblotting—HeLa nuclear extract preparation has been described earlier (28). MCF7 small scale nuclear extracts were prepared as previously described (29). For nuclear fractionation, the small scale nuclear extracts method was modified as follows. Pelleted nuclei were resuspended in saponin buffer in which ATP had been changed for 0.2 mM EDTA (30), allowed to swell on ice for 5 min, and centrifuged at 2000 rpm for 5 min at 4 °C. Supernatants were kept and represented the soluble nuclear fraction. Saponin pellets were resuspended in buffer C supplemented with 0.05% Nonidet P-40, stirred with magnetic bars for 45 min on ice, and centrifuged at 13,000 rpm for 5 min. Supernatants contained chromatin-bound proteins. 50 µg of total nuclear extract, soluble or chromatin fractions, were run on 6/15% SDS-PAGE and revealed by immunoblotting using antibodies against phosphorylated serine 2 (H5 from Babco), N-terminal region of RNAPII subunit Rpb1 (N20), Cdk9, TFIIH (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and H3 (from Upstate Biotechnology, Inc.). The same membrane was stripped (30 min at 50 °C in stripping buffer containing 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.7), blocked, and rebotted for a maximum of four times.

shRNA-mediated Knockdown of BRCA1 Expression—The shRNA sequence specific for BRCA1 (AATGCCAACAG-TAGCTAATGTA) and the scrambled sequence (GTCA- GATAAGACAATGATAT) were chosen according to previously published small interfering RNA sequences (31). These sequences were annealed with their complementary sequences and cloned in the pSUPER.retro.puro vector according to the manufacturer’s recommendations (OligoEngine). MCF7 cells were grown in 100-mm dishes at 60% confluence as described above and transfected with 6 µg of shRNA-expressing vector using the FuGENE transfection reagent (from Roche Applied Science). Puromycin was added at a final concentration of 2 µg/ml 24 h post-transfection. After 7 days in the presence of puromycin, survival cells were diluted to 0.5 cells/ml and plated to obtain stable clonal cell lines.

Expression and Purification of Recombinant Proteins from Escherichia coli—BRCA1-C derivatives were expressed in E. coli with the pET30a-expressing vector (Novagen), purified on Ni²⁺-nitrilotriacetic acid–agarose beads (from Qiagen), and chromatographed by anion exchange chromatography on a MonoQ column (Amersham Biosciences) as described earlier (26). The GST-CTD-expressing vector was a gift of A. Barberis. GST-CTD was expressed in E. coli and affinity-purified on glutathione-Sepharose beads (GE Biosciences) according to standard procedures. Details on plasmid constructions are available upon request.

Immunoprecipitation and Kinase Assays—Immunoprecipitations were performed essentially as described (32). Briefly, 80% confluent MCF7 cells (100-mm dishes) were lysed with 1 ml of TLB buffer. 5 µg of anti-Cdk9 were prebound to a 50-µl bed volume of protein A–agarose beads (from Roche Applied Science) and added to 1 ml of cell lysate. Immunocomplexes were washed as described (33). A 10-µl bed volume of immunoprecipitated Cdk9 was used for kinase assays. Kinase assays were performed in 25 µl of kinase buffer (32) at 30 °C for 30 min after the addition of 10 µCi of [γ³²P]ATP (3000 Ci/mmol) and 1 µM ATP, 0, 12, or 24 pmol of BRCA1-C derivatives were added along with 0.5 pmol of GST-CTD 5 min prior to ATP addition. Reactions were analyzed by 10% SDS–PAGE, colored with Coomassie Blue, detected with a PhosphorImager (Amer-
sham Biosciences), and quantified using the ImageQuant software (Amersham Biosciences). Immunoprecipitated material was migrated on 10% SDS-PAGE until the 25-kDa marker was run out of the gel to accurately separate Cdk8 (56 kDa), rabbit IgG (52 kDa), Cdk9 (43 kDa), and Cdk7 (40 kDa) and analyzed by immunoblotting with anti-Cdk9, anti-Cdk8, and anti-Cdk7 antibodies (from Santa Cruz Biotechnology). In Fig. 1C, 25 μg of HeLa nuclear extract were incubated with 5 μM ATP and 24 pmol of bovine serum albumin or 0, 6, 12, or 24 pmol of recombinant BRCA1-C for 30 min at 25 μl of kinase buffer and then migrated on 6% PAGE and revealed by immunoblotting with H5 and N20 antibodies.

In Vitro Transcription Assays—MCF7 cells were transfected with BRCA1 constructs, exposed to UV irradiation, and submitted to small scale nuclear extract preparation as described above. In vitro transcription assays were performed essentially as described (34) with the following modifications. 50 μg of a nuclear extract preparation was incubated with 100 ng of G5E4 template DNA bearing the adenovirus E4 early promoter downstream of five Gal4 binding sites (35) and with 200 ng of pGem3 (Promega) in a reaction mixture containing 9 μl of salt mix, 1 μl of 200 mM phospho(enol)pyruvic acid (from Sigma), 0.5 μl of RNasin (Promega), and buffer D (29) to complete the reaction volume to 24 μl. Preinitiation complexes were allowed to form for 20 min at 25 °C prior to the addition of 25 mM NTPs. 5 min following NTPs addition, Sarkosyl was added at 0.08% final concentration, and RNA synthesis was allowed to proceed for an additional 25 min. Reactions were stopped, and RNA was isolated and subjected to primer extension using a specific radiolabeled probe (34). Primer extension reactions were run on a 10% PAGE containing 5 M urea and 1× Tris-borate buffer (TBE), dried, and revealed by phosphorimaging (Molecular Dynamics).

Luciferase Assays—MCF7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. 70% confluent 6-well dishes were transfected with 0, 0.2, 0.4, or 0.8 μg of wild type BRCA1-C or BRCA1-C S1572E-expressing vectors (27), along with 0.8, 0.4, 0.2, and 0 μg of pcDNA3, respectively, 10 ng of the WWLUC (36), and pBgal plasmids bearing the p21-luciferase and SV40-LacZ reporter genes respectively, using the FuGENE 6 transfection reagent (from Roche Applied Science) according to the manufacturer’s instructions. 24 h following transfection, the medium was removed, cells were exposed to the indicated doses of UVC, and fresh medium was added. In Fig. 7B, old medium was replaced with fresh medium containing 0 or 50 μM DRB. Cells were harvested 8 h later. Luciferase assays were performed using to the Promega Luciferase Assay System, and β-galactosidase activity was measured according to standard procedures.

RT-PCR and Quantitative RT-PCR—Conventional RT-PCRs (Fig. 2A) were performed as described earlier (37), with a supplemental DNase I treatment following RNA extraction. Primer sequences for BRCA1 were as follows: forward, 5’-ggatctccattaagttgtagt-3’, reverse, 5’-gtcagcactctcttgtgaa-3’. 1 RNA was extracted using the GenElute RNA extraction kit from Sigma, and RNA was subjected to a reverse transcription reaction containing 0.5 μg oligo(dT), 500 μM dNTPs, 200 units of Moloney murine leukemia virus reverse tran-scriptase (from Promega), and 5 μl of 5× Moloney murine leukemia virus buffer in a final volume of 25 μl. The reaction mix was incubated for 10 min at 42 °C, 50 min at 55 °C, and 15 min at 70 °C. For DNA amplification, 2 μl of cDNA was added to the SYBR Green Master Mix prepared according to the manufacturer’s procedure (Stratagene) along with a 0.15 μM concentration of each primer. Sequences of primers were as follows: p21-forward, 5’-ggagctctaggctc-3′; p21-reverse, 5’-gattaggctctccct-3′; 36B4-forward, 5’-cgactggtagcactac-3′; 36B4-reverse, 5’-atctgcgactctggctc-3′. Amplifications were performed by real time PCR using the Stratagene Mx3000P apparatus and analyzed with the MxPro QPCR software. Amplification data for p21 were normalized to 36B4.

RESULTS

The BRCA1 COOH-terminal Region Inhibits P-TEFb Kinase Activity in Vitro—Earlier studies have shown that the BRCA1 COOH-terminal region, alone or fused to a DNA binding domain, is sufficient to stimulate the expression of target genes in vivo and in vitro (38–40), and we previously described that this region could inhibit TFIIH kinase activity in vitro (26). In an attempt to better understand the role of BRCA1 in transcription regulation, it is of interest to define the effect of BRCA1 on other important CTD kinases, such as the positive elongation factor P-TEFb. P-TEFb kinase activity is ensured by the cyclin-dependent kinase Cdk9 in association with cyclin T. We first performed an in vitro kinase assay using immunoprecipitated Cdk9 as a kinase source and the fusion protein GST-CTD as a substrate. Purified BRCA1-C protein was then added to the mixture, followed by the addition of radiolabeled ATP to allow the detection of CTD phosphorylation. Fig. 1A first shows that GST-CTD phosphorylation by immunoprecipitated Cdk9 leads to two differentially migrating forms, namely the hypophosphorylated (IIa) and hyperphosphorylated (IIo) forms, which is typical of efficient CTD phosphorylation (see lane 2) (41). The figure further shows that BRCA1-C strongly impedes P-TEFb kinase activity on the GST-CTD substrate (lanes 2–4). We also tested a BRCA1-C derivative bearing a point mutation at residue 1572 (S1572E), which we have previously shown to have a strongly reduced effect on serine 5 phosphorylation in comparison with wild type BRCA1-C (26). Interestingly, BRCA1-C S1572E also shows reduced inhibitory effect on P-TEFb kinase activity (Fig. 1A, lanes 5 and 6). Lane 1 shows that in the absence of anti-Cdk9, no CTD kinase activity could be observed. Furthermore, the gel has been stained with Coomassie Blue to ensure that equal amounts of immunoprecipitated material, GST-CTD, and BRCA1-C proteins were distributed in each reaction (data not shown). Fig. 1B shows an immunoblot analysis of the immunoprecipitated material used in the kinase assay to ensure that Cdk7 (40 kDa) and Cdk8 (56 kDa), the two other major CTD kinases, have not been precipitated along with Cdk9. Although we cannot completely exclude the possibility that other kinases are present in our immunoprecipitation, we can ascertain that Cdk9 is the principal CTD kinase present in our assay.

P-TEFb specifically phosphorylates RNAPII CTD on serine 2 of the consensus heptapeptide YSPTSPS (14). In order to verify the effect of BRCA1 on this target residue, we performed an
in vitro kinase assay using a HeLa nuclear extract as a kinase and substrate source, which contains endogenous P-TEFb and RNAPII. We added purified BRCA1–C protein to the nuclear extract preparation and incubated the mixture in the presence of ATP. Serine 2 phosphorylation and total RNAPII were monitored by immunoblotting using appropriate antibodies. Fig. 1C shows the addition of purified BRCA1–C efficiently inhibits serine 2 phosphorylation of RNAPII from a HeLa nuclear extract. Bovine serum albumin has been used here as a negative control (lane 1). We cannot exclude the possibility that BRCA1 would also modulate a CTD phosphatase activity, since the yeast homologue of human FCP1, the major CTD phosphatase described to date, has been shown to resist to the commonly used phosphatase inhibitors sodium vanadate (Na$_3$VO$_4$) and sodium fluoride (NaF) (42), which were nevertheless added to the kinase assay.

**BRCA1 Globally Decreases Serine 2 Phosphorylation in MCF7 Cells**—We next wanted to determine whether BRCA1 could modulate serine 2 phosphorylation in a cellular context. To address that issue, we transfected MCF7 cells with BRCA1 constructs expressing either the BRCA1 COOH-terminal region (BRCA1–C) that bears amino acids 1528–1863 (Fig. 2A) or full-length BRCA1 (Fig. 2B). Following transfection with BRCA1 constructs, we performed nuclear extractions of the transfected cells and analyzed serine 2 phosphorylation levels by immunoblotting. As can be seen in Fig. 2A (upper panels), wild type BRCA1–C (lanes 2–4) decreases serine 2 phosphorylation levels when overexpressed in MCF7 cells. MCF7 cells have also been transfected with a BRCA1–C construct bearing the S1572E mutation, and as expected, no significant variation of serine 2 phosphorylation levels was observed (lanes 6–8). The bottom panels show immunoblots using an antibody that recognizes the N-terminal part of Rpb1 and show that total RNAPII amounts do not significantly vary. RT-PCRs and immunoblots (right panels) have been performed to ensure that the wild type and S1572E constructs were equally expressed and that both proteins show similar stability. Fig. 2B shows that overexpressed full-length BRCA1 also efficiently decreases serine 2 phosphorylation levels in MCF7 cells. BRCA1 expression levels were monitored by immunoblotting in order to verify that the full-length protein is indeed overexpressed in the transfected cells.

MCF7 cells are able to express a significant amount of wild type BRCA1 (12). We thus made use of shRNA technology to knock down the expression of BRCA1 in those cells and examine the fate of serine 2 phosphorylation when BRCA1 levels are depleted. Fig. 2C shows that BRCA1 levels are significantly decreased in cells stably transfected with an shRNA directed against BRCA1 (BR-1.1 and BR-1.2 are two different clones) in comparison with a scrambled shRNA (CT) used as a control. A quantification of different nuclear extract preparations reveals that ~60% of BRCA1 protein is lost in the MCF7-shBR compared with MCF7-shCT (Fig. 2C, upper panel). Importantly, the assessment of total RNAPII levels revealed a significant increase of the hyperphosphorylated form of RNAPII in cells expressing the BRCA1–directed shRNA compared with cells transfected with the control shRNA. A graphic representation shows the ratio of RNAPIIo/RNAPIIa in MCF7-shBR relative to MCF7-shCT cells (Fig. 2C, lower panel). Furthermore, loss of BRCA1 expression correlates with an increase in global CTD phosphorylation levels as detected with the phosphoserine 2 and phosphoserine 5 antibodies. An antibody against TFII B was used as a loading control (bottom panel). Taken together, our results suggest that either cellular overexpression of BRCA1 or depletion of BRCA1 can directly affect phosphorylation levels of the serine 2 residue in the RNAPII CTD in a global fashion.

**UV-induced P-TEFb Kinase Activity Is Inhibited by BRCA1–C in Vitro**—Approximately 50% of cellular P-TEFb is found in a large, inactive complex comprising of, in addition to Cdk9 and cyclin T, the 7SK small nuclear RNA and the HEXIM1 and HEXIM2 proteins (20, 22). To verify whether BRCA1 can still decrease the CTD kinase activity when the small P-TEFb complex is predominant, we performed an immunoprecipitation against Cdk9 from a cellular extract treated with RNase A, a method previously used to precipitate exclusively the small, active complex (21). BRCA1–C protein was added to immunoprecipitated Cdk9 along with GST–CTD and radiolabeled ATP. Fig. 3A shows that P-TEFb kinase activity is increased when the extracts have been subjected to an RNase A digestion before sequestering the complex (compare lanes 1 and 5). The figure
FIGURE 2. BRCA1 decreases serine 2 phosphorylation in MCF7 cells. A, immunoblot analyses of nuclear extracts from MCF7 cells transfected with constructs expressing the BRCA1 COOH-terminal region (BRCA1-C; amino acids 1528–1863), either wild type or bearing the S1572E mutation. Right panels, RT-PCRs showing the expression of transfected BRCA1-C and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene as control and immunoblots against BRCA1-C. B, cells were transfected with full-length BRCA1 and analyzed as in A. C, knockdown of BRCA1 expression in MCF7 cells increases serine 2 phosphorylation levels. MCF7 cells were stably transfected with shRNA containing either a sequence specific for BRCA1 (BR) or a scrambled sequence (CT) as a negative control. BR-1.1 and BR-1.2 represent two different clones. Phosphorylated serine 2 and 5 and total Rpb1 were revealed using H5, H14, and N20 antibodies respectively. IIa and IIo, hypo- and hyperphosphorylated form of Rpb1, respectively. Graphics represent the quantification of BRCA1 expression (top graph) and the RNAPIIlo/RNAPIIla ratio (bottom graph) in MCF7-shBR relative to MCF7-shCT from two different nuclear extract preparations and immunoblots.
further shows that the addition of BRCA1-C decreases CTD phosphorylation levels to practically the same extent as with untreated cells (lanes 1–4 and 9–12). Immunoblot against Cdk9 were performed to ensure that equal amounts of Cdk9 were immunoprecipitated in untreated and treated cellular extracts (Fig. 3C). These data suggest that the inhibition of P-TEFb kinase activity by BRCA1 can occur upon a transcriptional stress that renders P-TEFb more active.

UV-induced P-TEFb Phosphorylates the CTD of Soluble as Well as Chromatin-bound RNAPII—As mentioned above, extensive CTD phosphorylation in response to UV exposure has been proposed to reduce transcription initiation by decreasing the pool of RNAPII competent for preinitiation complex assembly (24). Since BRCA1 is found in some RNAPII holoenzymes, we hypothesized that its ability to inhibit P-TEFb kinase activity could serve to limit the deleterious effects of CTD phosphorylation on transcription initiation, particularly under conditions of transcriptional stress, such as when subjected to UV irradiation. We were thus interested in determining whether UV-activated P-TEFb has the ability to phosphorylate RNAPII before its recruitment to promoters. A prediction of such a scenario is that following P-TEFb activation by UV irradiation, soluble RNAPII, as well as chromatin-bound RNAPII, would become hyperphosphorylated and would thus show an increase in serine 2 phosphorylation levels. To test this, we first subjected MCF7 cells to 25 J/m² of UV radiation and harvested the cells at different time points ranging from 0 to 6 h after exposure. We then performed nuclear fractionation analyses in order to separate soluble RNAPII from chromatin-bound RNAPII. Briefly, nuclei were resuspended in a physiological buffer that contains a mild detergent, saponin, which allows nuclear membrane lysis without affecting most protein-protein interactions. The supernatant is therefore enriched in soluble nuclear proteins (30), whereas the pellet contains chromatin-bound proteins. These two fractions, namely the soluble (Fig. 4, left panels) and chromatin fractions (Fig. 4, right panels) were thus analyzed by immunoblot using different antibodies, as indicated in Fig. 4.
shows serine 2 phosphorylation levels that correlate with a higher proportion of hyperphosphorylated RNAPII (Fig. 4A, lanes 1 and 7). Interestingly, serine 2 phosphorylation of soluble RNAPII rapidly increases upon UV treatment, and we observe a very important shift from the hypo- to the hyperphosphorylated state as early as 30 min after irradiation (Fig. 4A, lanes 1–6). Since the accumulation of serine 2-phosphorylated RNAPII in the soluble fraction is not correlated with depletion from the chromatin fraction, we assumed that it results from de novo phosphorylation of soluble RNAPII induced by UV irradiation. RNAPII located in the chromatin fraction displayed a similar phosphorylation pattern with the exception that no significant decrease of total RNAPII was observed, even 6 h after UV irradiation (Fig. 4A, lanes 7–12, and data not shown). We also performed the same experiment with cells treated with actinomycin D, a drug that has previously been shown to activate P-TEFb, and we observed an important shift from RNAPIIa to RNAPIIo in the soluble and chromatin fractions (data not shown). These results suggest that at least a portion of P-TEFb can efficiently phosphorylate soluble RNAPII and that, most likely, not all stress-induced P-TEFbs are recruited to genes. To further test our idea, we determined the abundance levels of soluble and chromatin-bound P-TEFb from our nuclear fractionation assays by performing an immunoblot against Cdk9. As shown in Fig. 4A, third panel, the amount of soluble and chromatin-bound Cdk9 does not significantly vary upon UV exposure. Furthermore, although Cdk9 is more abundant in the chromatin fraction (Fig. 4A, third panel, lanes 7–12), a large proportion is also found in the soluble fraction, even 6 h after UV exposure (Fig. 4A, third panel, lanes 1–6). This result further supports the idea that UV-induced P-TEFb can phosphorylate RNAPII prior to promoter binding. To ensure that the soluble and chromatin fractions do not contaminate each other, we examined the presence of H3, which should be restricted to the chromatin fraction, and TFIIB that should be equally distributed among the two fractions (30). As can be seen in Fig. 4A, H3 was only detected in the chromatin fraction, whereas TFIIB was present at comparable levels in both fractions. Importantly, H3 and TFIIB levels in each fraction from UV-irradiated cells do not significantly fluctuate.

A previous study reported that UV-induced serine 2 phosphorylation could be attributed to the stress-activated protein kinase c-Jun N-terminal kinase (JNK), as well as P-TEFb, as determined by the use of specific drugs (43). To verify that the increase in serine 2 phosphorylation that we observed in the soluble fraction (see Fig. 4A, upper panel, lanes 1–6) is at least in part due to P-TEFb kinase activity, we took advantage of an expression vector containing a dominant negative form of Cdk9 (Cdk9dn) that carries a point mutation in the kinase catalytic site. We reasoned that if P-TEFb is responsible for UV-induced serine 2 phosphorylation of soluble RNAPII, overexpression of Cdk9dn should restrain this effect. To test our hypothesis, MCF7 cells were first transfected with Cdk9dn and then treated with 25 J/m² of UV. 2 h later, soluble nuclear proteins were recovered and analyzed by immunoblotting with H5, N20, and anti-Cdk9 antibodies. IlIo and IlLa, hypo- and hyperphosphorylated form of Rbp1, respectively.
BRCA1 Inhibits UV-activated CTD Kinases

counters UV-induced serine 2 phosphorylation (compare lane 2 with lanes 3 and 4). A graphic representation shows a quantification of serine 2 phosphorylation, normalized with the TFIIB immunoblot (Fig. 4B, right). The immunoblot against total RNAPII (middle panel) reveals that Cdk9dn limits the shift from hypophosphorylation (IIa) to hyperphosphorylation (Ilo) catalyzed by UV irradiation. Indeed, quantification of RNAPIIo and RNAPIIa from lanes 2 and 4 revealed that although RNAPII is ∼30% less abundant in the extract prepared from cells previously transfected with Cdk9dn, we observed 20% more hypophosphorylated RNAPII (quantification not shown). Finally, the lower panel shows that Cdk9dn is efficiently overexpressed (lanes 3 and 4) in the soluble fraction. Taken together, data from Fig. 4 suggest that P-TEFb, once released from the 7SK small nuclear RNA-HEXIM1/2 upon UV irradiation, can phosphorylate serine 2 of the RNAPII CTD prior to recruitment to DNA.

BRCA1 Inhibition of Serine 2 Phosphorylation Is Constrained to Soluble RNAPII When Cells Are Treated with UV Irradiation—To test the idea that BRCA1 could help maintain a pool of RNAPII molecules competent for transcription initiation (i.e. in the IIa form), we addressed whether BRCA1 inhibition of serine 2 phosphorylation occurs before or after RNAPII association with DNA. Therefore, we investigated the effect of BRCA1 on serine 2 phosphorylation by comparing soluble and chromatin-bound RNAPII upon UV irradiation. MCF7 cells were first transfected with BRCA1 constructs and subsequently subjected to UV irradiation. In accord with the time course experiment shown in Fig. 4A, we chose a recovery time of 2 h after UV exposure, since serine 2 phosphorylation is considerable at this time point, whereas RNAPII degradation is not yet significant. Thus, 2 h following UV treatment, cells were harvested and subjected to nuclear fractionation as described above. Serine 2 phosphorylation and total RNAPII were monitored by immunoblotting with appropriate antibodies. Fig. 5A first shows that UV-induced serine 2 phosphorylation occurs on soluble RNAPII (lanes 1 and 4) as well as on chromatin-bound RNAPII (lanes 7 and 10), a result that is consistent with data shown in Fig. 4. Fig. 5A further shows that overexpressed BRCA1 decreases noninduced and UV-induced serine 2 phosphorylation levels of soluble RNAPII (lanes 1–3 and 4–6). Interestingly, BRCA1 does not have the same effect on chromatin-bound RNAPII. Although BRCA1 markedly decreases noninduced serine 2 phosphorylation levels (lanes 7–9), no significant effect can be observed on UV-induced serine 2 phosphorylation levels (lanes 10–12). We noticed in several experiments that the inhibition of CTD phosphorylation by BRCA1 was often correlated with a slight reduction of total RNAPII levels (see lanes 7–9). Since the BRCA1/BARD1 heterodimer bears E3 ligase function, one could argue that serine 2 phosphorylation inhibition could depend on RNAPII degradation by the proteasome pathway. Although we cannot totally dismiss such a possibility, the observation that BRCA1-C diminishes de novo serine 2 phosphorylation (Fig. 1C) suggests that inhibition of CTD phosphorylation is not an indirect effect of RNAPII degradation.

To further substantiate our hypothesis, we repeated a similar experiment, but with BRCA1-C, which does not possess the RING domain that is essential for BARD1 binding and E3 ligase activity. Fig. 5B (left) shows that overexpressed BRCA1-C decreases basal and UV-induced serine 2 phosphorylation of soluble RNAPII. On chromatin-bound RNAPII (Fig. 5B, right), we can observe that the inhibition of serine 2 phosphorylation by BRCA1-C is relieved upon UV irradiation. Importantly, the presence of BRCA1-C diminished the RNAPIIo/RNAPIIa ratio without significantly affecting the total amount of RNAPII (lanes 1–3 and 7–9, bottom panels). These results suggest that inhibition of serine 2 phosphorylation by BRCA1 that occurs on transcriptionally engaged RNAPII is alleviated following UV irradiation. In this latter situation, BRCA1 would preferentially inhibit UV-induced serine 2 phosphorylation prior to RNAPII binding to target chromatin regions.

BRCA1 Rescues in Vitro Transcriptional Defects of Nuclear Extracts from UV-irradiated Cells—Since BRCA1 preferentially inhibits UV-induced serine 2 phosphorylation of soluble RNAPII, we reasoned that it could therefore possibly reverse the negative outcome of UV irradiation on transcription. To address that question, we performed an in vitro transcription assay with nuclear extracts prepared from MCF7 cells that were previously transfected with BRCA1 and exposed to UV. The transcriptional activity of these nuclear extracts was monitored on a DNA template bearing five Gal4 binding sites located upstream to the adenoviral E4 promoter and coding region, namely G5E4 (35). Fig. 6A shows that nuclear extracts from UV-irradiated cells are less efficient in promoting in vitro transcription, as compared with extracts prepared from untreated cells (lanes 1 and 2 and lanes 4 and 5). Importantly, the transcriptional activity of nuclear extracts prepared from cells that were transfected with full-length BRCA1 or BRCA1-C before UV irradiation is partially rescued (lanes 2 and 3 and lanes 5 and 6). To further substantiate the possibility that BRCA1 counteracts the negative effect of UV on transcription, we transfected MCF7 cells with increasing amounts of BRCA1-C prior to...
exposing cells to UV and verified if the corresponding nuclear extracts showed a proportional recovery of in vitro transcription activity. As shown in Fig. 6B, increasing amounts of BRCA1-C in the reactions correlates with an increasing recovery rate of RNA synthesis. These nuclear extracts were then analyzed for serine 2 phosphorylation levels. Fig. 6B (bottom panels) shows that UV irradiation efficiently induced serine 2 phosphorylation and that increasing the amount of transfected BRCA1-C proportionally impairs this phosphorylation. These results show the existence of a correlation between inhibition of UV-induced serine 2 phosphorylation by BRCA1-C and recovery of a UV-infected transcription defect. As a control, we verified the serine 2 phosphorylation by BRCA1-C and recovery of a UV-mediated transcription defects.

In vitro transcription was performed as in A with nuclear extracts from cells previously transfected with wild type BRCA1-C or the S1572E mutant. llo and llo, hypo- and hyperphosphorylated form of Rpb1, respectively.

Taken together, these results suggest that BRCA1 can act as a...
CTD kinase inhibitor that modulates p21 expression, either under basal or stress-induced conditions.

**DISCUSSION**

In this report, we have shown that BRCA1 decreases RNAPII CTD phosphorylation on serine 2 and that recombinant BRCA1-C inhibits P-TEFb kinase activity in vitro. By performing nuclear extract fractionation assays, we demonstrated that UV-activated P-TEFb phosphorylates soluble as well as chromatin-bound RNAPII. Interestingly, in cells subjected to UV irradiation, BRCA1 no longer affects chromatin-bound RNA-II but preferentially decreases serine 2 phosphorylation of soluble RNAPII. This result correlated with the ability of BRCA1 to rescue the in vitro transcription defect of a nuclear extract from UV-injured cells. Finally, we showed that BRCA1 stimulates endogenous p21 expression before and after UV irradiation in a P-TEFb-dependent manner, as suggested by the S1572E BRCA1-C mutant and use of the pharmacological inhibitor DRB.

BRCA1 plays both positive and negative roles on gene expression, but the precise mechanism by which BRCA1 achieves these opposite functions is poorly understood. Our finding that BRCA1 inhibits serine 2 phosphorylation could represent an important step in resolving this conundrum. Since different promoters do not require the same set of transcription factors to undergo proper gene expression, it would be interesting to assess the CTD kinase requirement of genes that are positively and negatively modulated by BRCA1. Importantly, it has been recently shown that DRB-induced p21 transcriptional activation does not require serine 2 phosphorylation of elongating RNAPII (23). This observation may explain why BRCA1 can stimulate p21 expression while it inhibits CTD phosphorylation. In fact, inhibition of CTD kinases by BRCA1 could represent a manner by which it induces p21, by similarity to DRB. On the other hand, BRCA1 and COBRA1 (cofactor of BRCA1) have been shown to be negative regulators of ERα-responsive genes (47, 48). It was recently established that COBRA1 is the NELF-B subunit of the human NELF (negative elongation factor) and causes RNAPII pausing at the promoter-proximal region of an ERα-responsive gene (48). Interestingly, biochemical studies reported that the cooperative repression elicited by NELF and DSIF (DRB sensitivity-inducing factor) is alleviated by the P-TEFb kinase activity (50). Thus, by inhibiting P-TEFb kinase activity, BRCA1 could help NELF maintain RNAPII in a paused state at the promoter of ERα-responsive genes.

Data presented here suggest that UV-activated P-TEFb can phosphorylate the CTD prior to RNAPII recruitment to genes. We speculate that in such situations, it may act as a general transcription repressor by reducing the pool of RNAPII mole-
dolecules competent for preinitiation complex formation. We surmise that in cases of global transcription arrest, the induction of P-TEFb kinase activity would not only serve to activate specific stress-responsive genes, as has been suggested earlier (21), but could additionally contribute to globally restrain inappropriate transcription initiation by phosphorylating the CTD of soluble RNAPII. BRCA1 could participate in this regulatory pathway by modulation of P-TEFb activity. For example, the association of BRCA1 with the RNAPII holoenzyme could counteract the negative effect of P-TEFb, thus maintaining a pool of RNAPII competent for initiation and reinitiation steps of stress-responsive genes. This is consistent with our finding that BRCA1 constrains UV-induced serine 2 phosphorylation of soluble RNAPII, restores the transcription initiation defect of nuclear extracts from UV-irradiated cells, and stimulates UV-induced p21 expression.

Although BRCA1 strongly decreases global serine 2 phosphorylation in the absence of any particular cellular stress, we found that it cannot inhibit UV-induced serine 2 phosphorylation of the majority of chromatin-bound RNAPII. This phenomenon may be explained by the fact that BRCA1 could be dissociated from transcriptionally engaged RNAPII when cells are treated with UV irradiation. This dissociation would then lead to subsequent redistribution of BRCA1, perhaps to sites where DNA damage repair complexes are targeted. Consistent with this idea is the fact that BRCA1 association with processive RNAPII is disrupted following treatment with DNA-damaging agents (13). Moreover, the subnuclear localization of BRCA1 changes after treatment with UV or hydroxyurea, and BRCA1 accumulates with BARD1 and Rad50 to proliferating cell nuclear antigen-containing DNA structures (12).

Two recent studies reported that the BRCA1/BARD1 heterodimer ubiquitinates hyperphosphorylated RNAPII (51, 52). Therefore, one could argue that the increase in serine 2 phosphorylation that we observed when BRCA1 is knocked down by shRNA (Fig. 2C) is solely due to the prevention of RNAPII ubiquitination and degradation. Although we cannot completely rule out this possibility, it is worth noting that we have not been able to detect the slow migrating form of Rpb1 corresponding to the ubiquitinated counterpart, even in cells that were transfected with the control shRNA in the presence of the proteasome inhibitor MG132, and by using the H14 antibody, commonly used to detect ubiquitinated Rpb1 (data not shown). Consistently, RNAPII ubiquitination and degradation have been shown to occur mainly in response to cellular insults such as DNA damage (53). Kleiman et al. further suggested that a BRCA1/BARD1-containing complex functions to initiate degradation of stalled RNAPII following UV irradiation, thus facilitating repair of damaged DNA. In light of these results, we speculate that the BRCA1 COOH-terminal region, as an inhibitor of CTD kinases, and the BRCA1 N-terminal RING domain, as a ubiquitin E3 ligase once associated with BARD1, assume distinct functions in cells in order to modulate gene expression or promote a DNA damage response, respectively. It will be of great interest to investigate the overlap of those functions, particularly in cells subjected to transcriptional stress, in order to understand their relevance in the BRCA1 tumor suppressor role.

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BRCA1 Inhibits UV-activated CTD Kinases

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