Potential Role of BRCA2 in a Mitotic Checkpoint after Phosphorylation by hBUBR1

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Abstract

BRCA2, a gene responsible for inherited susceptibility to breast cancer in a number of families, is thought to be critical for replication and repair of DNA during S-phase. To elucidate the physiological functions of BRCA2, we used a yeast two-hybrid system to screen for proteins that could associate with BRCA2. Here we report interaction of BRCA2 with a mitotic checkpoint protein, hBUBR1, and its phosphorylation by hBUBR1 in vitro. After cotransfection of BRCA2 and hBUBR1 expression vectors into the COS7 cell line, both proteins were stained together in the nuclei of cells whose spindle fibers were disrupted, but not in undamaged cells. Treatment with vincristine, which disrupts microtubules, significantly increased expression of both hBUBR1 and BRCA2 in the MCF7 cells. The results suggest that BRCA2 protein might be involved in a mitotic checkpoint during mitosis and repair the spindles (13, 14). Mutations in any of these genes result in failure to arrest the cell cycle at G2-M, and cells exit mitosis prematurely (14). Inactivation of murine BUB1 also impairs cell-cycle arrest in the mouse, causing premature cell-cycle progression and aneuploidy (15). These facts suggest that mitotic checkpoint genes may be critical for preventing aneuploidy during chromosomal segregation of mammalian cells.

To investigate the function of BRCA2, we have used a yeast two-hybrid system to search for proteins in addition to Rad51 that can interact with BRCA2. Here we report the detection of one such protein, hBUBR1 (16). We also demonstrate that this association can lead to phosphorylation of BRCA2 protein.

Introduction

Approximately 5–10% of breast cancers can be attributed to inheritance of genetic defects that have occurred in certain genes (1). Among the genes conferring susceptibility to breast cancer, BRCA1 and BRCA2 already have been isolated (2,3), and germline mutations of one or the other have been identified in ~80% of patients with familial breast cancer (4,5). To date, frameshifts that yield truncated products, presumably nonfunctional, have been the most common mutations identified (6,7). Tumors from individuals carrying germline BRCA1 or BRCA2 mutations often have lost the wild-type allele, suggesting that the absence of functional BRCA1 or BRCA2 proteins is a direct cause of early-onset breast cancer (8).

The BRCA2 gene encodes a large molecule (3418 amino acids) that bears no significant sequence homology to any other known protein (3,9). In mammalian cells, BRCA2 interacts with Rad51, a homologue of Escherichia coli RecA, which plays key roles in homologous recombination and DNA repair after double-strand breakage (10–12). Moreover, deleted versions of the murine Brca2 gene cause increased sensitivity to DNA damage and spontaneous accumulation of chromosomal abnormalities in mice, but they do not impair checkpoint function or apoptosis in response to DNA damage (12). Despite these clues, however, the cellular function of BRCA2 has remained uncertain.

In yeast, chromosomal stability and segregation are monitored and maintained by a set of proteins that includes Bub1, Bub2, Bub3, Mad1, Mad2, Mad3, and Mps1 (13). Damage to mitotic spindle fibers can activate these mitotic checkpoint genes, whose products arrest mitosis and repair the spindles (13,14). Mutations in any of these genes result in failure to arrest the cell cycle at G2-M, and cells exit mitosis prematurely (14). Inactivation of murine BUB1 also impairs cell-cycle arrest in the mouse, causing premature cell-cycle progression and aneuploidy (15). These facts suggest that mitotic checkpoint genes may be critical for preventing aneuploidy during chromosomal segregation of mammalian cells.

Materials and Methods

Yeast Two-Hybrid Screening. We applied a yeast two-hybrid screening system (17) to isolate cDNAs that encode proteins that interact with the R7 domain (amino acids 2861–3176) of BRCA2. We constructed a vector by subcloning BRCA2-R7 into the EcoRI-XhoI site of pAS2-1 (Clontech). This vector was used as “bait” to screen an oligo(dT)-primed human testis cDNA library in pACT2 vector (Clontech), according to the manufacturer’s instructions. Positive clones were cotransfected into yeast with either the bait vector or the original pAS2-1 vector; β-galactosidase activity served as a marker to confirm the interaction.

Sequence Analysis and Homology Search. Each clone obtained by yeast two-hybrid screening was sequenced by the dideoxy nucleotide termination method using an ABI 377 DNA sequencer (Perkin-Elmer). These sequences were tested against known genes in the public database with the BLAST and FASTA programs (18,19).

Construction of Vector Expressing hBUBR1. The entire coding sequence of hBUBR1 was subcloned into the pcDNA3.1 (Invitrogen) expression vector. To construct myc- or HA-tagged hBUBR1, an oligonucleotide duplex that encodes Kozac and epitope sequences was inserted into the pcDNA3.1. The PCR-amplified fragment was subcloned into the EcoRI-XhoI site of pcDNA3.1. The final vector was humanized by addition of a hexahistidine tag at the 3′ end.

Cell Culture and Transfection. COS7 cells cultured in DMEM containing 10% fetal bovine serum and antibiotic/antimycotic solution (Sigma) were plated in 10-cm culture dishes (1 × 106 cells/dish) 24 h before transfection. Expression vectors of hBUBR1 (4 μg/dish) were transfected using LIPOFECTAMINE PLUS (Life Technologies, Inc.), according to the manufacturer’s instructions. Cells were harvested 24 h after transfection.

Immunoprecipitation. Transfected COS7 cells were collected and lysed in a lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP40, 1 μg/ml phenylmethylsulfonyl fluoride]. Lysates were precleared with protein G-Sepharose, and then incubated with anti-myc (Oncogene Research) or anti-HA (Boehringer Mannheim) antibody at 4°C for 2 h. Immune complexes were precipitated with 15 μl of protein G-Sepharose and washed five times with lysis buffer.

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incubated overnight at 4°C with rotation, and then washed six times with the lysis buffer described above. The precipitates were boiled in SDS sample buffer for 3 min, and eluted proteins were analyzed by SDS-PAGE on a 12% gel. Autoradiography was performed for 3 h at room temperature.

**In Vitro Kinase Assay.** Immunoprecipitates and immobilized GST-fusion proteins (GST-hBUB1A and C) were equilibrated in kinase buffer [50 mM NaCl, 50 mM HEPEs (pH 7.4), 20 mM β-glycerophosphate, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM sodium vanadate, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride], and then incubated with 10 μCi of [γ-32P]ATP (Amersham) and substrate at 30°C for 30 min (20). Histone H1 protein (Life Technologies) was used as a positive control. Each sample was boiled in SDS sample buffer for 3 min, and eluted proteins were analyzed by SDS-PAGE on an 8% gel. Autoradiography was performed for several hours at ~80°C with intensifying screens.

**Microinjection and Immunocytochemistry.** A BRCA2 expression vector (pCDNA3_HA/BRCA2 CORRECTtr1) was kindly provided by Dr. David M. Livingston (Dana-Farber Cancer Institute, Boston, MA). COS7 cells (1 × 10⁶) were plated in 10-cm culture dishes; 24 h later, myc-tagged hBUB1R and BRCA2 expression vectors (0.5 μg/ml of each) were injected into at least 10¹ COS7 cells by microinjection (Eppendorf). These cells were treated with

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1 The abbreviation used is: GST, glutathione S-transferase.
vincristine 24 h after injection, fixed 24 h later with PBS containing 4% paraformaldehyde, and then rendered permeable with PBS containing 0.1% Triton X-100 for 2.5 min at 4°C. Cells were incubated with blocking solution (3% BSA and 0.25% goat serum in PBS) for 1 h at room temperature, and then anti-myc mouse monoclonal antibody (PharMingen) diluted 1:1000 in blocking solution and/or anti-BRCA2 goat polyclonal antibody for 1 h at room temperature. Antibodies were stained with a horse antimouse secondary antibody conjugated to Texas Red and/or a rabbit antigoat secondary antibody conjugated to FITC, and viewed with an ECLIPSE E800 microscope (Nikon).

Isolation of RNA and Northern Blot Analysis. Total RNAs were prepared from the cell lines treated with vincristine using TRizol (Life Technologies). Poly(A)^+ RNA was purified from total RNA with Oligotex-dT30 (JSR, Japan). A 2-μg aliquot of each poly(A)^+ RNA was separated on a 1% agarose gel containing 1× 4-morpholinosulfonic acid and 2% formaldehyde and transferred to nylon membrane. The blots were hybridized with a random-primed ^32^P-labeled hBUBR1, BRCA2, and β-actin cDNA probe in a mixture of 5× saline-sodium phosphate-EDTA and 10× Denhardt’s-2% SDS-50% Formamide at 50°C, washed with 0.1× SSC-0.1% SDS at 65°C, and exposed for autoradiography at ~80°C.

Results and Discussion

During a search by two-hybrid screening for proteins capable of associating with BRCA2, we isolated several candidate clones that revealed specific interaction with the R7 domain of BRCA2 (amino acids 2861–3176) and determined their sequences. One 1193-bp cDNA included an open reading frame encoding a 265-amino acid peptide. Database analysis using BLAST and FASTA programs indicated that the predicted product was identical to the COOH-terminal sequence of hBUBR1 protein (16).

Recently published data have implied that BRCA2 may be involved in a mitotic checkpoint that maintains accurate chromosomal segregation and guards against chromosomal instability. For example, a targeted mutation to truncate mBrca2 at exon 11 resulted in an accumulation of chromosomal abnormalities in the mouse, including breaks and aberrant chromatid exchanges (12). However, the precise mechanism by which inactivation of this gene causes chromosomal instability is not clear. On the other hand hBUBR1, a human homologue of Saccharomyces cerevisiae BUB1, appears to monitor accurate chromosomal segregation and cell division during mitosis (16). In yeast, mutations of BUB1 lead to abnormal numbers of chromosomes, suggesting an important role of this protein in maintenance of genomic stability (14). hBUBR1 contains a kinase domain at the COOH terminus, and phosphorylates itself (20). Defects in cell-cycle checkpoints may be responsible for the genomic instability of cancer cells (13, 21). On the basis of that information, we considered that by associating with hBUBR1, BRCA2 might prevent abnormal chromosomal segregation. Thus, we decided to confirm its ability to interact with hBUBR1, a known mitotic checkpoint protein.

We performed a pull-down assay to examine whether the
BRCA2-R7 and hBUBR1A proteins would interact in vitro. Using a nucleotide sequence from the clone initially isolated by yeast two-hybrid screening, we synthesized the GST-fusion protein (GST-hBUBR1A; 56 kDa) in bacteria and immobilized it on Glutathione-Sepharose 4B (Fig. 2A). LacZ (119 kDa) and BRCA2-R7 (34 kDa) proteins, translated in vitro and labeled with 35S (Fig. 2B), were incubated with GST or purified GST-hBUBR1A proteins. After intensive washing, the pelleted beads were dissolved in SDS-loading buffer and analyzed by SDS-PAGE electrophoresis. Neither LacZ nor BRCA2-R7 had coprecipitated with the GST protein itself (Fig. 2C, Lanes 1 and 2), but the 35S-labeled BRCA2-R7 peptide clearly had coprecipitated with GST-fused hBUBR1A (Fig. 2C, Lane 4).

The region of hBUBR1 that interacts with BRCA2-R7 is considered to be its kinase domain. Moreover, the yeast homologue, BUB1, is known to exert a checkpoint function by phosphorylating target proteins (13). Hence, we speculated that hBUBR1 might phosphorylate BRCA2, and performed an in vitro kinase assay to investigate that possibility. GST-hBUBR1B and GST-hBUBR1C proteins (see Fig. 1) were incubated with GST-BRCA2-R7 protein or GST-protein in the presence of [γ-32P]ATP. A 61-kDa band representing phosphorylated GST-BRCA2-R7 (Fig. 3A, arrows) was detected, in addition to bands of 67 and 139 kDa that corresponded to autophosphorylated GST-hBUBR1B and GST-hBUBR1C, respectively (Fig. 3A, *). Incubation of GST-BRCA2-R7 protein with GST-protein in this assay did not cause phosphorylation of GST-BRCA2-R7 (data not shown).

We also investigated the phosphorylation of BRCA2-R7 by hBUBR1 protein in mammalian cells. myc- or HA-tagged hBUBR1 expression vectors were transfected into COS7 cells. hBUBR1 protein was precipitated from the cell extracts by anti-myc or anti-HA antibodies, and incubated with GST-BRCA2-R7 protein as a substrate. In addition to 139-kDa phosphorylated bands, we observed 61-kDa (Fig. 3B, Lane 2, arrow) and 35-kDa (Fig. 3B, Lane 4) bands that corresponded to phosphorylated GST-BRCA2-R7 and histone H1 (positive control). Interestingly, the intensity of the bands decreased when hBUBR1 protein was incubated with GST-BRCA2-R7 (Fig. 3B, Lane 2) or histone H1. These results implied that the immune complex that included hBUBR1 precipitated by HA- or myc-antibody could actually phosphorylate GST-BRCA2-R7 protein. On the basis of these results, we suggest that BRCA2 is likely to be a substrate of hBUBR1 in vivo. Incubation of GST-BRCA2-R7 protein with the precipitates that formed with myc- or HA-monoclonal antibody from the mock-transfected cell lysate did not influence phosphorylation status of GST-BRCA2-R7 (data not shown).

BRCA2 is now well known as an important player in the S-phase, where it is involved in replication and recombination processes associated with DNA repair (10–12). On the other hand, hBUBR1 is thought to play a significant role in a spindle-damage checkpoint during M-phase (22, 23). These observations seem inconsistent with our results that indicate interaction between these two proteins. However, because hBUBR1 does phosphorylate the BRCA2-R7 domain in vitro and because disruption of microtubules activates hBUBR1 kinase activity, we considered that hBUBR1 might phosphorylate BRCA2 in cells that had sustained damage to microtubules. Therefore, we examined the locations of hBUBR1 and BRCA2 within cells treated with vincristine, a drug that disrupts the microtubule structure. In this series of experiments, COS7 cells transfected with hBUBR1 and/or BRCA2 expression vectors were treated with vincristine for 24 h, fixed, and stained with anti-BRCA2 (Fig. 4A) or anti-myc (Fig. 4B) antibodies. When the cells were damaged with vincristine to arrest the cell-cycle in M-phase, BRCA2 was confined in the nuclei with hBUBR1 (Fig. 4C, arrows). The nuclear staining pattern of BRCA2 in damaged cells was diffuse and very different from that in undamaged cells. In the cells that probably were not much affected by vincristine and therefore not arrested at M-phase (Fig. 4C, without arrows), BRCA2 appeared as a spotty green stain in the nucleus (24). We estimated that 40% of the cells were damaged and that 60% were undamaged under the condition of Fig. 4 on the basis of the morphology of the cells and the fluorescence-activated cell-sorting data. Almost all damaged cells revealed nuclear containing of BRCA2 and hBUBR1 similar to that shown in Fig. 4C. This result suggests that damage to microtubules activates mitotic checkpoint genes, including BUBR1, causing a drastic change in the cellular location of BRCA2 protein as it becomes phosphorylated.

We examined the inducibility of BRCA2 by serial dosage (0.0001–1 μM) of vincristine and determined that BUBR1 was induced by vincristine in a dose-dependent manner and that BRCA2 was also induced by a dosage of 0.001–1 μM of vincristine (Fig. 5). Similar results were obtained in experiments using 293 cell lines (data not shown). To exclude the possibility that BRCA2 protein might accumulate in the nuclei of normal mitotic cells, we examined the expression and cellular localization of BRCA2 in M-phase by Northern analysis and by immunostaining in combination with a standard cell-cycle synchronization technique. We detected no significant increase of BRCA2 mRNA, but we did see the spotty staining pattern characteristic of BRCA2 protein in normal mitotic cells (data not shown). These results clearly indicate that BRCA2 might play a previously undocumented role in cells with damaged microtubules, and might be involved in a mitotic checkpoint along with hBUBR1.

We have demonstrated here that hBUBR1 interacts with and phosphorylates the BRCA2-R7 domain in vitro, and that a microtubule-disrupting drug can induce mRNA expression of both of BRCA2 and hBUBR1; this process causes simultaneous translocation of hBUBR1 from the cytoplasm to the nucleus and relocation of BRCA2 in the nucleus from spotty to diffuse. To date, a number of studies that focused on the role of BRCA2 in the S-phase (25, 26) have determined only that BRCA2 might play an important role in DNA damage repair with the participation of RAD51 (10). However, no evidence has explained a mechanism to account for the spontaneous accumulation of chromosomal abnormalities in cells lacking wild-type Brca2, including breaks and aberrant chromatid exchanges. Our findings provide significant clues for clarifying this phenomenon, leading us to speculate that hBUBR1 monitors accurate chromosomal segregation through phosphorylation of target proteins, of which BRCA2 protein is probably one. Although the actual outcome of BRCA2 phosphorylation is still uncertain, mutational loss of the function of BRCA2 might block hBUBR1 signaling pathways and result in genomic instability as a consequence of failure of a mitotic checkpoint.

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References


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