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PRINCIPAL INVESTIGATOR: James L. O'Kelly, Ph.D.

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
Los Angeles, California 90048

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6. AUTHOR(S)
James L. O'Kelly, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cedars-Sinai Medical Center
Los Angeles, California 90048

E-Mail: okellyj@cshs.org

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13. ABSTRACT (Maximum 200 Words)

Women who have familial breast cancer often have a germline mutation of the breast cancer susceptibility gene known as BRCA1. The function of BRCA1 is not totally understood. BRCA1 has a number of activities including DNA repair, growth inhibition, and as a transcription factor. The $p27^{kip1}$ is a member of the universal cyclin-dependent kinase inhibitor family. In this study we have shown that BRCA1 can transcriptionally activate the $p27^{Kip1}$ promoter. This transactivation is dependent on the presence of a functional C-terminal domain. The BRCA1-responsive element was defined from position -545 to -511. We next determined that within this region is also a potential binding site for the transcription factor FOXA1. In transient transfection reporter assays, FOXA1 could activate the $p27^{Kip1}$ promoter. Co-transfection of BRCA1 and FOXA1 resulted in a synergistic activation of the $p27^{Kip1}$ promoter. Mutation of the FOXA1 DNA binding site in the $p27^{Kip1}$ promoter-luciferase construct significantly diminished the activity of FOXA1 alone or in combination with BRCA1. EMSA analysis demonstrated that FOXA1 could bind to the p27kip1 promoter, but this binding was lost upon mutation of the FOXA1 binding site. Co-immunoprecipitation experiments indicated that FOXA1 and BRCA1 proteins interacted *in vivo*.

14. SUBJECT TERMS
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Annual Report
May 1, 2003 – April 30, 2004
Interaction of BRCA1 and p27^{kip1} pathway in breast cancer
***PI**: James O'Kelly*

Award Number: DAMD17-02-1-0337

Summary: Families with inherited breast and ovarian cancers frequently have mutations of the breast cancer susceptibility gene BRCA1. The BRCA1 gene encodes a 220 kDa nuclear protein whose precise biochemical function remains unclear, although multiple functions have been suggested. These include DNA repair, growth inhibition and as a transcription factor. Cell cycle progression is governed by a family of cyclin-dependent kinases (CDKs), whose activity is regulated by phosphorylation, activated by cyclin binding and inhibited by various inhibitors (CDKIs), such as p21$^{Waf1/Cip1}$ and p27^{Kip1}. It has been shown that p27^{Kip1} protein decreases during tumor development and progression in breast, colon, prostate and ovarian cancers. Previously we observed a correlation between the expression of BRCA1 and p27^{Kip1} in a series of breast cancer cell lines. In this study, we have analyzed the p27^{Kip1} promoter in order to determine if this CDKI is transcriptionally activated by BRCA1 and to elucidate the elements important for this activity. We determined that the BRCA1-responsive element of the p27^{Kip1} promoter was localized to a 35 bp region at positions -545 to -511. BRCA1 has been shown to interact with a wide variety of proteins but we were unable to show that any of these were involved with BRCA1 and the regulation of p27^{Kip1}. Therefore, we further analyzed the DNA sequence of the 35 bp BRCA1-responsive element of the p27^{Kip1} promoter. We identified a FOXA1 binding site within the BRCA1-responsive element of the p27Kip1 promoter and showed that FOXA1 activated the promoter alone and in conjunction with BRCA1 and that these two proteins interacted *in vivo*.

Results:

BRCA1 can transactivate expression of p27 ^{Kip1}: Using transient transfections we examined the effect of BRCA1 on both mouse and human p27^{Kip1} promoter reporter gene expression in Cos, MCF-7 breast cancer and HCT116 colon cancer cells (Figure 1a, b). pCR3-BRCA1 activated the mouse p27^{Kip1} promoter by 10-fold in Cos cells and 5-fold in MCF-7 and HCT116 cells (Figure 1b), as compared to the pCR3 vector. The specificity of p27Kip1 induction by BRCA1 was determined using various synthetic and tumor associated BRCA1 mutants. Four different tumor-associated BRCA1 Mutants and BRCA1 mutant (del-500-1863) were unable to significantly transactivate the p27Kip1 promoter reporter compared to wild-type BRCA1 (Figure 2a, b). However, a second mutant lacking only the RAD51-interacting domain but with a functional nuclear localization signal and C-terminal transactivation domain, BRCA1 (del 515-1091), was able to transactivate the p27 promoter nearly as efficiently as wild type BRCA1 (Figure 2b).

Identification of the BRCA1 response element in the promoter region of p27^{kip1}. Experiments were carried out using deletion mutants of the mouse p27^{Kip1} promoter. Deletion up to position -774 increased the activity of BRCA1 on the p27^{Kip1} promoter reporter constructs (Figure 4a), which is consistent with a previous report showing that the mouse p27^{Kip1} promoter contains negative regulatory elements in the region -1609 to -925. Further deletion to -615 did not significantly decrease the response of the p27^{Kip1} promoter to BRCA1 (Figure 4a). However, BRCA1 responsiveness was lost by deletions up to position -511. These results suggested that a putative BRCA1-responsive element was located between positions -615 and -511 of the p27^{Kip1} promoter. To determine whether BRCA1 binds directly to the p27^{Kip1} promoter we performed EMSA analysis using oligonucleotides spanning the region of the mouse promoter containing the putative BRCA1-responsive element. Nuclear extract from MCF7 produced a slowly migrating band with oligo C but did not show any significant binding to oligo A or oligo B (Figure 4b). The slowly migrating complex observed with MCF7 nuclear extract and oligo C could be competed by a 10-fold excess of cold oligo C and an antibody against BRCA1 resulted in a supershifting of the complex (Figure 4b). Therefore it appears that the BRCA1-responsive element is located at position -545 to -511 of the mouse p27^{Kip1} promoter, which corresponds to -714 to -680 of the human p27^{Kip1} promoter.

$p27^{Kip1}$ is regulated by breast cancer susceptibility gene 2 (BRCA2): We performed deletion analysis of the $p27^{Kip1}$ promoter in transient transfection assays suggests that BRCA2-responsiveness is in the region –988 to –925 (Figure 5). We are currently making constructs to delete this region from the full-length promoter. Possible further experiments are gel shift/EMSA analysis to demonstrate BRCA2 binding and identification of other co-factors which may be involved with BRCA2.

FOXA1 activates the $p27^{Kip1}$ promoter: Analysis using a number of transcription factor data bases suggested that the region -544 to -536 corresponded to a binding site for the transcription factor FOXA1, a member of the forkhead family of transcription factors. BRCA1 alone activated the $p27^{Kip1}$ promoter 12-14-fold (Figure 6A), FOXA1 alone activated the $p27^{Kip1}$ promoter up to 75-fold and a combination of FOXA1 and BRCA1 appeared to be synergistic in activating the $p27^{Kip1}$ promoter (Figure 6A). Mutation of the potential FOXA1 DNA-binding site in the $p27^{Kip1}$ promoter decreased the activation by FOXA1 either alone or in combination with BRCA1 (Figure 6B). Therefore, these results suggest that FOXA1 can strongly activate the $p27^{Kip1}$ promoter, and this activity is increased dramatically in the presence of BRCA1. Only wild-type BRCA1 and the transcriptionally active mutant, BRCA1del515-1091, were able to synergize with FOXA1 on the $p27^{Kip1}$ promoter (Figure 6C).

FOXA1 binds to the $p27^{Kip1}$ promoter: In order to show that FOXA1 can bind the $p27^{Kip1}$ promoter, we carried out EMSA analysis . We observed a protein-DNA complex in MCF-7 extracts that we had previously determined expressed FOXA1 protein (Figure 7) Confirmation that FOXA1 was present in this complex came when a FOXA1 antibody was included in the binding reaction, causing a supershift of the protein-DNA complex (Figure 7, lane 11). Therefore, these results show that FOXA1 is capable of binding an element in the $p27^{Kip1}$ promoter and directly activating this promoter.

FOXA1 protein expression is enhanced by co-expression with BRCA1: Analysis of the nuclear extracts from transiently transfected cells showed that when FOXA1 was co-transfected with BRCA1, greater expression of FOXA1 protein occurred compared to cells transfected with FOXA1 alone (Figure 8a, b). We determined that in cells transiently transfected with FOXA1 alone, the half-life of the FOXA1 protein was approximately 8 hours (Figure 8a). By comparison, the half-life of the FOXA1 protein co-transfected with BRCA1 was greater than 24 hours (Figure 8b). Therefore this result suggests that expression of BRCA1 may indeed stabilize FOXA1 protein.

9: FOXA1 and BRCA1 proteins interact *in vivo*. Since the data suggested that BRCA1 could stabilize FOXA1 protein, we hypothesized that this was due to a physical interaction between these two proteins *in vivo*. Immunoprecipitation of nuclear lysates with a BRCA1 antibody and subsequent probing with an antibody against FOXA1 indicated that *in vivo* BRCA1 and FOXA1 were in a protein complex together in these two breast cancer cell lines (Figure 9).

Conclusions: Recent studies have shown that decreased expression of p27Kip1 correlates either with both the presence of a BRCA1 mutation in breast tumor tissue as well as with BRCA1 promoter methylation. Thus, loss of functional BRCA1 might be expected to result in impaired growth inhibition due to ineffective regulation of p27Kip1. Therefore, understanding the mechanisms controlling p27Kip1 expression in breast tumors may provide new strategies to inhibit tumor growth. We have identified FOXA1 as a binding partner for BRCA1, that both can regulate the expression of $p27^{Kip1}$ alone or in concert with BRCA1 and that BRCA1 stabilizes FOXA1 protein.

Reportable Outcomes: BRCA1 Transactivates the cyclin dependent kinase inhibitor $p27^{kip1}$. Oncogene (2002) 21, 3199-3206.

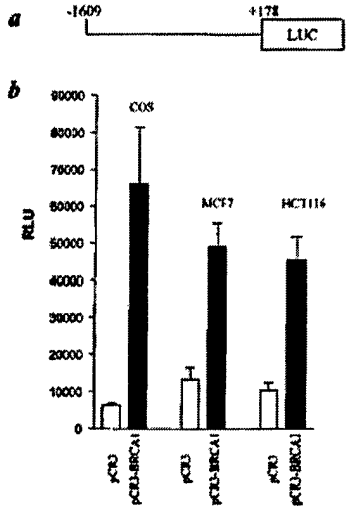

Figure 1 BRCA1 transactivates the mouse $p27^{Kip1}$ promoter. (a) Schematic representation of the $p27^{Kip1}$ promoter luciferase promoter. The 3' boundary is 178 bp downstream of the translation start site. (b) COS, MCF7 and HCT116 were co-transfected with p27ptr-luc and either pCR3 or pCR3-BRCA1 and luciferase activity was measured 48 h later. These results represent three independent experiments. Results are shown as RLU which is the ratio of the luciferase units observed for the promoter construct compared to that seen for the pRL-SV40

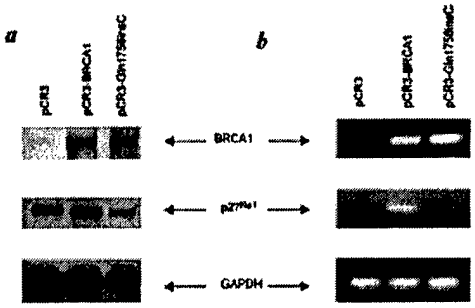

Figure 3 BRCA1 transcriptionally regulates $p27^{Kip1}$ expression. (a) HCT116 colon cancer cells were transfected with pCR3, pCR3-BRCA1 or pCR3-BRCA1(Gln1756insC). Proteins were harvested 48 h post-transfection and analysed by SDS PAGE and Western blot. Expression of endogenous $p27^{Kip1}$ protein in up-regulated only in the presence of wild-type BRCA1. Equal protein loading is demonstrated by re-probing the blot with an antibody against GAPDH. (b) HCT116 cells were transfected as in (a) and RNA harvested by Trizol 48 h post-transfection. Reverse transcription-PCR demonstrated that both wild-type and mutant BRCA1 were being expressed. Expression of $p27^{Kip1}$ was observed only in the cells transfected with wild-type BRCA1. Equality between samples was shown by using primers for GAPDH

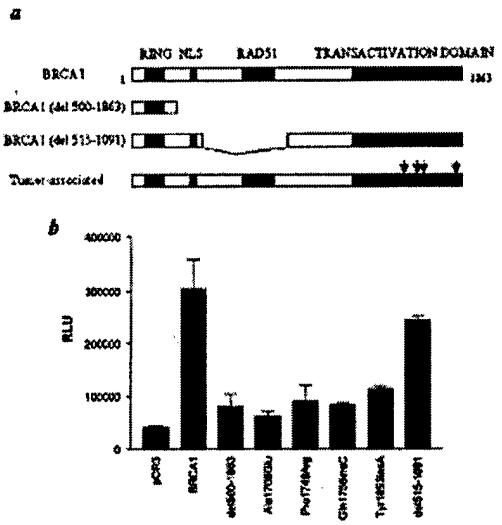

Figure 2 BRCA1 mutants lacking the C-terminal transactivation domain are defective for activation of $p27^{Kip1}$. (a) Schematic representation of the BRCA1 mutants indicating the important domains for BRCA1 function. The arrows indicate the position of the C-terminal mutations of the BRCA1 gene. (b) COS cells were co-transfected with p27ptr-luc and pCR3 or either wild-type or mutant pCR3-BRCA1 expression plasmids as indicated. Luciferase activity was measured 48 h post-transfection as in Figure 1. These results represent two independent experiments

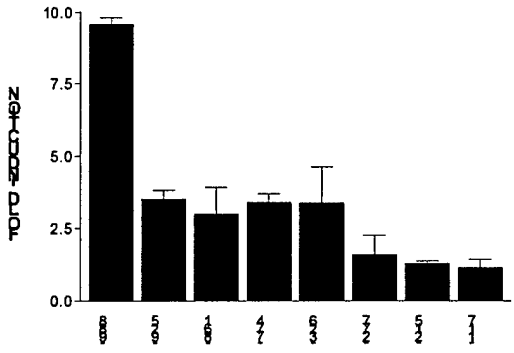

Figure 5. Deletion analysis of the $p27^{Kip1}$ promoter. HCT116 cells were transfected with p27ptr-luc and pCR3 with either wild-type of mutant pCR3-BRCA2 expression plasmids as indicated. Luciferase activity was measured 48 h post-transfection. BRCA2 responsiveness is in the region –988 to -925

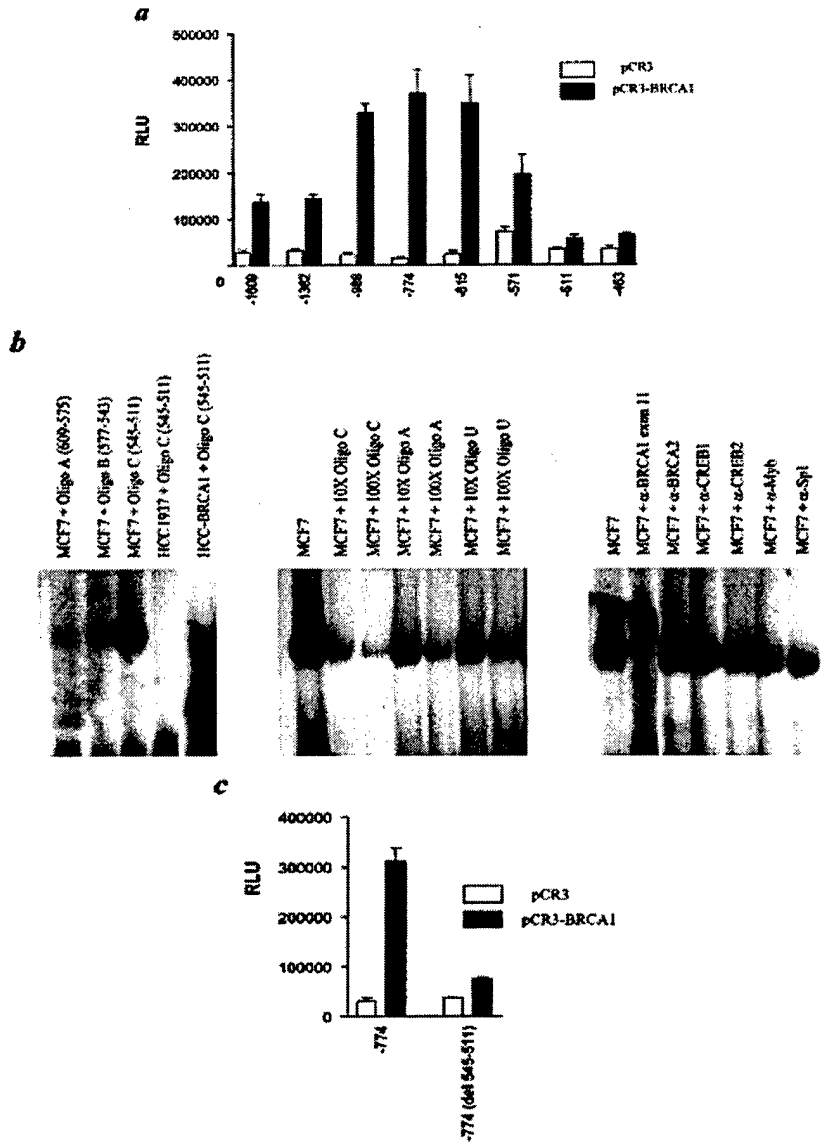

Figure 4 Region of mouse $p27^{Kip1}$ promoter containing the putative BRCA1-responsive element identified by 5′ deletion mapping. **(a)** COS cells were co-transfected with the 5′-deletion $p27^{Kip1}$ promoter reporter constructs and either pCR3 or pCR3-BRCA1, and luciferase activity was measured 48 h later. These results represent three independent experiments. **(b)** Nuclear lysate from MCF7 was incubated with oligo A (−609 to −575), oligo B (−577 to −543) and oligo C (−545 to −511) of the $p27^{Kip1}$ promoter. Nuclear lysate from HCC1937 and HCC-BRCA1 was incubated with oligo C. Cold competition was carried out with an excess of oligo C, oligo A or oligo U (an unrelated 35 bp sequence). The complex is supershifted by an antibody against exon 11 of BRCA1. **(c)** MCF7 cells were transfected with a 5′-deletion $p27^{Kip1}$ promoter construct (−774) or with the construct minus the putative BRCA1-responsive element (−774 (del 545-511)). Luciferase activity was measured as in Figure 1. These results represent three independent experiments

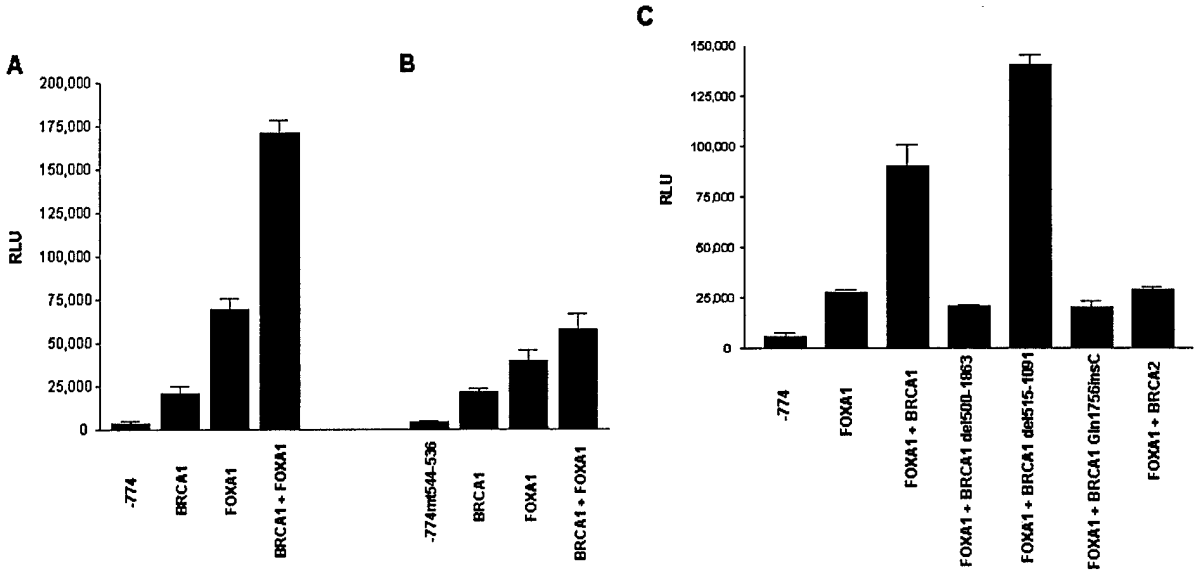

Figure 6. FOXA1 and BRCA1 together activate the $p27^{Kip1}$ promoter-luciferase reporter construct. (A) HCT116 cells were transiently transfected with either FOXA1 and/or BRCA1. The $p27^{Kip1}$-774 promoter-luciferase construct was used in these assays since this construct was previously shown to have the greatest induction of activity by BRCA1. Lysates were harvested 48 hours post-transfection. The results shown here represent the results from 5 independent experiments. (B) The potential FOXA1 binding site in the $p27^{Kip1}$ promoter was mutated and the transient transfection reporter assays were repeated as described above. These results represent 5 independent experiments. (C) Transient transfection reporter assays were repeated using FOXA1 in combination with wild type and mutant BRCA1 and BRCA2 for activation of the $p27^{Kip1}$ promoter.

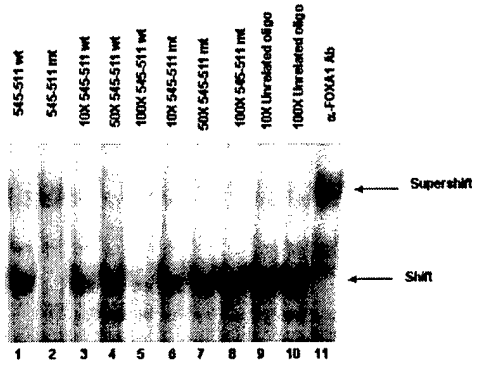

Figure 7. FOXA1 protein binds to the potential DNA binding site in the $p27^{Kip1}$ promoter. EMSA analysis using nuclear protein extracts from the MCF7 breast cancer cell line and the following oligonucleotides: $p27^{Kip1}$ promoter 545-511 wild-type, $p27^{Kip1}$ promoter 545-511 mutant (544-536), and an unrelated DNA sequence. The mutation of the FOXA1 site in the oligonucleotide was identical to the mutation made of this site in the $p27^{Kip1}$ promoter-luciferase reporter construct. The protein-DNA complex observed with the MCF7 nuclear protein extracts could be supershifted by the inclusion of an antibody directed against FOXA1 in the binding reaction.

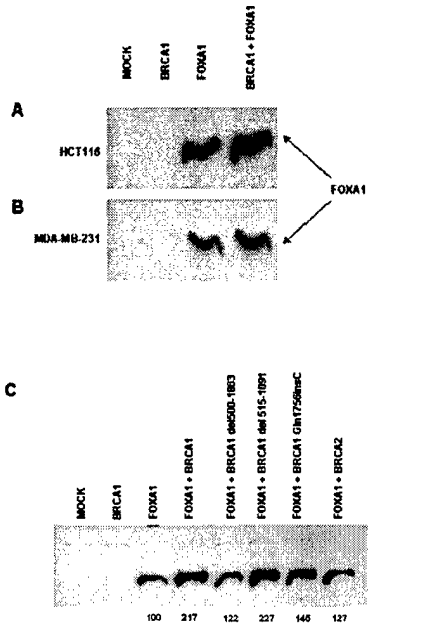

Figure 8. Co-transfection of wild type BRCA1 increases the expression of FOXA1 protein. Nuclear extracts were prepared from (A) HCT116 and (B) MDA-MB-231 transiently transfected with either FOXA1 and/or BRCA1. The extracts were immunoblotted for FOXA1 protein. (C) Nuclear extracts from HCT116 transiently co-transfected with FOXA1 and either wild-type or mutant BRCA1. Equal loading of the nuclear extracts was visualized by Ponceau S staining of the membrane prior to immunoblotting.

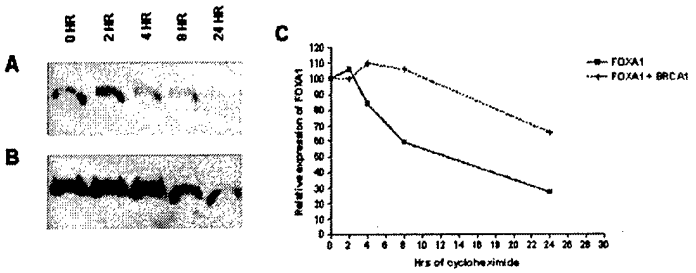

Figure 9. Co-transfection of BRCA1 with FOXA1 increases the half-life of FOXA1 protein. HCT116 cells were transiently transfected with FOXA1 in the absence (A) and presence (B) of BRCA1. These cells were treated with cycloheximide (10 µg/ml) and cells harvested for nuclear protein extraction at time points up to 24 hours. Nuclear extracts were separated by SDS-PAGE and immunoblotted for FOXA1 protein. (C) Ratio of protein concentration and densitometry from the Western blot determined the relative amounts of FOXA1 protein in the samples during the treatment with cycloheximide.

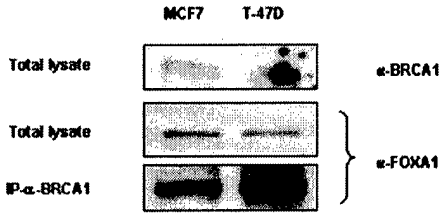

Figure 10. BRCA1 and FOXA1 occur together in a protein complex *in vivo*. Nuclear extracts were prepared from MCF7 and T-47D breast cancer cell lines, immunoprecipitated with an antibody against BRCA1, and the resulting Western blot probed with an antibody against FOXA1. Total nuclear lysate was also probed for the expression of these two proteins. FOXA1 protein is observed in the BRCA1-immunoprecipitated samples.

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BRCA1 transactivates the cyclin-dependent kinase inhibitor $p27^{Kip1}$

Elizabeth A Williamson*,[1], Farnaz Dadmanesh[2] and H Phillip Koeffler[1]

[1]*Department of Medicine, Cedars-Sinai Medical Center, UCLA School of Medicine, 8700 Beverly Blvd., Los Angeles, California, CA 90048, USA;* [2]*Department of Anatomic Pathology, Cedars-Sinai Medical Center, UCLA School of Medicine, 8700 Beverly Blvd., Los Angeles, California, CA 90048, USA*

The $p27^{Kip1}$ is a member of the universal cyclin-dependent kinase inhibitor family. Previously, immunochemical analysis of a series of breast cancer cell lines demonstrated a correlation between the expression of $p27^{Kip1}$ and the breast cancer susceptibility gene BRCA1. BRCA1 has a number of activities including DNA repair, growth inhibition and as a transcription factor. Here we demonstrate that BRCA1 transactivates expression of $p27^{Kip1}$. This transactivation is dependent on the presence of a functional C-terminal transactivation domain. Promoter-deletion analysis identified the presence of a putative BRCA1-responsive element located at position −615 to −511 of the $p27^{Kip1}$ promoter. These results suggest that the transcriptional regulation of $p27^{Kip1}$ by BRCA1 may be a mechanism for BRCA1- induced growth inhibition.
Oncogene (2002) **21**, 3199–3206. DOI: 10.1038/sj/onc/1205461

Keywords: BRCA1; $p27^{Kip1}$; transcriptional regulation

Introduction

Families with inherited breast and ovarian cancers frequently have mutations of the breast cancer susceptibility gene BRCA1 (Futreal *et al.*, 1994). The BRCA1 gene encodes a 220 kDa nuclear protein whose precise biochemical function remains unclear, although multiple functions have been suggested. These include DNA repair, growth inhibition and as a transcription factor (Aprelikova *et al.*, 1999; Chapman and Verma, 1996; Chen *et al.*, 1999; Haile and Parvin, 1999). BRCA1 does not share any significant homology to any known proteins. However it does contain several well-defined functional domains: an N-terminal RING finger domain important for protein–protein interactions, including BARD1 and ATF1 (Houvras *et al.*, 2000; Wu *et al.*, 1996); a domain in the middle of BRCA1 associates with the DNA repair protein RAD51 (Scully *et al.*, 1997); and the C-terminal contains two repeats of the BRCT domains. These BRCT domains appear to be involved in many of the functions ascribed to BRCA1.

Various studies have suggested a role for BRCA1 in the transcriptional activation of specific genes. Overexpression of wild-type BRCA1, but not tumor-derived mutants, results in a G1 cell cycle arrest mediated via the transcriptional activation of $p21^{Waf1/Cip1}$ in a p53-independent manner (Somasundaram *et al.*, 1997). However, BRCA1 has also been shown to co-activate the transcription of p53-regulated genes (Jin *et al.*, 2000; Ouchi *et al.*, 1998; Zhang *et al.*, 1998). These studies demonstrated that the C-terminus of BRCA1 is required for its function as a transcriptional activator. Two transactivation domains have been identified in the C-terminus; one localized to amino acids 1560–1863 including the BRCT domain; and more recently, a second transactivation domain was mapped to amino acids 1293–1558 (Chapman and Verma, 1996; Hu *et al.*, 2000). Most cancer-predisposing mutations of BRCA1 results in gross truncation of the protein, thus disrupting the C-terminal transactivation domain and compromising this function of BRCA1.

Although BRCA1 has been shown to inhibit cell cycle progression via activation of $p21^{Waf1/Cip1}$, this may not be the only mechanism growth inhibition by BRCA1.

Cell cycle progression is governed by a family of cyclin-dependent kinases, whose activity is regulated by phosphorylation, activated by cyclin binding and inhibited by various inhibitors, such as $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (Sherr, 1994; Sherr and Roberts, 1999). The $p27^{Kip1}$ was demonstrated to bind to cyclin E-ckd2 complexes and inhibit the kinase function of cdk2 (Polyak *et al.*, 1994). A number of other functions have been suggested for $p27^{Kip1}$ including as a promoter of apoptosis, as a regulator of drug resistance in solid tumors and having a role in cell differentiation (Katayose *et al.*, 1997; St. Croix *et al.*, 1996; Durana *et al.*, 1997; Onishi and Hruska, 1997). A number of studies have also examined $p27^{Kip1}$ expression in a series of tumors to determine if there is any diagnostic or prognostic significance. It has been shown that $p27^{Kip1}$ protein decreases during tumor development and progression in breast, colon, prostate and ovarian cancers (Catzevalos *et al.*, 1997; Ciaparrone *et al.*, 1998; Cordon-Cardo *et al.*, 1998; Masciullo *et al.*,

*Correspondence: EA Williamson, Department of Medicine-Hematology/Oncology, Davis Research Building, Room 5016, Cedars-Sinai Medical Center, 8700 Beverly Blvd., Los Angeles, CA 90048, USA; E-mail: williamsonc@cshs.org
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1999). The demonstration that even a heterozygous reduction of $p27^{Kip1}$ leads to spontaneous and radiation-induced tumors in mice further suggests that $p27^{Kip1}$ may play an important role in neoplastic progression (Fero *et al.*, 1998).

Previously we observed a correlation between the expression of BRCA1 and $p27^{Kip1}$ in a series of breast cancer cell lines (Elstner *et al.*, 2002). In this study, we have analysed the $p27^{Kip1}$ promoter in order to determine if this cyclin-dependent kinase inhibitor (CDKI) is transcriptionally activated by BRCA1 and the elements important for this activity.

Results

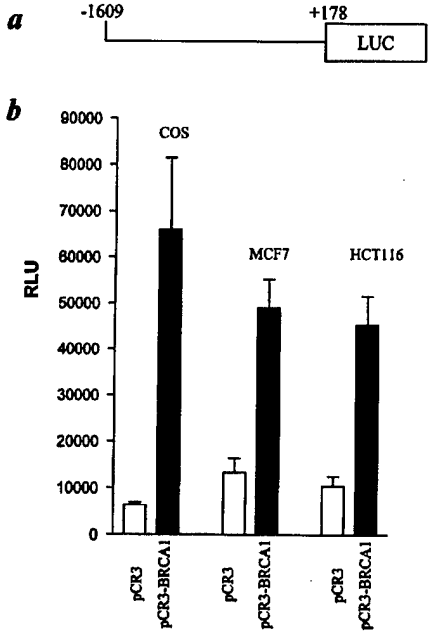
Transcriptional activation of the $p27^{Kip1}$ promoter

By transient transfections we examined the effect of BRCA1 on both mouse and human $p27^{Kip1}$ promoter reporter gene expression in COS, MCF7 breast cancer and HCT116 colon cancer cells (Figure 1a,b). pCR3-BRCA1 activated the mouse $p27^{Kip1}$ promoter by 10-fold in COS cells and fivefold in MCF7 and HCT116 cells (Figure 1b), as compared to the pCR3 vector. Similar fold activation was also observed for the human $p27^{Kip1}$ promoter in these cell lines (data not shown).

BRCA1 is frequently mutated in hereditary breast and ovarian cancer. The consequence of these mutations is the generation of a truncated protein which is either non-functional or unstable. Therefore to investigate further the specificity of $p27^{Kip1}$ induction by BRCA1 we studied the effect of various synthetic and tumor-associated BRCA1 mutants on $p27^{Kip1}$ promoter reporter gene expression (Figure 2a). In these reporter assays four different tumor-associated BRCA1 mutants and a synthetic BRCA1 mutant (del 500–1863) were unable significantly to transactivate the $p27^{Kip1}$ promoter reporter compared to the wild-type BRCA1 (Figure 2b). However, a second synthetic mutant lacking only the RAD51-interacting domain but with a functional nuclear localization signal and C-terminal transactivation domain, BRCA1 (del 515–1091), was able to transactivate the $p27^{Kip1}$ promoter nearly as efficiently as the wild-type BRCA1 (Figure 2b).

We next investigated the possible mechanism for regulation of $p27^{Kip1}$ by BRCA1. It has been shown in other studies that the regulation of expression of $p27^{Kip1}$ is primarily via post-translational mechanisms.

Western immunoblot analysis of HCT116 cells demonstrated that endogenous $p27^{Kip1}$ protein expression was up-regulated by wild-type BRCA1 but not by either the control vector pCR3 or a mutated BRCA1 (Gln1756insC) (Figure 3a). Wild-type and mutant forms of BRCA1 were expressed at similar levels



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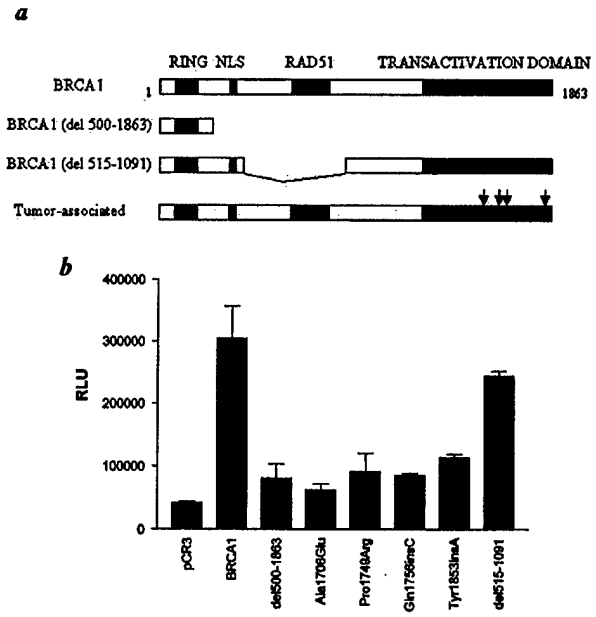

Figure 2 BRCA1 mutants lacking the C-terminal transactivation domain are defective for activation of $p27^{Kip1}$. (**a**) Schematic representation of the BRCA1 mutants indicating the important domains for BRCA1 function. The arrows indicate the position of the C-terminal mutations of the BRCA1 gene. (**b**) COS cells were co-transfected with p27ptr-luc and pCR3 or either wild-type or mutant pCR3-BRCA1 expression plasmids as indicated. Luciferase activity was measured 48 h post-transfection as in Figure 1. These results represent two independent experiments

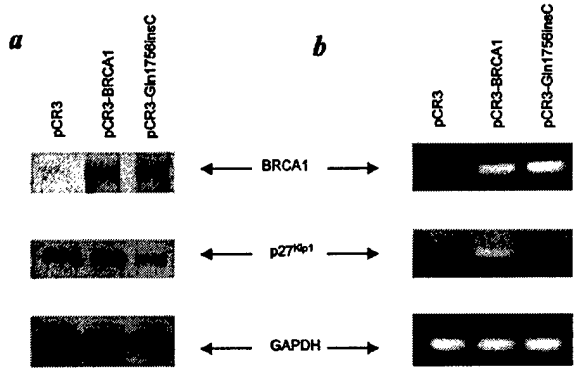

Figure 3 BRCA1 transcriptionally regulates $p27^{Kip1}$ expression. (**a**) HCT116 colon cancer cells were transfected with pCR3, pCR3-BRCA1 or pCR3-BRCA1(Gln1756insC). Proteins were harvested 48 h post-transfection and analysed by SDS–PAGE and Western blot. Expression of endogenous $p27^{Kip1}$ protein in up-regulated only in the presence of wild-type BRCA1. Equal protein loading is demonstrated by re-probing the blot with an antibody against GAPDH. (**b**) HCT116 cells were transfected as in (**a**) and RNA harvested by Trizol 48 h post-transfection. Reverse transcription-PCR demonstrated that both wild-type and mutant BRCA1 were being expressed. Expression of $p27^{Kip1}$ was observed only in the cells transfected with wild-type BRCA1. Equality between samples was shown by using primers for GAPDH

(Figure 3a). Thus BRCA1 does up-regulate $p27^{Kip1}$ protein expression.

To determine the mechanism for this up-regulation (transcriptional versus post-translational) we isolated RNA from cells transiently transfected with control vector, wild-type BRCA1 or mutated BRCA1 and proceeded with reverse transcription-PCR. PCR for BRCA1 used primers for exons 14 and 15 so that both wild-type and mutant BRCA1 could be detected. At 25 cycles a PCR product for both BRCA1 constructs was observed (Figure 3b). However, at 30 cycles a PCR product for $p27^{Kip1}$ was detected only in the cells transfected with wild-type BRCA1 (Figure 3b). By 35 cycles the $p27^{Kip1}$ PCR product was equivalent in all samples (data not shown). Thus these results suggest that the regulation of $p27^{Kip1}$ by BRCA1 is transcriptional.

Identification of a putative BRCA1-responsive element in the $p27^{Kip1}$ promoter

These results suggested that the $p27^{Kip1}$ promoter contained a putative BRCA1- responsive element. Initial experiments were carried out using deletion mutants of the mouse $p27^{Kip1}$ promoter. Deletion up to position −774 increased the activity of BRCA1 on the $p27^{Kip1}$ promoter reporter constructs (Figure 4a), which is consistent with a previous report showing that the mouse $p27^{Kip1}$ promoter contains negative regulatory elements in the region −1609 to −925 (Kwon *et al.*, 1996). Further deletion to −615 did not significantly decrease the response of the $p27^{Kip1}$ promoter to BRCA1 (Figure 4a). However, BRCA1 responsiveness was lost by deletions up to position −511. These results suggested that a putative BRCA1-responsive element was located between positions −615 and −511 of the $p27^{Kip1}$ promoter.

BLAST analysis determined that this region of the mouse $p27^{Kip1}$ promoter (−774 to the translation start site) was 94% identical to this region of the human $p27^{Kip1}$ promoter, which starts at position −943. Subsequent transient transfection assays with deletion mutants of the human $p27^{Kip1}$ promoter demonstrated that the putative BRCA1-responsive element identified in the mouse $p27^{Kip1}$ promoter was located to the same region of the human $p27^{Kip1}$ promoter, position −784 to −680 (data not shown).

These results in conjunction with the immunoblot analysis of the breast cancer cell lines suggest that the effect of BRCA1 on $p27^{Kip1}$ is p53-independent. The correlation of BRCA1 and $p27^{Kip1}$ protein expression was observed in breast cancer cell lines expressing either wild-type p53 (MCF7) or mutant p53 (MDA-MB-231, T-47D). Also the putative p53 elements in the $p27^{Kip1}$ promoter are 5′ to position −774, the $p27^{Kip1}$ promoter deletion construct having the greatest induction by co-transfection with BRCA1 (Figure 4b).

However these results do not determine whether $p27^{Kip1}$ activation by BRCA1 is a direct or indirect effect by BRCA1. To investigate the effect of BRCA1 on the $p27^{Kip1}$ promoter we generated oligonucleotides spanning the region of the mouse promoter containing the putative BRCA1-responsive element as identified by the promoter deletion analysis. These oligonucleotides (oligo A −609 to −575, oligo B −577 to −543, oligo C −545 to −511) were used in EMSA with nuclear lysates from two breast cancer cell lines MCF7 and HCC1937, a breast cancer cell line which is unizygous for the BRCA1 5382insC mutation, resulting in termination of BRCA1 protein translation at codon 1829 (Tomlinson *et al.*, 1998). Nuclear extract from MCF7 produced a slowly migrating band with oligo C but did not show any significant binding to oligo A or oligo B (Figure 4b). In contrast, nuclear extract from HCC1937 did not produce a band shift with any of the oligonucleotides (Figure 4b and data not shown). Subsequently we determined by immunohistochemistry that HCC1937 did express BRCA1 protein (Figure 5b).

The slowly migrating complex observed with MCF7 nuclear extract and oligo C could be competed 90% by a 10-fold excess of cold oligo C (Figure 4b). Some competition was observed with cold oligo A but there was no competition observed with an excess of an unrelated oligo (oligo U; STAT site from MUC1 promoter). This suggests that the DNA-protein complex is specific.

BRCA1 has been shown to interact with a number of other proteins. We used a number of antibodies in the EMSAs to determine the proteins involved in the slowly migrating complex. Of all the antibodies tested, only an antibody against exon 11 of BRCA1 resulted in a supershifting of the complex (Figure 4b). The antibody against the C-terminus of BRCA1 decreased the protein-DNA complex by 40–50%, suggesting that this antibody might interfere with BRCA1 binding to

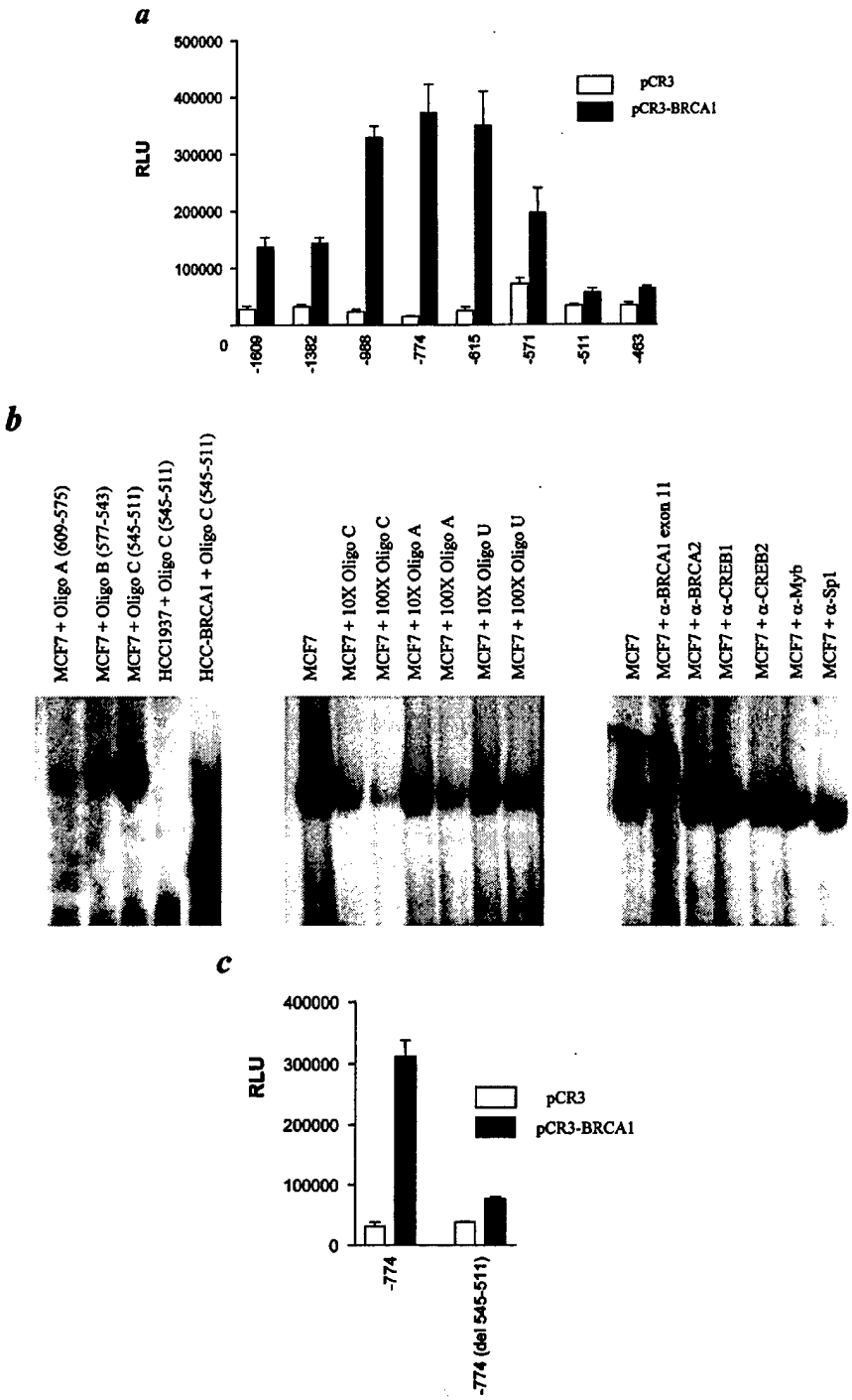

Figure 4 Region of mouse $p27^{Kip1}$ promoter containing the putative BRCA1-responsive element identified by 5′ deletion mapping. (**a**) COS cells were co-transfected with the 5′-deletion $p27^{Kip1}$ promoter reporter constructs and either pCR3 or pCR3-BRCA1, and luciferase activity was measured 48 h later. These results represent three independent experiments. (**b**) Nuclear lysate from MCF7 was incubated with oligo A (−609 to −575), oligo B (−577 to −543) and oligo C (−545 to −511) of the $p27^{Kip1}$ promoter. Nuclear lysate from HCC1937 and HCC-BRCA1 was incubated with oligo C. Cold competition was carried out with an excess of oligo C, oligo A or oligo U (an unrelated 35 bp sequence). The complex is supershifted by an antibody against exon 11 of BRCA1. (**c**) MCF7 cells were transfected with a 5′-deletion $p27^{Kip1}$ promoter construct (−774) or with the construct minus the putative BRCA1-responsive element (−774 (del 545-511)). Luciferase activity was measured as in Figure 1. These results represent three independent experiments

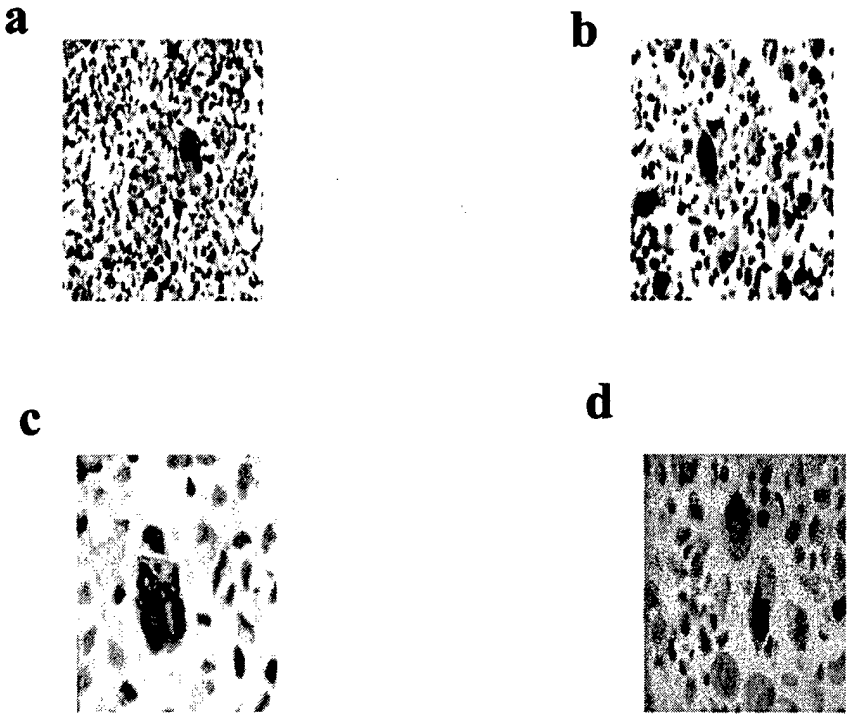

Figure 5 Expression of $p27^{Kip1}$ is decreased in cells expressing mutated BRCA1. (a) (c) Formalin-fixed paraffin-embedded MCF7. (b) (d) Formalin-fixed paraffin-embedded HCC1937. (a) (b) N-terminal anti-BRCA1 (Calbiochem). (c) (d) Anti-$p27^{Kip1}$ (Transduction Labs). (a) (b) are 4× magnification. (c) (d) are 10× magnification

the DNA (data not shown). Together these results suggest that BRCA1 is interacting directly with the DNA sequence from the $p27^{Kip1}$ promoter.

Since nuclear extract from the mutant BRCA1 cell line HCC1937 did not bind the DNA sequence from the $p27^{Kip1}$ promoter, we re-introduced wild-type BRCA1 into this cell line (HCC-BRCA1) and repeated the EMSA analysis. In this experiment a slowly migrating complex was observed with oligo C and nuclear extract from HCC-BRCA1 (Figure 4b). This slowly migrating complex with HCC-BRCA1 was less than that observed for MCF7 but the level of expression of wild-type BRCA1 in HCC-BRCA1 was lower than that observed for MCF7 (data not shown). Thus introduction of wild-type BRCA1 did restore binding to the $p27^{Kip1}$ promoter sequence.

Since it appeared that the BRCA1-responsive element could be localized to a 35 bp region of the $p27^{Kip1}$ promoter, we generated a construct in which this region was deleted. Transient transfection assays using the deletion construct −774 and the construct without the putative BRCA1-responsive element −774 (del 545−511) demonstrated that the removal of this 35 bp region decreased BRCA1 responsiveness of the $p27^{Kip1}$ promoter by 80% (Figure 4c). Therefore it does appear that the BRCA1-responsive element is located at position −545 to −511 of the mouse $p27^{Kip1}$ promoter, which corresponds to −714 to −680 of the human $p27^{Kip1}$ promoter. This region is 100% identical between the mouse and human promoter. Previous deletion analysis of the human $p27^{Kip1}$ promoter suggested that a region from −774 to −435 contained the essential transcription factor binding sites (Minami *et al.*, 1997).

Decreased $p27^{Kip1}$ protein expression in mutant BRCA1-expressing breast cancer cell line

Since wild-type BRCA1 increases $p27^{Kip1}$ protein levels in contrast to the tumor-associated BRCA1 mutants, we decided to compare the endogenous $p27^{Kip1}$ protein levels in cells expressing wild-type BRCA1 (MCF7) versus mutated BRCA1 (HCC1937). Immunohistochemistry demonstrated reactivity of both MCF7 and HCC1937 with an N-terminal BRCA1 antibody (Figure 5a,b). As expected from our transfection assay results immunoreactivity for $p27^{Kip1}$ was weak for the HCC1937 cell line, as compared to MCF7 which showed strong expression of $p27^{Kip1}$ protein (Figure 5c,d).

Discussion

It has previously been demonstrated that BRCA1 can transcriptionally upregulate $p21^{Waf1/Cip1}$ and GADD45 (Somasundaram *et al.*, 1997; Jin *et al.*, 2000). This study demonstrates another target for BRCA1 transcriptional activation, namely the CDI $p27^{Kip1}$. Wild-type BRCA1 transactivated the $p27^{Kip1}$ promoter in a

number of cell lines. However, tumor-associated BRCA1 mutants were defective in transcriptionally regulating $p27^{Kip1}$ indicating that a functional C-terminal transactivation domain of BRCA1 is required for modulating $p27^{Kip1}$. This study suggests that another mechanism for growth inhibition by BRCA1 may be mediated via the upregulation of $p27^{Kip1}$.

BRCA1 contains two BRCT motifs within the C-terminal transactivation domain and these BRCT regions are also found in several proteins involved in DNA repair and cell cycle checkpoints. (Koonin *et al.*, 1996; Callebaut and Mornon, 1997). These BRCT motifs have been shown to bind DNA in a sequence specific manner (Halligan *et al.*, 1995; Yamane *et al.*, 2000). The results shown here suggest that the mutated BRCA1 in HCC1937 cells is not able to bind DNA because the mutation results in a truncated protein lacking intact BRCT domains. Furthermore, an antibody against the C-terminus of BRCA1 decreased the slowly migrating complex observed with the MCF7 nuclear extract by 40–50%. Thus, these results suggest that the tumor-derived BRCA1 mutations may not be able to efficiently transactivate gene transcription due to an inability to bind DNA.

The region of the $p27^{Kip1}$ promoter containing a putative BRCA1 responsive element was mapped to positions −545 to −511 of the mouse or positions −714 to −680 of the human $p27^{Kip1}$ promoter. A comparison of this region to those identified as being important for BRCA1 regulation of the $p21^{Waf1/Cip1}$ promoter (−143 to −93) and the GADD45 promoter (−121 to −75) did not demonstrate any homology between these promoters regarding the identified BRCA1-responsive elements. However, gel mobility shift analysis did demonstrate that all three promoter regions could bind BRCA1, that the complex could be supershifted by an antibody against BRCA1, and that binding to one promoter region could be competed by an excess of the other promoter regions (Williamson, unpublished results). These studies together suggest that BRCA1 might have different target motifs for activation. It has been suggested that another $p21^{Waf1/Cip1}$ promoter motif, the STAT binding serum-inducible element can be activated in response to BRCA1 (Ouchi *et al.*, 1998). Thus the choice of motifs for activation by BRCA1 may vary according to the presence of transcription factors in different cell types, as well as other signaling events within the cell.

The $p27^{Kip1}$ is decreased in various human cancers but specific mutations have only rarely been reported (Spirin *et al.*, 1996). Transcriptional, translational and post-translational mechanisms contribute to $p27^{Kip1}$ regulation. It had been considered that the major mechanism for regulating $p27^{Kip1}$ at the protein level was post-translational. However, transcriptional upregulation of $p27^{Kip1}$ by the Forkhead transcription factors has been reported recently (Medema *et al.*, 2000). For breast tumors, this decrease in $p27^{Kip1}$ expression might be a reflection of a loss of functional BRCA1, resulting either from a mutation in the BRCA1 gene or by methylation of the BRCA1 promoter. Recent studies have shown that decreased expression of $p27^{Kip1}$ does indeed correlate either with both the presence of a BRCA1 mutation in breast tumor tissue as well as with BRCA1 promoter methylation (Chappuis *et al.*, 2000; Niwa *et al.*, 2000). Thus, loss of functional BRCA1 might be expected to result in impaired growth inhibition due to ineffective regulation of $p27^{Kip1}$. Therefore, understanding the mechanisms controlling $p27^{Kip1}$ expression in breast tumors may provide new strategies to inhibit tumor growth.

Materials and methods

Plasmid constructs

The promoter region of $p27^{Kip1}$ and the enzyme generated 5′ deletions were subcloned into pGL2-basic as previously described (Kwon *et al.*, 1996; Minami *et al.*, 1997). These were provided by J Wyke. The $p27^{Kip1}$ promoter construct −774 (del 545–511) was generated by two rounds of PCR and subcloned into the *Hind*II/*Sac*I site of the pGL2 vector. Removal of the 35 bp was confirmed by sequencing. The pCR3 vectors encoding wild-type BRCA1 and the synthetic and tumor-associated mutants of BRCA1 were constructed as previously described and provided by B Weber (Somasundaram *et al.*, 1997). The Zn^{2+}-inducible BRCA1 was generously provided by J Holt (Abbott *et al.*, 1999).

Cells, transfections and luciferase assays

All cell lines were obtained from ATCC. COS, MCF7 and HCT116 were transfected using 1 μg of each expression plasmid by Geneporter according to the manufacturer's instructions (Gene Therapy Systems). Lysates were harvested 48 h post-transfection and luciferase assays carried out according to the manufacturer's protocol (Promega). Transfection efficiency was normalized using pRL-SV40 at 1/10 of total DNA concentration.

Generation of stably transfected HCC1937 cells

Full-length BRCA1 was ligated into the *Not*I/*Hind*III of the pMT vector, which contains a Zn^{2+}-inducible promoter and the neomycin resistance gene (Abbott *et al.*, 1999). HCC1937 was transfected with 1 μg of pMT-BRCA1. Cells were then selected in 50 μg/ml G418 (Sigma) for 4 weeks. Five cell lines were generated and analysed for expression by Western blotting.

Reverse transcription and PCR

HCT116 cells were transiently transfected with either pCR3, pCR3-BRCA1 or pCR3-BRCA1 (Gln1756insC). After 48 h the cells were harvested for RNA using Trizol according to the manufacturer's instructions (Invitrogen). 2.5 μg total RNA was used for reverse transcription followed by 1/50 of the reverse transcription reaction being used for PCR. PCR for BRCA1 and $p27^{Kip1}$ was as follows: BRCA1 primers (FOR 5′-GATTTGACGGAAACATCTTAC and REV 5′-CCAGCAGTATCAGTAGTATGA) spanning exons 14 and 15 resulting in a 236 bp product; $p27^{Kip1}$ primers (FOR 5′-CCATGTCAAACGTGCGAGTGT and REV 5′-CGTTTGACGTCTTCTGAGG) which give a product of

594 bp. Results were visualized by an ethidium bromide stained agarose gel. Integrity of the RNA was assessed by PCR for GAPDH.

Western blot analysis

Cells were lysed in a buffer containing 20 mM Tris-Cl pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM Na_3VO_4 and protease inhibitors. Lysates were analysed with the following antibodies: 1:200 anti-C-terminal BRCA1 (C-20, Santa Cruz), 1:500 anti-N-terminal BRCA1 (Ab-1, Calbiochem) and 1:2500 monoclonal anti-p27^{Kip1} (Transduction Labs). Equal protein loading was determined with an antibody against GAPDH (Research Diagnostics). Results were visualized by Enhanced Chemiluminescence (Amersham).

Nuclear protein preparation and electrophoretic mobility shift assays (EMSA)

Double stranded consensus oligonucleotides were end-labeled with γ-^{32}P-ATP by T4 polynucleotide kinase. Nuclear extracts were prepared from MCF7 and HCC1937 cells. Ten μg of nuclear extract was incubated with 20000 c.p.m. of labeled oligonucleotide. Binding reactions were also carried out in the presence of a number of antibodies: BRCA1 exon 11 (Pharmingen); BRCA1 C-terminal (Zymed); BRCA2 Ab-2 (Calbiochem); CREB1 (C-21, Santa Cruz); CREB2/ATF4 (C-20, Santa Cruz); Myb (M-19, Santa Cruz); Sp1 (IC6, Santa Cruz). The binding reactions were separated on a 4% polyacrylamide gel. The gel was dried and the results visualized by autoradiography.

Immunohistochemistry

MCF7 and HCC1937 were harvested, fixed in 4% formalin and embedded in paraffin blocks. The sections were analysed with the following antibodies: monoclonal anti-p27^{Kip1} (Transduction Labs) and N-terminal BRCA1 (Ab-1, Calbiochem). The positive control for these antibodies was an invasive mammary carcinoma. The negative control for all antibodies was the detection process without the primary antibody. Dilutions were as recommended by the manufacturers. Results were visualized by horseradish peroxidase and assessed by light microscopy.

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