Regulation of BRCA1 Expression by the Rb-E2F Pathway*

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Inheritance of a mutant allele of the breast cancer susceptibility gene BRCA1 confers increased risk of developing breast and ovarian cancers. Likewise, inheritance of a mutant allele of the retinoblastoma susceptibility gene (RB1) results in the development of retinoblastoma and/or osteosarcoma, and both alleles are often mutated or inactivated in sporadic forms of these and other cancers. We now demonstrate that the product of the RB1 gene, Rb, regulates the expression of the murine Brca1 and human BRCA1 genes through its ability to modulate E2F transcriptional activity. The Brca1 gene is identified as an in vivo target of E2F1 in a transgenic mouse model. The Brca1 promoter contains E2F DNA-binding sites that mediate transcriptional activation by E2F1 and repression by Rb. Moreover, ectopic expression of cyclin D1 and Cdk4 can stimulate the Brca1 promoter in an E2F-dependent manner, and this is inhibited by coexpression of the p16INK4a cyclin-dependent kinase inhibitor. The human BRCA1 promoter also contains a conserved E2F site and is similarly regulated by E2F1 and Rb. This functional link between the BRCA1 and Rb tumor suppressors may provide insight into the mechanism by which BRCA1 inactivation contributes to cancer development.

The mechanism by which loss of BRCA1 function leads to breast and ovarian cancer is unclear. A general role for BRCA1 in cell growth control is suggested by BRCA1’s growth-regulated and ubiquitous expression pattern (1–4). Involvement of BRCA1 in DNA repair, replication, and transcriptional regulation have all been suggested. The BRCA1 protein associates with the RAD51 DNA repair factor as well as the BRCA2 and BARD1 proteins (5, 6). These proteins colocalize in nuclear foci, termed “dots,” that dissipate upon DNA damage and may reappear at replication structures containing PCNA (7). BRCA1 is also found in complexes containing RNA polymerase II and has a carboxyl-terminal acidic region that can function as a transcriptional activation domain (8–10). Several recent reports have demonstrated that BRCA1 physically associates with the p53 tumor suppressor protein and functions as a transcriptional coactivator for p53 (11, 12). BRCA1 can induce apoptosis and this activity is enhanced by coexpression of p53 (11, 13, 14). This correlates with the ability of BRCA1 to significantly augment transcriptional activation of the bax gene by p53 (11). BRCA1 can also induce apoptosis through the p53-independent stimulation of GADD45 expression and the activation of the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway (14).

Inactivation of the Rb tumor suppressor, through mutation of the RB1 gene or deregulation of cyclin-D-associated kinase activity, is a common event in many cancers (15). Recent data suggests that the resultant activation of E2F transcription factors contributes to tumor development (16–18). The Rb-E2F pathway regulates the expression of many genes whose products are required for DNA synthesis and cell cycle progression. Transcriptional activation of at least some of these genes, such as cyclin E, is associated with the loss of proliferation control as a consequence of Rb inactivation (19–21). In addition to unchecked proliferation, inactivation of Rb can also lead to p53-dependent and -independent apoptosis (22, 23). This also appears to involve deregulation of E2F-dependent transcription. At least one member of the E2F family, E2F1, can induce both p53-dependent and -independent apoptosis when overexpressed (24–28). It has been suggested that the ability of E2F1 to transcriptionally activate the p15ARF/p14ARF tumor suppressor gene may be involved in E2F1’s ability to induce apoptosis (25, 29). The p19ARF/p14ARF protein activates p53 by inhibiting the action of Mdm2 (30, 31). Although it is likely that E2F1 regulates the expression of additional pro-apoptotic genes, these targets remain to be identified.

As a model to study the role of deregulated E2F activity in cancer, we have developed a transgenic mouse model in which E2F1 expression is targeted to squamous epithelial tissue by a keratin 5 (K5) promoter. Increased E2F1 activity results in hyperplasia, hyperproliferation, and p53-dependent apoptosis in the epidermis of K5 E2F1 transgenic mice (16, 17). Depending on the experimental context, K5 E2F1 transgenic mice are found to be either predisposed or resistant to tumor development (16, 17, 32). To determine the molecular mechanisms underlying these effects, we analyzed alterations in gene expression in transgenic epidermis and primary keratinocytes. We found that the murine homologue of BRCA1 is up-regulated in K5 E2F1 transgenic cells and tissue. Analysis of the murine Brca1 promoter demonstrates that this is a direct effect and that cell cycle regulatory factors such as Rb, cyclin D1, and p16INK4a control Brca1 gene expression through modulation of E2F transcriptional activity. The human BRCA1 gene promoter is also found to be regulated by E2F1 and Rb.

EXPERIMENTAL PROCEDURES

Northern Blot Analysis—Primary keratinocytes were cultured from newborn K5 E2F1 transgenic and wild type sibling mice as described previously (16). Total RNA was isolated from keratinocytes using Tri Reagent (Molecular Research Center, Cincinnati, OH) per the manufacturer’s protocol, separated by gel electrophoresis, and transferred to
nylon membrane. Epidermal RNA was isolated from dorsal skin by submerging skin in DEPC-treated H2O at 55 °C for 30 s and transferring skin to DEPC-treated H2O at 4 °C for 30 s. Skin was then placed in Tri Reagent on ice, secured by a glass slide, and the epidermis was scraped off using a scalpel. Epidermis scrapes in Tri Reagent were transferred to eppendorf tubes and extracted per the manufacturer’s protocol. Epidermal RNA was poly(A)-selected using the Fast Track kit (Invitrogen) per manufacturer’s instructions. Murine cDNA probes were received from ATCC (c-myc and γ-actin), Dr. Joe Nevins (cdc2), and Dr. Susan Fischer (glyceraldehyde-3-phosphate dehydrogenase).

The Brca1 probe (nucleotides 5913–6179) was generated using reverse transcription-PCR and confirmed by sequencing.

**Electrophoretic Mobility Shift Assays (EMSA)—**Double-stranded oligonucleotides corresponding to wild type Brca1 promoter sequences or a version containing a mutated E2F binding site were generated and end-labeled for use as probe. The E2F binding site in the Brca1 promoter oligonucleotide is underlined and the mutated nucleotides are shown in bold: Brca1 wt, 5′-TCTATCTAAAAATTTCCGCCGCTTCCGCT-3′; Brca1 E2F−, 5′-TCTATCTAAAAATTTCCAAACGCTTCCGCT-3′.

Alternatively, an end-labeled, 100-bp pair DNA fragment derived from the adenosvirus E2 gene promoter was used as probe and unlabeled Brca1 oligonucleotides used as competitors. The E2F promoter contains two classical E2F sites (TATCCCAG) in inverse orientation 17 base pairs apart. NIH3T3 nuclear extract or recombinant E2F1/DP1 (containing equal amounts of GST-E2F1 and GST-DP1) were incubated with the radiolabeled probe in binding buffer (4 mM Tris-HCl, 12 mM Heps, pH 7.9, 60 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 12% glycerol) containing about 0.2 ng of the radiolabeled DNA fragment and 0.5 μg of salmon sperm DNA for 25 min at room temperature in a final volume of 20 μl. Samples were fractionated on a 4% polyacrylamide gel in 0.25 × TBE (0.0225 M Tris borate, 0.0065 M EDTA) at 4 °C, the gel was dried, and autoradiography was performed.

**Plasmids and Luciferase Reporter Assays—**Murine Brca1 promoter sequences from +6 to −1003 were isolated by PCR and cloned into pGL3 vector (Promega) to create Brca1-luc (wt). Brca1-luc (E2F−) was made by introducing mutations in the E2F sites at +6 to −74 with a double-stranded oligonucleotide containing appropriate base changes. Brca1-luc (+6 to −539) wild type and E2F− versions were made by removing Brca1 sequences from −539 to −1003 from Brca1-luc (+6 to −1003). The human Brca1 luciferase plasmid (−567) was a kind gift from Bernard Fuchscher (Arizona Cancer Center) (33). Cytomegalovirus expression vectors encoding E2F1, DP1, cyclin D1, Cdk4, Rb, and p16INK4a have been described (34, 35).

NIH3T3 and C3A cells were cultured in DMEM with 10% fetal bovine or calf serum and replated 24 prior to transfection. NIH3T3 cells were transfected using pfx-3 lipid (Invitrogen). The cells were starved of bovine or calf serum and replated 24 prior to transfection. NIH3T3 and C33A cells were cultured in DMEM with 10% fetal bovine serum; PCNA, proliferating cell nuclear antigen; wt, wild type.

**RESULTS**

**Brca1 Is Overexpressed in K5 E2F1 Transgenic Cells—**Previsously we demonstrated that the cyclin E gene is overexpressed approximately 6-fold in primary keratinocytes isolated from K5 E2F1 transgenic mice (17). In contrast, we find that several other genes previously identified as E2F targets, such as cdc2 and c-myc, are only minimally up-regulated in these transgenic cells (Fig. 1). The expression of several control genes, including γ-actin, is similar in primary keratinocytes isolated from either K5 E2F1 transgenic mice or wild type siblings. On the other hand, the murine homologue of the Brca1 tumor suppressor gene, Brca1, is expressed in K5 E2F1 transgenic keratinocytes at a level at least 4-fold over that found in nontransgenic control epidermis after correction using glyceraldehyde-3-phosphate dehydrogenase expression as a control (data not shown).

**The Brca1 Gene Promoter Contains an E2F DNA-binding Site—**Previous studies have demonstrated that Brca1 gene expression is highest in tissues containing rapidly proliferating cells such as the testes, thymus, and epidermis (1, 4). The transcription factors responsible for this proliferation-associated expression of the Brca1 gene have not been identified. Examination of the Brca1 gene promoter (36) identified two potential E2F binding sites immediately upstream of exon 1 (ATTTCCCGC at −27 and TTTCGCCGC at −62) that closely match the consensus sequence identified as optimal for E2F1 binding (37). To determine whether E2F transcription factors could bind the Brca1 promoter, an EMSA was performed using recombinant E2F1 and DP1 proteins and a double-stranded oligonucleotide corresponding to the Brca1 promoter region containing the putative proximal E2F site as probe. Recombinant E2F1/DP1 was found to bind the probe containing the wild type Brca1 promoter sequences but not a similar probe containing a mutated E2F site (Fig. 2A). An EMSA was also performed using nuclear extract from NIH3T3 cells and a fragment from the adenosvirus E2 gene promoter. In this experiment the double-stranded oligonucleotide containing the wild type Brca1 promoter sequence was found to efficiently compete for cellular E2F DNA-binding complexes, while the Brca1 oligonucleotide containing the mutant E2F site did not compete (Fig. 2B).

**Regulation of the Brca1 Gene Promoter by the Rb-E2F Pathway—**To examine transcriptional regulation of the Brca1 gene by E2F, we isolated sequences 5′ to exon 1 of the Brca1 gene from mouse genomic DNA using PCR and cloned the Brca1 promoter region upstream of a luciferase reporter gene. In serum-starved NIH3T3 cells, overexpression of E2F1, in conjunction with its heterodimerization partner DP1, transcriptionally activated the Brca1 promoter and this response was lost when the E2F binding sites were mutated (Fig. 3A). The

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1 The abbreviations used are: DEPC, diethyl pyrocarbonate; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; PCNA, proliferating cell nuclear antigen; wt, wild type.
activity of the mutant Brca1 promoter was approximately 5-fold higher than the wild type promoter in these Rb-positive cells forced into quiescence by serum withdrawal, indicating that the E2F sites can function as negative regulatory elements. A similar type of regulation has been shown for several other E2F target genes and is due to the binding of E2F repressors complexes containing Rb or Rb family members (34, 35, 38–40).

The repressive effect of Rb on the activity of the Brca1 promoter could be demonstrated more directly in the Rb-deficient cell line C33A. Brca1 promoter activity was significantly repressed by expression of wild type Rb but not by expression of a Rb mutant defective in E2F binding (Fig. 3B). Mutation of the E2F binding sites in the Brca1 promoter abolished repression by Rb.

Regulators of Rb activity were also found to modulate expression of the Brca1 promoter through the E2F sites. Cotransfection of expression plasmids encoding cyclin D1 and Cdk4 caused a derepression of the wild type Brca1 promoter in quiescent NIH3T3 cells but had only a modest effect on the E2F site mutant promoter (Fig. 3C). Coexpression of the cyclin-dependent kinase inhibitor p16INK4a blocked the ability of cyclin D1/Cdk4 to stimulate Brca1 promoter activity (Fig. 3D).

**Fig. 2. E2F complexes bind the Brca1 gene promoter. A**, an EMSA was performed using recombinant E2F1/DP1 protein (lanes 2, 3, 5, and 6) and end-labeled double-stranded oligonucleotides (17 fmol) corresponding to the Brca1 promoter region (–21 to –43) containing either wild type sequence (Brca1 wt, lanes 1–3) or a mutation in the putative E2F site (Brca1 E2F mut, lanes 4–6). B, an EMSA was performed using the adenovirus E2 gene promoter as probe and NIH3T3 nuclear extract (lanes 2–5). Unlabeled double-stranded oligonucleotides (10 and 100 ng) containing either wild type Brca1 promoter sequences (wt, lanes 3 and 4) or a version containing a mutation in the putative E2F site (mut, lanes 5 and 6) were used as competitors.

**Fig. 3. Regulation of the Brca1 promoter by the p16-cyclin D-Rb-E2F pathway. A**, 2 μg of Brca1-luc (+6 to –1003) wt or E2F– was transfected into NIH3T3 cells using pfx-3 reagent (Invitrogen) either alone or with 20 or 200 ng of expression plasmids encoding E2F1 and DP1 where indicated. Cells were incubated in medium containing 0.3% FBS for 48 h before harvesting and performing luciferase assays. The average luciferase activity from triplicate plates is presented. B, triplicate plates of C33A cells were transfected using the calcium phosphate method with 5 μg of either Brca1-luc (+6 to –539) wt or E2F– mutant plasmid and 10 μg of expression vector encoding wild type or mutant (exon 22 deletion) Rb where indicated. Cells were incubated in medium containing 10% FBS for 48 h, harvested, and luciferase assays were performed. C, triplicate plates of NIH3T3 cells were transfected with 2 μg of Brca1-luc (+6 to –1003) wild type or E2F– mutant plasmid either alone or with expression plasmids encoding cyclin D1 (1.5 μg) and Cdk4 (200 ng). Cells were incubated in medium containing 0.3% FBS 48 h prior to harvesting extract for luciferase assays. D, transfections were performed as in C using 2 μg of Brca1-luc (+6 to –1003) wild type and either vector alone or cyclin D1 (1 μg) and Cdk4 (200 ng) with or without an expression plasmid encoding p16 (800 ng) as indicated.

The data presented establish the regulation of the human BRCA1 gene by the Rb-E2F pathway, a BRCA1 promoter-luciferase plasmid (33) was used in similar cotransfection experiments as presented in Fig. 3. The human BRCA1 promoter was transactivated in a dose-dependent manner by coexpression of E2F1 and DP1 much like the murine Brca1 promoter (Fig. 4B). At the highest concentration of E2F1 expression vector, BRCA1 promoter activity was induced 5-fold in these quiescent cells. Moreover, BRCA1 promoter activity was repressed 15-fold by expression of wild type Rb, but not by a mutated Rb unable to bind E2F transcription factors, in the Rb-negative C33A cell line. These findings demonstrate that the Rb-E2F pathway also regulates the human BRCA1 gene.

**DISCUSSION**

The data presented establish the Brca1/BRCA1 gene as a target for E2F-dependent transcriptional regulation. The Brca1 promoter behaves very similarly to several other E2F-regulated promoters such as B-myb and E2F1 (34, 35, 38, 39). In quiescent cells, E2F is found in association with Rb and Rb-related proteins and these complexes function as active repressors of transcription (40, 42–45). This is likely why the wild type Brca1 promoter has lower activity than the E2F site mutant promoter in serum-starved NIH3T3 cells but not in C33A tumor cells that lack Rb. This model is supported by the finding that expression of cyclin D1/Cdk4, which phosphoryl-
E2F Links the Rb and BRCA1 Tumor Suppressors

A

Consensus E2F1 binding site: G C G G G A A A

Mouse: (−37) A G C G G G A A T T T A G A T A (−19)

Human: (−23) A G C G G G A A T T A C A G A T A (−5)

B

C

Fig. 4. Regulation of the human BRCA1 promoter by E2F1 and Rb.

A, a conserved E2F1 binding site is shared between the murine Brca1 and human BRCA1 promoters. B, BRCA1-luc (2 µg) and cytomegalovirus β-galactosidase (1 µg) were transfected into NIH3T3 cells using pfx-3 reagent with increasing amounts of expression plasmids (0, 20, 100, or 200 ng) encoding E2F1 and DP1. Cells were incubated in medium containing 0.3% calf serum for 48 h before harvesting and performing luciferase assays. The average luciferase activity after correcting for β-galactosidase activity from triplicate plates is presented. C, triplicate plates of C3A cells were transfected with BRCA1-luc (5 µg) and 10 µg of expression plasmid encoding wild type Rb or mutant Rb where indicated. Cells were incubated in medium containing 10% calf serum for 48 h, harvested, and luciferase assays performed.

downstream targets of p53, as well as other genes involved in apoptosis (49), in agreement with this suggestion. If this is the case, then BRCA1 would be an additional effector, along with p19ARF and perhaps others, of the apoptotic response that occurs in cells that have lost Rb function. Loss of BRCA1 function, as occurs in familial breast cancer, may therefore increase the survival of cells that have acquired mutations resulting in Rb inactivation. This role for BRCA1 does not rule out additional functions for BRCA1, such as a role in the cellular response to DNA damage. In fact, it is quite possible that BRCA1 is induced and augments p53 and JNK/SAPK activities in response to both cell cycle deregulation and DNA damage and that both functions are important for BRCA1’s tumor suppressive activity.

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