Regulation of BRCA1 Phosphorylation by Interaction with Protein Phosphatase 1α

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Abstract

Numerous reports have revealed that the tumor suppressor BRCA1 may play an important role in DNA damage repair. BRCA1 is expressed and phosphorylated during cell cycle progression and after DNA damage. BRCA1 is hypophosphorylated in G0-G1 and probably during mitosis as well. Kinases known to phosphorylate BRCA1 include cyclin-dependent kinase 2, as well as ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related kinase (ATR), which function in G2 checkpoint control. However, protein phosphatases responsible for dephosphorylation of BRCA1 had yet to be identified. hCds1, which acts downstream of ATM, also phosphorylates a BRCA1 fragment containing amino acids 759-1064 [BRCA1 fragment 4 (BF4)]. We have used a GST-BF4 protein phosphatase by hCds1 [glutathione S-transferase (GST)-BF4-P] as a substrate to identify potential phosphatases responsible for BRCA1 dephosphorylation. Data presented here show that both recombinant protein phosphatase 1α (PP1α) catalytic subunit and endogenous PP1α dephosphorylate GST-BF4-P. Inhibitor 2 abolishes this activity. Overexpression of PP1α partially inhibits hyperphosphorylation of BRCA1 after ionizing radiation, indicating that PP1α dephosphorylates BRCA1 in vivo. BRCA1 and PP1α reciprocally immunoprecipitate, and a glutathione S-transferase pull-down assay shows that PP1α catalytic subunit associates directly with the BF4 region of BRCA1. In addition, BRCA1 inhibits PP1α activity. Therefore, BRCA1 is both a substrate and a regulator of PP1α. The interaction between BRCA1 and PP1α thus may play a role in DNA damage repair and cell cycle progression.

Introduction

Inherited BRCA1 mutations are responsible for the majority of familial breast and ovarian cancer cases (reviewed in Ref. 1). BRCA1 is a tumor suppressor protein consisting of 1863 amino acids with an apparent molecular weight of Mr ~220,000. It is mainly localized to the nucleus where it displays a nuclear dot pattern (2). BRCA1 is believed to play an important role in DNA repair and/or cell cycle regulation. Evidence indicates that BRCA1 associates with DNA repair proteins such as Rad51 and Rad50 (3, 4), which participate in homologous recombination after DNA damage (18–20). Lee et al. (21) have identified Ser 988 in BRCA1 as the major hCds1 phosphorylation site. We have confirmed this finding, demonstrating that hCds1 phosphorylates BRCA1, primarily within BF4, which spans amino acids 759-1064. Extending these results, we have used hCds1-phosphorylated BF4 to demonstrate that PP1α interacts with and dephosphorylates BRCA1. Interestingly, BRCA1 inhibits PP1α activity and may serve as a regulator of PP1α.

Materials and Methods

Construction of Vectors. The full-length hCds1 cDNA sequence was obtained from GenBank (GenBank Accession No. 4206720). The hCds1 cDNA was PCR amplified from a human colon phage cDNA library using primer pairs 5’-GGGGAATTCATGCTGGGAGTGCAGGTTTT-3’ and 5’-GGCTCGAGTCAAAACGAGCAGACACACA-3’. The resulting EcoRI-HindIII fragment containing the hCds1 cDNA was cloned into pBluescript, sequence verified, and then subcloned into pGEX-5X-1 (Amersham Pharmacia Biotech) to generate an expression construct encoding a GST-hCds1 fusion protein. To generate a PP1αGFP expression vector, PP1α cDNA was PCR

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3 The abbreviations used are: Ser, serine; ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3-related; PP, protein phosphatase; I-2, inhibitor 2; BF, BRCA1 fragment; GST, glutathione S-transferase; S988-P, phosphorylated Ser 988; IR, irradiation radiation.

* L-C. Hsu and R. L. White, unpublished data.

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amplified using primer pairs 5′-gggggccggccacagcagcagcagc-3′ and 5′-ggggaaaattttcttggcagagtt-3′ and a rabbit PPIα cDNA as a template and subjected into the XhoI and HindIII sites of pEGFP-N1-KS(−) (22)

Cell Culture and Transfection. COS-7 and 293T cells were grown in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and fed twice a week. 293T cells were transfected overnight with PPIα-GFP or the pEGFP-N1-KS(−) control vector using the calcium phosphate method. Twenty-four hours after transfection, cells were either untreated or irradiated with 5 Gy of γ-radiation and harvested for Western analysis 1–3 h after ionizing irradiation.

GST-Fusion Proteins. GST-fusion proteins were expressed in Escherichia coli and purified using glutathione Sepharose beads (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Constructs of GST-BFs were subcloned into the pEGFP-N1-KS(−) vector using the calcium phosphate method. At least 5 g of GST-hCds1, 2 g of GST-BF4, 0.2 g of GST-BF5, or GST-BF6 was cotransfected with 500 ng of COS-7 cell lysate and glutathione Sepharose beads in GST fusion buffer containing 0.1 mM EDTA, 1 mg/ml BSA, 20 mM imidazole-HCl (pH 7.4), 1 mM MgCl2, 5 mM DTT and 0.01% Brij 35. One unit of PP1α is defined as the amount of enzyme that hydrolyzes 1 nmol of p-nitrophenyl phosphate (50 mM) in 1 min at 30°C in a total volume of 50 μl. PP1α reaction buffer contained 50 mM Tris (pH 7.0), 0.1 mM Na₂ EDTA, 5 mM DTT, 0.01% Brij 35, and 1 mM MgCl₂. The catalytic subunit of rabbit skeletal muscle (2.5 units/μl, specific activity: 15 units/μg; New England BioLabs) and 20 or 180 ng of purified PP2A catalytic subunit in 25-μl reactions. One unit of PP1α defined as the number of enzyme that hydrolyzes 1 nmol of p-nitrophenyl phosphate per minute. The kinase reaction was performed as described previously (22), and Western blot analysis was performed using PP1α catalytic subunit antibody (Santa Cruz Biotechnology). To determine whether BF4 and PP1α catalytic subunit interact directly, 1 μg of GST-BF4, GST-BF4-P, GST-BF5, or GST-BF6 was incubated with 1 μg of recombinant PPIα catalytic subunit (2.5 units/μg) and glutathione beads for 2 h at 4°C. Beads were washed with L buffer and resuspended in SDS sample buffer. Precipitates were subjected to SDS-PAGE and followed by Western analysis using PPIα antibody.

BRCA1 S988-P-Specific Antibody. The antibody was generated as previously described by Lee et al. (21). Briefly, Ser 988 (CRIPLPFPKSFVKTK) and S988-P (CRPLPFPSCPFPVKTK) peptides were custom synthesized by Bob Schackmann at the University of Utah. S988-P peptide was coupled to keyhole limpet hemocyanin and used for raising rabbit antisera. S988-P specific antibody was affinity-purified using Ser 988 and S988-P affinity columns. Production and purification of the antibody was a custom service provided by Covance Research Products, Inc. Results

Recombinant PPIα Catalytic Subunit Dephosphorylates BF4 of BRCA1. BRCA1 hyperphosphorylated in response to DNA damage (11, 17) and is involved in S-phase and G2-phase checkpoints (23). It is likely that BRCA1 is mainly hypophosphorylated in M phase (11). We have also reported that a hypophosphorylated isoform of BRCA1 is associated with the centromere during mitosis (12). Hypophosphorylated BRCA1 may be generated by de novo synthesis or dephosphorylation of BRCA1. A working hypothesis is that dephosphorylation of BRCA1 may serve as a signal for completion of DNA repair and play a role in cell cycle progression into mitosis. Therefore, we are interested in identifying phosphatases involved in dephosphorylation of BRCA1. It has been reported that hCds1 phosphatase can dephosphorylate BRCA1 at Ser 988 of BF4 (amino acids 759-1064) after DNA damage. Phosphatase at Ser 988 is required for the release of BRCA1 from hCds1 and is important for BRCA1 to restore survival after DNA damage in BRCA1-mutated breast cancer cell line HCC1937 (21). We first confirmed that hCds1 phosphorylated BRCA1 mainly on BF4 and then used GST-BF4 phosphorylated by GST-hCds1 (GST-BF4-P) as a substrate to search for PP5s that could dephosphorylate BRCA1.

We first tested whether a recombinant PPIα catalytic subunit or a purified PP2A catalytic subunit could dephosphorylate GST-BF4-P. As shown in Fig. 1A, 0.67 ng (0.01 units) of recombinant PPIα dephosphorylated GST-BF4-P efficiently (10% of the signal remained compared with the buffer control). In contrast, 20 ng of PP2A had no effect on GST-BF4-P phosphorylation level. Although 180 ng of PP2A did dephosphorylate GST-BF4-P (~30% signal remained), it was not as effective as 0.67 ng of PPIα. To exclude the possibility that the purified PP2A might have lost its phosphatase activity, we conducted a PP assay comparing the activity of 0.67 ng of recombinant PPIα to 20 ng of purified PP2A using 32P-labeled phosphatase a as a substrate. Twenty ng of PP2A exhibited ~5-fold phosphorylase phosphatase activity than 0.67 ng of PPIα (Fig. 1B). Taken together, these results suggest that GST-BF4-P may be a specific substrate of PPIα.

Endogenous PPIα Dephosphorylates BF4 of BRCA1. The action of PP catalytic subunit is modulated by formation of heteromeric complexes with regulatory subunits that target the catalytic subunit to specific subcellular compartments and localize the catalytic subunit close to its substrates. Therefore, regulatory subunits of PP5s can determine their substrate specificities. Results shown in Fig. 1 were obtained using recombinant or purified catalytic subunits of PPIα and PP2A, which lack regulatory subunits. To establish that PPIα holoenzymes were also active on GST-BF4-P. COS-7 cell extract and PPIα immunoprecipitates were used as sources for endogenous PPIα complex. A COS-7 cell extract removed the phospho groups on GST-BF4-P to the same extent as recombinant PPIα catalytic subunit (Fig. 2A). Four hundred ng/ml (~17.5 nm) I-2, which specifically inhibits PPI1 (13, 14), reduced the phosphatase activity of the COS-7 cell extract toward GST-BF4-P (~70% inhibition), indicating that the
Taken together, these data indicate that endogenous PP1α specifically dephosphorylates BRCA1, which is phosphorylated by hCds1.

**PP1α Partially Inhibits Hyperphosphorylation of BRCA1 Induced by IR.** To provide evidence that PP1α could dephosphorylate BRCA1 in vivo, 293T cells were transfected with pEFGP-N1-KS(−) or PP1αGFP, followed by ionizing irradiation. As shown in Fig. 3, PP1αGFP (Lanes 1 and 2) but not pEFGP-N1-KS(−)-transfected cells (Lanes 3 and 4) expressed PP1αGFP, which was detected by PP1α antibody. BRCA1 was hyperphosphorylated in pEFGP-N1-KS(−)-transfected cells after exposure to IR, as illustrated by slower migrating bands (Lane 4) compared with that in the untreated control (Lane 5). The mobility shift of BRCA1 in PP1αGFP-transfected cells (Lane 2) was less profound than that in pEFGP-N1-KS(−)-transfected cells (Lane 4). In addition, a BRCA1 band in Lane 2 comigrating with that in the control (Lane 1) was obvious and equally intense as the slower migrating band induced by IR (Lane 2). These results suggest that hyperphosphorylation of BRCA1 after IR is partially dephosphorylated by overexpression of PP1αGFP and PP1α is capable of dephosphorylating BRCA1 in vivo.

**Direct Interaction between BRCA1 and PP1α Catalytic Subunit.** We next investigated whether there was a physical interaction between endogenous PP1α and BRCA1. Fig. 4A demonstrates that PP1α antibody specific for PP1α catalytic subunit communoprecipitated PP1α catalytic subunit (M₃ ~ 37,500) and BRCA1 from a COS-7 cell lysate. Communoprecipitation of PP1α and BRCA1 was blocked by addition of the immunogenic PP1α peptide. Conversely, BRCA1 immunoprecipitation using C-20 antibody also precipitated PP1α catalytic subunit but rabbit immunoglobulin did not (Fig. 4A). The reciprocal communoprecipitation of BRCA1 and PP1α supports an association between the two proteins. To identify the domain in BRCA1 responsible for the interaction with PP1α, we performed a GST pull-down assay using GST-BFs 1–6 and COS-7 whole cell lysate. As shown in Fig. 4B, only the GST-BF4 brought down endogenous PP1α catalytic subunit from COS-7 cell lysate. Coomassie Blue staining of GST-BFs on the blot indicates that approximately equal amounts of GST-fusion proteins were used (Fig. 4B, bottom panel).

GTP-BF4 may interact with PP1α catalytic subunit directly, or it may interact with another subunit in the PP1α holoenzyme and bring down PP1α catalytic subunit indirectly. To answer this question, GST-BF4 was incubated with recombinant PP1α catalytic subunit. As shown in Fig. 4C, GST-BF4 but not GST-BFs 5 or 6 interacted directly with recombinant PP1α catalytic subunit. Because unphosphorylated GST-BF4 is capable of interacting with PP1α in vitro, we next tested whether hyperphosphorylated BRCA1 also associated with PP1α by communoprecipitation. BRCA1 is hyperphosphorylated after ionizing irradiation indicated by slower gel mobility. PP1α antibody consistently communoprecipitated BRCA1 in both control and irradiated COS-7 cell lysates, although BRCA1 levels varied slightly.

**Fig. 1.** Recombinant PP1α catalytic subunit but not purified PP2A catalytic subunit dephosphorylates BF4. A, PP1α dephosphorylates BF4 much more efficiently than PP2A. The recombinant catalytic subunit of PP1α (0.67 ng or 0.01 units) and purified catalytic subunit of PP2A (20 and 180 ng) were incubated with GST-BF4-P. GST-BF4-P in reaction buffer was used as a negative control. The top panel shows phosphorylation of GST-BF4, and the bottom panel shows relative intensity of GST-BF4-P (% of the negative control). B, protein phosphatase assay of PP1α and PP2A. Activities of recombinant PP1α and purified PP2A catalytic subunits were determined by the release of 32P from 32P-labeled phosphatase activity of 20 ng of PP2A is 5.6-fold of that of 0.67 ng of PP1α.

**Fig. 2.** Endogenous PP1α specifically dephosphorylates BF4. A, crude extract from COS-7 cells (0.1 μg protein) was incubated with GST-BF4-P in the absence or presence of 400 ng/ml I-2. GST-BF4-P in reaction buffer was used as a negative control. B, immunoprecipitated PP1α was incubated with GST-BF4-P. Precipitate with protein G beads alone was used as a negative control. The top and middle panels show phosphorylation of GST-BF4, and the bottom panel show relative intensity of GST-BF4-P (percentage of the negative control).

**Fig. 3.** PP1α dephosphorylates BRCA1 in vivo. 293T cells transfected with PP1αGFP (Lanes 1 and 2) or the pEFP-N1-KS(−) control vector (Lanes 3 and 4) were untreated (Lanes 1 and 3) or irradiated with 5 Gy of γ-radiation (Lanes 2 and 4). Cells were harvested 1–3 h after IR, lysed in radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors, and subjected to SDS-PAGE and Western analysis. BRCA1 was detected by MS110 antibody and PP1αGFP was detected by PP1α antibody.
in different experiments (Fig. 4D). These results indicate that PP1α may associate with BRCA1 regardless of its phosphorylation state.

Data shown above suggest that PP1α may associate with BRCA1 all of the time, raising one question regarding how BRCA1 becomes phosphorylated. PP1α may be regulated by an inhibitor in the BRCA1-PP1α complex and activated only when BRCA1 needs to be dephosphorylated. To test this hypothesis, recombinant PP1α catalytic subunit and I-2 were included in a kinase assay with GST-BF4 and GST-hCds1. Phosphorylation of GST-BF4 by GST-hCds1 was detected by an S988-P specific antibody. As illustrated in Fig. 4E, S988-P antibody recognized GST-BF4 phosphorylated by GST-hCds1 (Lane 2) much better than unphosphorylated GST-BF4 (Lane 1). In the presence of recombinant PP1α, the phosphorylation of GST-BF4 by GST-hCds1 was removed (Lane 3). However, in the presence of recombinant PP1α and I-2 (Lane 4), PP1α was unable to dephosphorylate GST-BF4-P. These in vitro data suggest that PP1α activity can be inhibited by an inhibitor while associated with BRCA1, allowing BRCA1 to be phosphorylated, for example, after DNA damage.

**BRCA1 Regulates PP1α Activity through the BF4 Region.** It has been reported that the tumor suppressor pRB interacts with PP1α catalytic subunit. pRB is a substrate as well as a regulator of PP1α. By binding to PP1α, pRB inhibits PP1α catalytic activity (24–26). To determine whether BRCA1 regulated PP1α activity, BRCA1 was generated by *in vitro* transcription/translation and incubated with recombinant PP1α. PP activity was then measured using phosphorylase *a* as a substrate. Phosphatase activity of PP1α was inhibited by 30% in the presence of full-length BRCA1 or BRCA1CT, which lacks the COOH-terminal 11 amino acid residues, but still contains PP1α binding region, compared with reticulocyte lysate control (Fig. 5A). Many proteins are present in reticulocyte lysate that may interfere with the PP assay and attenuate the inhibitory effect of BRCA1. We then used purified GST-BF4 to repeat the experiment. As shown in Fig. 5B, GST-BF4 clearly inhibited PP1α activity in a dose-dependent manner (up to 80% inhibition at 500 nM of GST-BF4), whereas GST-BF5, which does not interact with PP1α, did not have this inhibitory effect. GST-BF4 inhibited phosphorylase phosphatase activity of PP1α with IC₅₀ of ~150 nM (3 nM recombinant PP1α). Therefore, BRCA1 is a regulator as well as a substrate of PP1α.

**Discussion**

We have demonstrated that hCds1 phosphorylates BRCA1 mainly on BF4 (amino acids 759-1064), confirming the report by Lee et al. (21). In addition, we have used hCds1-phosphorylated BF4 (GST-BF4-P) to show that PP1α is involved in dephosphorylation of GST-BF4-P. We used different sources of PP1α, including recombinant catalytic subunit COS-7 cell lysate, and a PP1α immunoprecipitate to...
demonstrate that PP1α specifically dephosphorylates GST-BF4-P. This activity was reduced by I-2, a specific inhibitor of PP1. Furthermore, PP2A did not exhibit the same activity toward GST-BF4-P. Overexpression of PP1α-GFP partially inhibits hyperphosphorylation of BRCA1 induced by IR, suggesting that indeed BRCA1 is a substrate of PP1α in vivo. It is not yet known whether other subtypes of PP1 can dephosphorylate GST-BF4-P or whether PP1α is involved in dephosphorylation of BRCA1 phosphorylated by kinases other than hCds1.

One intriguing question is whether the association between BRCA1 and PP1α is initiated after DNA damage. A GST pull-down assay demonstrated that unphosphorylated GST-BF4 interacted with PP1α. Coimmunoprecipitation experiments also indicated that endogenous BRCA1 associated with PP1α either with or without DNA damage induced by ionizing radiation. These results suggest that BRCA1 and PP1α may associate stably, independent of DNA damage. It is possible that PP1α is constantly active and BRCA1 phosphorylation is primarily controlled by kinase activity. Alternatively, because the catalytic subunit of PP1 is controlled by numerous regulatory units, it is more likely that PP1α activity is inhibited in this complex by a separate inhibitor. Activation of PP1α to dephosphorylate BRCA1 may only occur after DNA repair is completed. Our in vitro data support that indeed I-2 can inhibit PP1α and allowing BRCA1 to be phosphorylated by hCds1. Further study is needed to identify signal transduction pathways regulating PP1α activity on dephosphorylation of BRCA1 and to delineate its correlation with cell cycle progression. Interestingly, the tumor suppressor pRB also interacts with PP1α catalytic subunit. This interaction is detected in G2-M-enriched cells. Hypophosphorylated pRB has been shown to coimmunoprecipitate with PP1α catalytic subunit, and pRB is also a substrate for dephosphorylation by PP1α (24, 25). By binding to PP1α, pRB also serves as a PP1α regulator and inhibits PP1α catalytic activity (26). A similar scenario may also apply to BRCA1. Indeed, the BF4 region of BRCA1 showed profound inhibition of PP1α activity.

Many kinases have been shown to phosphorylate BRCA1. We have demonstrated for the first time that the PP1α associates with and dephosphorylates BRCA1. Furthermore, by binding to PP1α, BRCA1 inhibits PP1α activity, probably serves as a feedback regulation of BRCA1 phosphorylation state or modulates PP1α activity toward other substrates. Our work lays the foundation for additional investigation regarding how the phosphorylation state of BRCA1 is involved in DNA damage repair and cell cycle progression.

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