BRCA1 Augments Transcription by the NF-κB Transcription Factor by Binding to the Rel Domain of the p65/RelA Subunit

Miriam Benezra‡, Nathalie Chevallier‡, Debra J. Morrison‡, Timothy K. MacLachlan§, Wafik S. El-Deiry§, and Jonathan D. Licht¶

From the ‡Department of Medicine, Mount Sinai School of Medicine, New York, New York 10029 and the §Laboratory of Molecular Oncology and Cell Cycle Regulation, Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

BRCA1 is a tumor suppressor gene mutated in cases of hereditary breast and ovarian cancer. BRCA1 protein is involved in apoptosis and growth/tumor suppression. In this study, we present evidence that p65/RelA, one of the two subunits of the transcription factor NF-κB, binds to the BRCA1 protein. Treatment of 293T cells with the cytokine tumor necrosis factor-α induces an interaction between endogenous p65/RelA and BRCA1. GST-protein affinity assay experiments reveal that the Rel homology domain of the p65/RelA subunit of NF-κB interacts with multiple sites within the N-terminal region of BRCA1. Transient transfection of BRCA1 significantly enhances the ability of the tumor necrosis factor-α or interleukin-1β to activate transcription from the promoters of NF-κB target genes. Mutation of the NF-κB-binding sites in the NF-κB reporter blocks the effect of BRCA1 on transcription. Also the ability of BRCA1 to activate NF-κB target genes is inhibited by a super-stable inhibitor of NF-κB and by the chemical inhibitor SN-50. These data indicate that BRCA1 acts as a co-activator with NF-κB. In addition, we show that cells infected with an adenovirus expressing BRCA1 up-regulate the endogenous expression of NF-κB target genes Fas and interferon-β. Together, this information suggests that BRCA1 may play a role in cell life-death decisions following cell stress by modulation of the activity of NF-κB.

BRCA1 is a tumor suppressor gene involved in 50% of hereditary breast cancers and 80% of families with hereditary breast/ovarian cancers (1, 2). The BRCA1 gene encodes a nuclear protein of 1863 residues, which contains two main motifs as follows: a zinc-binding RING finger domain at the N terminus, which mediates protein-protein interactions (3, 4); and two tandem repeats of BRCT in the C terminus (5, 6). The latter domain is involved in a variety of processes such as DNA repair, cell cycle control, and transcriptional regulation (7). The RING finger motif mediates heterodimerization with BARD1, and together the pair exhibits ubiquitin ligase activity (8).

A considerable body of data supports a role for BRCA1 in gene transcription. First, it was shown in both yeast and mammalian cells that a GAL4-BRCA1 fusion protein activates transcription of target genes, whereas tumor-derived missense mutants in the C terminus of BRCA1 failed to do so (9). Second, the C-terminal domain of BRCA1 binds to the RNA helicase, a component of the RNA polymerase II holoenzyme, as well as the p300/CBP1 co-activator (10–12). In addition, chromatin-modifying proteins, such as the SWI/SNF-related complex, bind to the N terminus of BRCA1 (13). Third, BRCA1 is able to bind transcription factors, such as p53, ATF1, STAT-1, and the Jun family of proteins, to co-activate transcription (14–17). BRCA1 also binds to Myc to inhibit transcription (18). Finally, BRCA1 was shown to regulate cell cycle progression by activating the p21CIP1/Cip1 promoter in cells that express wild type and mutant p53 (17, 19). BRCA1 also was found to transactivate the expression of p21CIP1/Cip1, a member of the universal cyclin-dependent kinase inhibitor family (20). Additionally, BRCA1 appears to regulate transcriptionally the expression of GADD45, a protein that is involved in G1 arrest. Cells that are null for BRCA1 or are deficient in GADD45 have a G/M cell cycle checkpoint defect as well as genomic instability (21–23). Recently, it was shown that BRCA1 is essential for activating the Chk1 kinase, which represses the expression of cyclin B1 and induces G2/M arrest (24).

In this study, we show that BRCA1 interacts with another transcription factor, p65/RelA. p65/RelA is a member of the NF-κB family of transcription factors, which consists of five members: p65/RelA, c-Rel, RelB, p50/p105, and p52/p100. The p105 and p100 proteins are processed into smaller, active ones (25–27). All of the members have a conserved N-terminal region of ~300 amino acids termed the Rel homology domain (RHD). The DNA-binding site, dimerization domain, nuclear localization signal, and 1κB-binding site reside within this domain. Three of the family members (p65/RelA, c-Rel, and RelB) have a transcriptional activation in the C terminus (25, 26). The NF-κB transcription factor is found as an inactive homodimer or heterodimer within the cytoplasm. The most abundant form is the p65/p50 heterodimer. The dimer associates through the Rel homology domain with an inhibitory molecule, 1κBα, a member of the 1κB protein family (26, 28). The inactive NF-κB-1κB complex also associates with the catalytic subunit of cAMP-dependent protein kinase, whose activity is inhibited by its association with the NF-κB-1κB complex (29). NF-κB is activated by exogenous stimuli, such as oxidative (H2O2) or radia-

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† To whom correspondence should be addressed: Box 1130, Mount Sinai School of Medicine, New York, NY 10029. Tel.: 212-659-5487; Fax: 212-849-2523; E-mail: jonathan.licht@mssm.edu.

‡ The abbreviations used are: CBP, CREB-binding protein (CREB, cAMP-response element-binding protein); RHD, Rel homology domain; TNFα, tumor necrosis factor-α; GST, glutathione S-transferase; STAT, signal transducers and activators of transcription; aa, amino acids; HA, hemagglutinin; IL, interleukin; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; IFN, interferon.

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tion stress (UV and γ-ray), inflammatory cytokines (TNFα or IL-1β), T cells activation, and growth factor stimulation (28, 30, 31). Upon activation, IκBα is phosphorylated by the IκB kinase complex, leading to the ubiquitination of IκBα and its subsequent degradation by the 26 S proteosome (32). Once phosphorylated by cAMP–dependent protein kinase on serine 276, the NF-κB dimer translocates to the nucleus (33, 34), binds to sequence-specific promoter elements, and activates the transcription of target genes that are involved in immune and inflammatory reactions, anti- and pro-apoptotic processes, and cell cycle regulation (35).

In the present study, we show by both in vitro and in vivo assays that the p65/RelA interacts through the Rel homology domain with BRCA1. The association between these two molecules enhances the transcriptional activation of NF-κB target genes, such as Fas and interferon β (IFNβ). This process is dependent upon the phosphorylation of p65/RelA at serine 276 and the presence of NF-κB-binding sites in the reporter gene. The transcriptional effect can be diminished by a super-stable inhibitor IκBα-B, a chemical inhibitor of NF-κB, and BRD1, and a BRCA1 RING domain mutant.

MATERIALS AND METHODS

Plasmids—The BRCA1 expression vectors, pc3-3BRCA1, pc3-3BRCA1 (C61G), and pc3-3BRCA1 (C64G), were generously provided by Dr. Barbara Weber, University of Pennsylvania Medical School (19). pSSG5, pSSG-CBP, and pSSG-CBP-ΔHAT were the gifts of Dr. J. Bieker, Mount Sinai School of Medicine. pGEX-N1 (a 1-100 ng) was a gift from Dr. W. C. Greene, J. D. Gladstone Institutes. The p65 mutant (serine 276 to alanine) was the gift from Dr. S. Ghosh, Yale University. A deletion mutant of IκBα encoding amino acids 37–317 (IκBαΔN) was the gift from Dr. W. Ballard, Vanderbilt University, Nashville, TN. The first 65/RelA (1–550) p65 fragments amino acids 1–352 and 299–550 were subcloned into pcDNA3-HA or pGEX-5x-1 constructs by PCR, using the following primers: 5′-GGAATTCTAATGGCAGAAGATGATCCATAT-3′ for 1–352 p65 and 5′-GAATTCTAATGGACGATCTGTTTC-3′ for 260–550 p65; 5′-p65 and 1–100 ng of pCR-3 infection. For Western blot analysis the nuclear and the cytoplasmic fractions (3.3% (w/v) sodium deoxycholate and 6.6% (v/v) Tween 40) was added to 100 ng of each fraction was blotted on nitrocellulose (Invitrogen) and probed with a polyclonal antibody (Santa Cruz Biotechnology). The blots were visualized using ECL (Amerham Biosciences). Membranes were developed with enhanced chemiluminescence (Amersham Biosciences).

For endogenous immunoprecipitations, cellular extracts were lysed in 1% Nonidet P-40 lysis buffer and incubated with polyclonal anti-BRCA1 antibodies (Santa Cruz Biotechnology) or IgG as a control. Western blotting for detection of endogenous BRCA1 and endogenous p65/RelA was performed with mouse monoclonal anti-BRCA1 (AB1; Oncogene, Boston) and anti-p65 antibody (F-6; Santa Cruz Biotechnology, respectively). Detection of IκBα in the cytoplasm after TNFα treatment was performed with an anti- IκBα antibody (Santa Cruz Biotechnology).

Subcellular Fractionation—Subcellular fractionation was described previously (36). Briefly, cells were rinsed twice in ice-cold phosphate-buffered saline, pH 7.4, and gently resuspended in lysis buffer B (10 mM Tris, pH 8.4, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 1 mM dithiothreitol, and complete protease inhibitors (Roche Applied Science)). The lysate was centrifuged at 1000 × g for 3 min at 4 °C, and the supernatant was saved as the cytosolic fraction. The pellets were resuspended in lysis buffer B, and a one-tenth volume of the detergent (3.3% (w/v) sodium deoxycholate and 6.6% (v/v) Tween 40) was added under slow vortexing, and the suspension was incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 1000 × g for 3 min at 4 °C. The supernatant and the nuclear fraction were resuspended in the cytoplasmic fraction. Nuclei were rinsed once in lysis buffer B. For Western blot analysis the nuclear and the cytosolic fractions were lysed in RIPA buffer supplemented with complete protease inhibitor (Roche Applied Science) on ice. A 50-μg aliquot of each fraction was analyzed. This protocol yielded intact nuclei as determined by the analysis of RNF120 protein (Santa Cruz Biotechnology). The presence of intact cytoplasmic contamination as determined by the absence of β-actin (Sigma).}

Reverse Transcription and Real Time PCR—MCF-7 cells were infected with an adenovirus containing BRCA1 or GFP (multiplicity of infection = 30). Forty hours after infection cells were treated (6 h, 4 mg/mL) with TNFα and 4 mg/mL cycloheximide. Cells were resuspended with 100 ng of each fraction was blotted on nitrocellulose (Invitrogen) and probed with a polyclonal antibody (Santa Cruz Biotechnology). The blots were visualized using ECL (Amerham Biosciences). Membranes were developed with enhanced chemiluminescence (Amersham Biosciences). Reverse Transcription and Real Time PCR—MCF-7 cells were infected with an adenovirus containing BRCA1 or GFP (multiplicity of infection = 30). Forty hours after infection cells were treated (6 h, 4 mg/mL) with TNFα and 4 mg/mL cycloheximide. Cells were resuspended with 100 ng of each fraction was blotted on nitrocellulose (Invitrogen) and probed with a polyclonal antibody (Santa Cruz Biotechnology). The blots were visualized using ECL (Amerham Biosciences). Membranes were developed with enhanced chemiluminescence (Amersham Biosciences). Reverse Transcription and Real Time PCR—MCF-7 cells were infected with an adenovirus containing BRCA1 or GFP (multiplicity of infection = 30). Forty hours after infection cells were treated (6 h, 4 mg/mL) with TNFα and 4 mg/mL cycloheximide. Cells were resuspended with 100 ng of each fraction was blotted on nitrocellulose (Invitrogen) and probed with a polyclonal antibody (Santa Cruz Biotechnology). The blots were visualized using ECL (Amerham Biosciences). Membranes were developed with enhanced chemiluminescence (Amersham Biosciences). Reverse Transcription and Real Time PCR—MCF-7 cells were infected with an adenovirus containing BRCA1 or GFP (multiplicity of infection = 30). Forty hours after infection cells were treated (6 h, 4 mg/mL) with TNFα and 4 mg/mL cycloheximide. Cells were resuspended with 100 ng of each fraction was blotted on nitrocellulose (Invitrogen) and probed with a polyclonal antibody (Santa Cruz Biotechnology). The blots were visualized using ECL (Amerham Biosciences). Membranes were developed with enhanced chemiluminescence (Amersham Biosciences).
using 2 µl of cDNA product from the reverse transcription reaction. The PCR contained 0.5 units of AmpliTaq Gold, 1× Syber buffer, 2 mM MgCl₂, 1 mM dNTP with dUTP (PE Biosystems, Warrington, UK), and 0.4 µM of each 5′ and 3′ primer. Reactions were performed with an initial hot start incubation at 95 °C for 10 min, followed by denaturation at 95 °C for 30 s, annealing at various temperatures depending upon the primer pairs for 30 s, and extension at 72 °C for 30 s. Forty amplification cycles were performed. The data were collected on an ABI Prism 7700 (PerkinElmer Life Sciences) and analyzed using the Sequence Detector version 1.7 program. The PCR end products were checked on 1.8% agarose gel. The primers pairs used for amplification of specific NF-κB target genes are as follows: IFNγ 5′-CAGGACAGCTCTTCTCCATGA-3′ and 3′-AGCCAGCTGCTGATTGATTCA-5′; Fas 5′-GGACATGGAACTCAGTATCTC-3′ and 3′-GCATTTGGATCTTCTGTCGCG-5′; actin 5′-GCCAGAGAAGAGAGGATTA-3′ and 3′-GGCCATCTCTTGCTCGAAGTT-5′.

RESULTS

**BRCA1 Activates NF-κB Target Promoters—**Cytotoxic stress and DNA damage activate the NF-κB transcription factor, leading to cell cycle arrest, apoptosis, or DNA repair (30, 31). Furthermore, it was shown that BRCA1 is also involved in apoptosis or DNA repair following DNA damage (21, 37). In order to establish whether BRCA1 could modulate the transcriptional activity of NF-κB, **BRCA1** was co-transfected into 293T or MCF-7 cells with luciferase reporter genes linked to the promoters of NF-κB target genes: IgK, IFNβ, and Fas. Thirty six hours after transfection cells were treated with TNFα, a cytokine activator of NF-κB. Control cells were transfected with equimolar amounts of empty expression vector and/or were mock-induced. Treatment of cells with TNFα in the absence of BRCA1 induced a 2–3-fold increase in the reporter activity (Fig. 1A and data not shown). Addition of **BRCA1** to TNFα-treated cells induced a further 3-fold increase in reporter activity. Similar results were also observed with the human immunodeficiency virus promoter and a construct containing multimerized NF-κB-binding sites in 293T cells (data not shown). Addition of the IL-1β cytokine to the co-transfected cells had the same effect on reporter activity (data not shown).

Because treatment of transfected cells with TNFα induces other transcription factors in addition to NF-κB (38), subsequent experiments were performed to determine whether the increase in reporter activity was due to the specific activation of NF-κB by TNFα. For this purpose, we used a truncated version (Fas 0.4 kb) of the above-mentioned Fas promoter that only contains two NF-κB-binding sites and a mutant form of this short promoter (Fas-mut 1.2), in which both NF-κB sites were mutated (36). Cells transfected with the wild-type promoter showed a 4-fold increase in transcriptional activity in the presence of TNFα and an almost 5-fold increase in the presence of **BRCA1**. The mutant Fas promoter was not stimulated by addition of TNFα, and co-transfection of **BRCA1** did not enhance the transcriptional activity (Fig. 1B). Next, a deletion mutant of IκBα (IκBαΔN) was used as a super-stable inhibitor of NF-κB. This inhibitor lacks two phosphorylation sites that are important for targeting IκB for degradation, as a result, IκBαΔN fails to dissociate from NF-κB and blocks the translocation of NF-κB to the nucleus after treatment of the cells with TNFα (Fig. 1C, compare lanes 1 and 2 to lanes 3 and 4). In the presence of IκBαΔN, the ability of **BRCA1** to augment transcription in the presence of TNF was reduced by 40% (Fig. 1D). IκBαΔN had no affect on the localization of **BRCA1** (Fig. 1C).

Similar results were observed when transfected cells were treated with SN-50 (Calbiochem), a peptide that competes with the binding of NF-κB to the nuclear membrane and thus blocks NF-κB translocation into the nucleus (Fig. 1E). Collectively, these results indicate that the effect of **BRCA1** on these reporter genes is mediated by NF-κB.

**Association of BRCA1 with the Rel Homology Domain of p65/RelA and BRCA1**—To determine whether **BRCA1** binds to the p65/RelA subunit of NF-κB in response to treatment with TNFα, we performed endogenous co-immunoprecipitation experiments. 293T cells were seeded at low concentration on 10-cm² plates and 24 h later treated with TNFα, whereas control cells were left untreated. Lysates from these cells were subjected to immunoprecipitation with either rabbit polyclonal anti-BRCA1 antibody or with IgG as a control. Western blot analysis performed with a mouse monoclonal anti-BRCA1 antibody revealed that endogenous **BRCA1** was immunoprecipitated from treated and untreated cells by the **BRCA1** antibody but not with the control IgG (Fig. 2). In contrast, the p65/RelA subunit was only co-immunoprecipitated with the **BRCA1** antibody from cells treated with TNFα (Fig. 2). The endogenous expression of **BRCA1** and p65/RelA is shown by Western blot analysis (Fig. 2). The co-immunoprecipitation of BRCA1 and p65/RelA was also observed in MCF-7 cells (data not shown). As a marker for the translocation of p65/RelA into the nucleus, the reduction of IκBα levels was demonstrated after treatment with TNFα (Fig. 2).

The p65/RelA subunit of NF-κB consists of two main regions, the RHD located at the N terminus (aa 1–332) and the transactivation domain located at the C terminus of the molecule (aa 299–550) (Fig. 3A). To identify which region of p65/RelA interacts with **BRCA1**, full-length p65/RelA and each individual domain of the molecule were HA-tagged (Fig. 3A) and expressed in 293T cells in the absence or presence of FLAG-**BRCA1**. The cells were treated with TNFα, and lysates were immunoprecipitated with a FLAG antibody, and precipitated proteins were immunoblotted with FLAG antibody to detect **BRCA1** and an HA-antibody to detect p65. As shown, full-length p65/RelA and RHD were co-immunoprecipitated with **BRCA1** in TNFα-treated cells (Fig. 3B, lanes 4 and 6). In contrast, the fragment containing only amino acids 299–550 of p65/RelA failed to associate with **BRCA1** (Fig. 3B, lane 8), even though this protein was expressed in the cells (Fig. 3C, lanes 7 and 8). Taken together, these results indicate that NF-κB interacts through the Rel homology domain with **BRCA1**.

**Identification of the p65-binding Region within the BRCA1 Molecule—**To determine which region of the **BRCA1** molecule binds to p65/RelA, six fragments of **BRCA1** (1–304, 260–553, 502–802, 758–1064, 1005–1313, and 1314–1863) were expressed as glutathione S-transferase (GST) fusion proteins and used for in vitro protein affinity assays with three fragments of p65/RelA metabolically labeled with [35S]methionine (Fig. 4A). Equivalent levels of GST fusion proteins were used in each protein affinity assay (data not shown). The GST-**BRCA1** fragments 1–304, 260–553, and 502–802 bound to the full-length p65/RelA protein as well as to the 1–332 p65/RelA fragment. p65/RelA bound most strongly to GST-**BRCA1** fragment 260–553 (Fig. 4A). A very weak binding was observed with the GST-**BRCA1**-1314–1863 fragment. In accordance with our earlier results, the 299–550 p65/RelA did not bind to any of the **BRCA1** fragments (Fig. 4A, **bottom panel**). The inverse experiment was also performed. Specifically, the 1–332 p65/RelA fragment was expressed as a GST fusion protein and used in an in vitro protein affinity assay with four fragments of **BRCA1** (1–802, 1–260, 261–553, and 554–802) metabolically labeled with [35S]methionine. All the **BRCA1** fragments bound to GST-(1–332)-p65/RelA but not to GST alone (Fig. 4B). These data demonstrate that a broad, N-terminal region of **BRCA1** is important for binding to the Rel homology domain of p65/RelA.

The RING finger domain, located in the N terminus of **BRCA1**, is important for protein-protein interactions. It is characterized by the presence of a conserved C3HC4 sequence,
which coordinates the binding of two zinc atoms. Missense mutations in \textit{BRCA1} occur, with varying frequencies, at four out of seven cysteine residues of the \textit{BRCA1} RING domain (cysteine 29, 34, 61, and 64). Mutation of cysteine 61 to glycine (C61G) was shown to disrupt the zinc binding and formation of \textit{BRCA1} N-terminal homodimers \textit{in vitro} (39). In order to determine whether mutation in the RING finger domain influences the transcriptional effect of \textit{BRCA1} on NF-\kappaB target genes, 293T cells were transfected with two concentrations (100 or 150 ng/ml) of either wild type \textit{BRCA1} or \textit{BRCA1} mutated at cysteine 61. A 293T cell line was transfected with IgK, IFN\gamma, or Fos reporter gene (100 ng), tk-Renilla (5 ng), and with either pcr-3 (25 ng) or pcr-3-BRCA1 (50 ng). Thirty six hours after transfection, cells were induced with TNF\alpha (10 ng/ml) for 12 h. Each luciferase point represents the mean of triplicates (± S.D.). B, 293T cells were transfected with the 0.4 kb of Fos promoter construct or the mutated form Fos-mut1,2 (25 ng) tk-Renilla (5 ng), and with either pcr-3 (50 ng) or pcr-3-BRCA1 (100 ng). Thirty six hours post-transfection, cells were treated with TNF\alpha (10 ng/ml). Luciferase measurement was performed as above. C, Western blot (WB) of nuclear and cytoplasmic fractions from TNF\alpha-induced cells transfected with FLAG-BRCA1 (9 \mu g/plate) and either I\kappaB\alpha or control (Cont) vector (3 \mu g/plate). Detection of \beta-actin and RNA polII in the cytoplasmic and nuclear fraction, respectively, demonstrates purity of fractionation. D, 293T cells were transfected with IgK promoter (100 ng), tk-Renilla (5 ng), pcr-3 (25 ng), or pcr-3-BRCA1 (50 ng) in the absence or presence of super-stable I\kappaB\alpha (I\kappaB\alpha\text{CN}) (50 ng). Thirty six hours post-transfection, cells were treated with TNF\alpha (10 ng/ml). Luciferase measurement was performed as above. E, 293T cells were transfected with IgK promoter (100 ng), tk-Renilla (5 ng), and with either pcr-3 (25 ng) or pcr-3-BRCA1 (50 ng). Thirty six hours after transfection, the cells were treated with SN-50 (20 \mu M) or mock-treated. One hour later, all cells were treated with TNF\alpha (10 ng/ml). All of the above experiments were performed at least three times with similar results being obtained.
The cysteine mutants were 30 and 50% less active than the wild type BRCA1 transfected at the same concentration (Fig. 5A). The wild-type BRCA1 and mutant forms were expressed at comparable levels at both concentrations (Fig. 5B).

BARD1 Inhibits Transcriptional Activation by BRCA1—BARD1 is a RING finger protein that binds to the N-terminal domain of BRCA1, including the RING finger and stabilizes the molecule (40). Because BARD1 and NF-κB both bind the N-terminal portion of BRCA1, we determined whether BARD1 could affect the functional interaction between BRCA1 and NF-κB. BARD1 was co-transfected into 293T cells with or without BRCA1. BRCA1 induced a 4-fold increase in reporter activity when cells were treated with TNFα (Fig. 5C). However, BARD1 blocked the ability of BRCA1 to augment transcription by 50% (Fig. 5D), even though the level of BRCA1 was elevated in cells co-transfected with BARD1 (Fig. 5D). BARD1 had no significant effect on the ability of TNFα to activate the reporter gene in the absence of BRCA1 (Fig. 5C). A truncated form of BARD1, which lacks the RING finger domain, did not inhibit the transcriptional activity of BRCA1 (data not shown). To determine whether BARD1 blocked co-activation by BRCA1 by inhibiting the formation of a BRCA1-p65 complex, HA-p65/RelA and FLAG-BRCA1 were expressed in 293T cells in the absence or presence of BARD1. In this triple transfection experiment,
BARD1 did not decrease the level of expression of BRCA1 in transfected cells (Fig. 5E, lanes 2 and 6) nor did it alter the subcellular localization of BRCA1 or p65 (data not shown). We consistently noted that p65/RelA expression decreased expression of BRCA1 in transfected cells, an effect possibly due to promoter or competition or squelching due to the overexpression of p65/RelA. Despite this, several important pieces of information could be determined from the experiment. When p65/RelA alone was transfected into cells, it could not be detected in BARD1 precipitates (Fig. 5E, lane 2). When cells were transfected with HA-p65/RelA and BARD1, HA-p65/RelA was detected in the BARD1 immunoprecipitates (Fig. 5E, lane 7). Curiously, a similar level of HA-p65 was also immunoprecipitated in the presence of FLAG-BRCA1 using BARD1 antibodies. This presumably occurred through endogenous BARD1 present in the cell (Fig. 5E, lane 4) and may be due to the mutual stabilization of BRCA1 and BARD1. Importantly, when BARD1 and BRCA1 were expressed together along with p65, the amount of p65 found in BARD1 immunoprecipitates was strongly enhanced. Similar results were obtained when BRCA1 was used to immunoprecipitate the proteins (data not shown). Therefore, the ability of BARD1 to inhibit trans-activation of TNFα-mediated transcription by BRCA1 could not be explained by blocking the formation of the BRCA1-p65-RelA complex. On the contrary, BARD1 and BRCA1 together more readily interacted with p65 than alone.

**Fig. 5.** The mutated form of BRCA1 and BARD1 inhibits the transcriptional effect of BRCA1 on NF-κB target genes. A, 293T cells were transfected with the IgK promoter (100 ng), tk-Renilla (5 ng), and with either pcr-3 (50 or 75 ng), pcr-3-BRCA1 (100 or 150 ng), pcr-3-BRCA1 (C61G; 100 or 150 ng), or pcr-3-BRCA1 (C64G; 100 or 150 ng). Thirty six hours after transfection, cells were induced with TNFα (10 ng/ml) for 12 h. This experiment was performed three times in triplicate (±S.D.). B, immunoblot with anti-BRCA1 antibody of cellular extracts from experiment above. C, 293T cells were transfected with the IgK promoter (100 ng), tk-Renilla (5 ng), and with either pcr-3 (25 ng) or pcr-3-BRCA1 (50 ng) and either pCMV4 (25 ng) or pCMV4-BARD1 (36 ng). Thirty six hours after transfection, the cells were induced with TNFαs (10 ng/ml) for 12 h. Each luciferase point represents the mean of triplicates (±S.D.). The experiment was performed at least three times. D, immunoblot to detect the level of BRCA1 and BARD1 expression. E, 293T cells were transfected with empty vector (lanes 1–4) or BARD1 (lanes 5–8), and FLAG-BRCA1 (lanes 2, 4, 6, and 8) or HA-p65 (lanes 3, 4, 7, and 8). Co-immunoprecipitations were performed with an anti-BARD1 antibody, and the immunoblot was probed with FLAG antibody to detect BRCA1, anti-HA to detect p65/RelA, and anti-BARD1 to detect BARD1. Western blot (WB) analysis was performed on the lysozyme samples described above. The blot was probed with anti-FLAG, anti-HA, and anti-BARD1 to detect BARD1.

Mutation of Serine 276 on the p65/RelA Subunit Abolishes the Effect of BRCA1—The p65/RelA subunit is phosphorylated by cAMP-dependent protein kinase at serine 276 in the Rel homology domain. This phosphorylation event is important for the binding of CBP. In order to determine whether the phosphorylation of the Rel homology domain is also important for the enhancement of the transcriptional activity by BRCA1, 293T cells were co-transfected with p65/RelA, p65/RelA (S276A) mutant, or empty vector in the presence or absence of BRCA1. In the presence of TNFα and wild-type p65/RelA, there is a 3-fold enhancement in the transcriptional activity induced by BRCA1. Transfection of the mutated form of p65/RelA was found to abolish this effect (Fig. 6A). To verify if this mutated form also influences the physical interaction between BRCA1 and p65/RelA, we performed a co-immunoprecipitation experiment. The mutant p65/RelA (S276A) was HA-tagged and expressed in 293T cells in the absence or presence of FLAG-BRCA1. Lysates from cells treated with TNFα were immunoprecipitated with anti-FLAG antibody. Precipitated proteins were analyzed by Western blot with anti-FLAG antibody to detect BRCA1 and an anti-HA antibody to detect mutated p65/RelA. As shown, mutant p65/RelA was immunoprecipitated only in the presence of BRCA1 (Fig. 6B, top panel). This suggests that phosphorylation at serine 276 is not required for the physical interaction between BRCA1 and p65/RelA but is important for the enhancement of the transcriptional activity induced by BRCA1.

It was shown previously (11) that CBP also binds to BRCA1. Therefore, we next determined whether co-expression of CBP and BRCA1 would have a greater effect on transcription of NF-κB target genes than either one alone. Whereas BRCA1 stimulated transcription from the reporter gene by nearly a factor of 3 and CBP stimulated transcription by 2.5-fold, the combination of BRCA1 plus CBP led to a 10-fold increase in transcriptional activity (Fig. 6C). Co-transfection of BRCA1 with a deletion mutant of CBP devoid of the histone acetyltransferase domain (CBPAHAT) resulted in significantly less stimulation of transcription. BRCA1 appeared to stabilize CBP, and in the presence of BRCA1, CBP and CBPAHAT were expressed at equivalent levels (Fig. 6D, lanes 4 and 6). In contrast, expression of CBP had no effect on the level of expression of BRCA1 (Fig. 6D, lanes 2, 4, and 6). We next wondered whether BRCA1, CBP, and p65/RelA could be found as a complex. For this purpose, 293T cells were co-transfected with HA-p65 and CBP in the absence or presence of FLAG-BRCA1. The cells were treated with TNFα; lysates were immunoprecipitated with FLAG antibody, and precipitated proteins were immunoblotted with FLAG antibody to detect BRCA1, HA antibody to detect p65/RelA, and CBP antibody to detect CBP. CBP or p65/RelA alone could be co-precipitated with BRCA1 (Fig. 6E, lanes 4 and 7, respectively) and also when all three proteins were expressed, both HA-p65 and CBP could be co-immunoprecipitated with FLAG-BRCA1 (Fig. 6E, lane 8). Immunoprecipitation with a CBP antibody also precipitated both BRCA1 and p65 (data not shown). Together, these results suggest that CBP, by complexing with the activated and phosphorylated form of NF-κB as well as with BRCA1, may mediate the transcriptional effects of BRCA1.

**BRCA1 Stimulates Expression of Endogenous NF-κB Target Genes**—Because BRCA1 enhanced the TNFα-induced activity of IFNβ and Fas reporter genes, we determined whether BRCA1 could affect expression of endogenous NF-κB target genes. RNA was isolated from MCF-7 cells infected with an adenovirus expressing BRCA1 or GFP and treated with TNFα. Quantitative real time PCR analysis revealed that BRCA1 significantly augmented the expression of IFNβ and Fas...
enhancement of the activity of IgK promoter by BRCA1. A, 293T cells were transfected with the IgK promoter (100 ng), tk-Renilla (5 ng), pCDNA 3.1, p65, or p65/S276A (100 ng), and with either pcr-3 (25 ng) or pcr-3-BRCA1 (50 ng). Thirty six hours after transfection the cells were induced with TNFα (10 ng/ml). Quantitative real time PCR was performed on the RNA. The quantification of FAS and IFNβ mRNA is relative to the amount of actin RNA. The experiment was performed at least three times in triplicate.

B, which also contains a Rel homology domain of the p65/RelA subunit, but not the C-terminal domain which contains the transactivation domain, interacts with the N-terminal region of BRCA1 (aa 1–802). This result was confirmed by a GST protein affinity assay, which further revealed that the major site of interaction on BRCA1 is between amino acids 260 and 553. The interaction between BRCA1 and the p65/RelA was also detected by co-immunoprecipitation of endogenous proteins in 293T cells, as well as in MCF-7 cells. The p50 subunit of NF-κB, which also contains a Rel homology domain, interacts similarly with BRCA1 (data not shown).

Tumor-associated BRCA1 RING finger mutants were partially defective for trans-activation, suggesting that the RING finger motif has a role in the functional interaction between BRCA1 and p65/RelA. BRCA1 can be found complexed with RNA polymerase II holoenzyme (41), an interaction dependent on the N-terminal portion of the protein including the RING finger. Furthermore, interaction of BRCA1 and CBP also requires the N-terminal portion of the protein including the RING finger. Point mutants of BRCA1 are known to preserve the interaction with BARD1 while disabling the ubiquitin ligase activity of BRCA1 (42, 43). This suggests a link between the transcriptional activity of BRCA1 and ubiquitination, and is consistent with emerging data indicating a link between transcriptional activation, ubiquitination, and degradation of transcription factors (reviewed in Ref. 44). An increasing number of ubiquitin-protein isopeptide ligases like Rep5/

mRNA in infected cells above the increase mediated by TNFα alone (Fig. 7). Importantly, these results were in agreement with those obtained in reporter experiments and suggest that the ability of BRCA1 to interact with the NF-κB system has relevance in vivo.

DISCUSSION

In this study, we present data demonstrating the interaction of the p65/RelA subunit of NF-κB with the N-terminal domain of BRCA1. By both in vitro binding assays and co-immunoprecipitation analysis, we demonstrate that the Rel homology domain of the p65/RelA subunit, but not the C-terminal domain which contains the transactivation domain, interacts with the N-terminal region of BRCA1 (aa 1–802). This result was confirmed by a GST protein affinity assay, which further revealed that the major site of interaction on BRCA1 is between amino acids 260 and 553. The interaction between BRCA1 and the p65/RelA was also detected by co-immunoprecipitation of endogenous proteins in 293T cells, as well as in MCF-7 cells. The p50 subunit of NF-κB, which also contains a Rel homology domain, interacts similarly with BRCA1 (data not shown).

Tumor-associated BRCA1 RING finger mutants were partially defective for trans-activation, suggesting that the RING finger motif has a role in the functional interaction between BRCA1 and p65/RelA. BRCA1 can be found complexed with RNA polymerase II holoenzyme (41), an interaction dependent on the N-terminal portion of the protein including the RING finger. Furthermore, interaction of BRCA1 and CBP also requires the N terminus of BRCA1 (11). Point mutants of BRCA1 are known to preserve the interaction with BARD1 while disabling the ubiquitin ligase activity of BRCA1 (42, 43). This suggests a link between the transcriptional activity of BRCA1 and ubiquitination, and is consistent with emerging data indicating a link between transcriptional activation, ubiquitination, and degradation of transcription factors (reviewed in Ref. 44). An increasing number of ubiquitin-protein isopeptide ligases like Rep5/
hPRF1 \((45)\) and E6-AP \((46)\), which can now include BRCA1, have been identified as transcriptional co-activators. What proteins of the transcription complex might be modified by BRCA1 is unknown.

The importance of the N-terminal region of BRCA1 for transcriptional activity was also highlighted by the ability of BARD1 to inhibit trans-activation by BRCA1. BARD1 and BRCA1 interact in their N terminus in a region immediately adjacent to each RING finger \((47)\). The RING fingers of the proteins then together form a structure with considerable ubiquitin ligase activity \((40)\). BARD1 expression, although generally increasing BRCA1 protein accumulation within the cell, actually inhibited the ability of BRCA1 to activate transcription through NF-xB. The N terminus of BARD1 including the RING finger and sequences required for heterodimerization with BRCA1 was required for this effect. BARD1 did not change the subcellular localization of BRCA1 or p65/RelA and did not inhibit the formation of the BRCA1-p65 complex. On the contrary BRCA1, BARD1, and p65 readily interacted when co-expressed. This suggests that the transcriptional function of BRCA1 is altered when complexed by BARD1. This may reflect the ability of BARD1 to bind with BRCA1 in nuclear dots which might represent sites of DNA repair rather than transcription \((48)\). The reduced transcriptional function of BRCA1 when co-expressed with BARD1 might also be related to the fact that BARD1 enhances the ubiquitin ligase activity of BRCA1 in vitro \((43, 49)\), potentially directing the activity of the complex to histones, particularly H2AX, found at sites of DNA damage. Alternatively, BARD1 might inhibit transcriptional activity of BRCA1 by association with the polymerase II holoenzyme, inhibiting polyadenylation though interaction with the CstF factor \((50)\).

NF-xB is primarily found in the cytoplasm in a complex with IxB and the catalytic subunit of protein kinase A. Upon activation by the cytokines TNF-x or IL-1B, p65/RelA is phosphorylated on serine 276 by protein kinase A and is translocated to the nucleus \((55, 56)\). This phosphorylation facilitates the docking of the transcriptional co-activator CBP, which enhances the ability of NF-xB to activate transcription \((33, 34)\). It has been shown that the KIX domain of p300/CBP interacts with both the N- and the C-terminal domains of BRCA1 \((11)\). We demonstrated by co-immunoprecipitation that BRCA1 can complex with both p65/RelA and CBP and cooperates with these proteins to activate NF-xB target genes. Mutation of serine 276 to alanine in the p65/RelA subunit abrogates the ability of BRCA1 to enhance transcription, even though the interaction between p65 \((S273A)\) and BRCA1 is not disrupted. This reduction in the transcriptional activity may be due to the inability of CBP to bind to the unphosphorylated form of p65/RelA. An analogous type of complex interaction occurs with another BRCA1-binding protein, ATF1. Like p65, the DNA-binding C-terminal domain of ATF1 is required for interaction with BRCA1. The N-terminal KID domain of ATF1, which is also phosphorylated by protein kinase A, mediates an interaction between ATF1 and CBP \((14)\). The phosphorylated form of ATF1 is important for its transcriptional activity but is not important for the physical interaction between BRCA1 and ATF1 \((14)\). This theme is found in yet another system. Stimulation of cells by another cytokine, IFN-z, leads to serine phosphorylation of STAT-1z and ultimately activation of IFN-z-inducible genes. Phosphorylation at Ser-727 in the C-terminal region of STAT-1z enhances transcription by recruiting p300/CBP and also BRCA1. BRCA1 acts in concert with STAT-1 to differentially regulate a subset of IFN-z target genes \((16, 51)\) involved in growth control. Similarly, BRCA1 may only activate a subset of NF-xB genes. How specificity in BRCA1-dependent gene regulation is achieved remains unclear.

Apart from binding to co-activators, such as CBP/p300 or to transcription factors, such as ATF1 and p65/RelA, BRCA1 also binds to RNA polymerase II through its C-terminal domain \((10, 12)\). Based on these multiple interactions, we propose a model whereby BRCA1 functions as a scaffold protein by tethering together the various transcriptional elements (Fig. 8). In the absence of BRCA1 (Fig. 8A), CBP binds to the p65/RelA subunit and to the TATA box-binding protein and TFIIB, which binds to RNA polymerase II \((52–54)\) and co-activates the transcription of NF-xB target genes. Addition of BRCA1, which interacts physically with p65/RelA, CBP, and RNA polymerase II as demonstrated in Fig. 8A, enhances the transcriptional activation of NF-xB target genes (Fig. 8B).

Both BRCA1 and NF-xB have been reported to be involved in pro-apoptotic functions in various cell types. We show BRCA1 significantly increases the transcriptional activity of NF-xB target promoters, such as those of IgK, IFN-b, and Fas, above the activity observed by stimulation with TNF-x alone. This activity is inhibited by a super-stable inhibitor of NF-xB, as well as by a chemical inhibitor of NF-xB. The mutated form of the Fas gene could not be activated by BRCA1, demonstrating that BRCA1 is exerting its effect through NF-xB. The potential physiological relevance of this finding was heightened by our result showing that overexpression of BRCA1 increased expression of the endogenous FAS and IFN-b genes above the levels seen with TNF-x treatment alone. The FAS receptor along with FASL activate a pathway leading to apoptosis. IFN-b is a cytokine that can promote apoptosis in response to viral infection \((51)\). Hence, through the induction of NF-xB targets genes BRCA1 might induce an antiviral and anti-proliferative state in the cell.

In multiple systems, NF-xB has been implicated in the apoptotic response. In T cells, topoisomerase poisons and UV irradiation activate the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway, which leads to the induction of the NF-xB transcription factor. NF-xB activates
NF-κB, which in turn binds to its receptor, FAS/CD95, thus resulting in apoptosis (55, 56). In addition, in neuronal cells glutamate induces cell death by activating the NF-κB transcrip-
tion factor (57). Overexpression of a dominant-negative p65/RelA protein inhibits apoptosis induced by serum starva-
tion in HEK 293 cells (58). Moreover, incubation of osteoblastic mcl73/E1 cells with TNFα induces apoptosis and stimulates nuclear localization of NF-κB. Treatment with an anti-TNFα
antibody prevents apoptosis and decreases NF-κB activation, indicating that activation of NF-κB through TNFα may be an
important apoptotic signaling pathway in osteoblasts (59, 60).

BRCA1 has also been implicated in the SAPK/JNK pathway leading to apoptosis (21). Thangaraju et al. (37) reported that
overexpression of BRCA1 in breast and ovarian cancer cell lines induces expression of FAS and FASL, ultimately leading
to apoptosis after serum withdrawal through the activation of the SAPK/JNK pathway. Because both NF-κB and BRCA1 were shown to activate the expression of FAS through the JNK path-
way, it is possible that BRCA1 is functioning as a transcrip-
tional co-activator with NF-κB in this system.

NF-κB has also been linked to the prevention of apoptosis.
NF-κB is often constitutively active in breast cancer cell lines and
diverse solid tumor-derived cell lines (61–63). NF-κB can induce expression of anti-apoptotic genes, such as inhibitor of
apoptosis protein (IAP) family and superoxide dismutase, and genes that contribute to proliferation, such as c-MYC
and BCL-XL (64). Therefore, persistent activation of NF-κB could provide a mechanism that protects cancer cells from apoptosis. In breast cancers where the tumor suppressor BRCA1 is mut-
tated, the balance between pro-apoptotic and anti-apoptotic pathways may be shifted toward the latter. Future studies will
be aimed at determining whether BRCA1 acts as a co-activator of only pro-apoptotic genes or as a general activator of all
NF-κB target genes.

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Miriam Benezra, Nathalie Chevallier, Debra J. Morrison, Timothy K. MacLachlan, Wafik S. El-Deiry and Jonathan D. Licht

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