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13. ABSTRACT (Maximum 200 words) The purpose of the studies outlined herein was to map phosphorylation sites on the BRCA1 protein as well as study the effects of these modifications on BRCA1 function. In numerous metabolic labeling studies, it has proven impossible to incorporate sufficient ³²Phosphate into the protein to properly carry out phosphopeptide mapping studies. In order to determine the mechanism of aberrant subcellular localization of BRCA1 in breast cancer, a series of deletion mutants of the protein, all containing the nuclear localization sequences, were prepared as green fluorescent protein fusions, expressed in both normal and transformed human breast cell lines and identified by fluorescence microscopy. The results indicate that the amino terminus of the protein may contain a motif that must be modified before the protein translocates from the cytoplasm to the nucleus. The development of a BRCA1 somatic cell knockout breast epithelial cell line through targeted disruption was begun. Screening of 100 drug resistant colonies generated one homologously targeted clone. Unfortunately, screening of several hundred clones in a second round of targeting failed to detect targeting of the second allele, indicating that BRCA1 expression is required for survival in cell culture, at least in the cell line being targeted.				
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FOREWORD

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Introduction

The discovery of the *BRCA1* tumor suppressor gene in 1994 was a significant milestone in breast cancer research (1). Mutations at this locus are responsible for nearly 50% of inherited breast cancer (2). Surprisingly, no *BRCA1* mutations have to date been found in sporadic breast tumors, which occur with much greater frequency than inherited neoplasms, prompting speculation that this gene may not play a role in the pathogenesis of sporadic breast cancers (3). Recent work in our laboratory suggests, however, that *BRCA1* dysfunction may indeed play an important role in the etiology of sporadic breast tumors (4, 5). Using polyclonal antisera raised against three different fragments of the protein, we demonstrated that *BRCA1* is a ubiquitously expressed 220 kDa phosphoprotein. In addition, we showed by indirect immunofluorescence staining that, in the majority of breast tumors examined, the protein was mislocalized in the cytoplasm. In normal breast epithelial cells, as well as most other cell types examined, *BRCA1* is a nuclear protein. The aberrant localization phenotype was particularly prevalent in advanced metastatic breast cancer. These findings have led us to speculate that the *BRCA1* protein, while not mutated in sporadic breast tumors, may contribute to their genesis and/or progression by virtue of its cytoplasmic sequestration away from its presumptive site of tumor suppression - the nucleus.

To date, only two cancer-related properties of the *BRCA1* protein have been reported: 1) its aberrant subcellular localization in breast tumor cells (4, 5), and 2) its ability to suppress growth (6). There is ample precedent to suggest that one or both of these properties may be related to the phosphorylation status of this protein.

In the case of protein transport to the nucleus, protein phosphorylation has been shown in several instances to play a central role. For example, the subcellular location and attendant biological activity of the phosphate-regulated yeast transcription factor PHO4 is controlled through phosphorylation by the cyclin-cdk complex PHO80-PHO85 (7). The phosphorylation of PHO4 on multiple serine residues by PHO80-PHO85 results in its exclusion from the nucleus. However, inhibition of PHO80-PHO85 kinase activity by the cdk inhibitor PHO81 results in the accumulation of underphosphorylated PHO4, which in turn localizes to the nucleus (8). The nuclear import of another yeast transcription factor - SW15 - has been shown to be sequestered in the cytoplasm upon phosphorylation by the *cdc2*/CDC28 complex (9).

Phosphorylation-mediated nuclear translocation of a number of mammalian and viral proteins has also been demonstrated. For example, treatment of cells with Interferon- γ results in the phosphorylation of the transcription factor Stat91 on a single tyrosine residue (Tyr 701), a modification shown to be essential for the transport of Stat91 to the nucleus (10). Similarly, the rate of nuclear transport of proteins fused to the SV40 T-antigen is highly dependent upon the phosphorylation by casein kinase II of two serine residues in this protein (11).

The relationship between protein phosphorylation and growth suppression has also been well-documented, particularly with respect to the Retinoblastoma (Rb) tumor suppressor protein (12). Rb is phosphorylated in a cell cycle-dependent manner. Hypophosphorylated forms predominate in G₀ and G₁, while hyperphosphorylated forms predominate in S, G₂ and M phases. Much of this phosphorylation is believed to be mediated by cyclin-dependent kinases, such as *cdk2* and *cdc2* (13, 14). The hypophosphorylated form of Rb is capable of inducing cell cycle arrest in G₁ by binding to and inactivating transcription factors such as E2F that regulate genes responsible for the G₁ to S transition. Phosphorylation of Rb results in its dissociation from E2F, which in turn mediates progression of the cell cycle (12).

Previously, our laboratory demonstrated that the *BRCA1* protein is expressed in a cell cycle-dependent manner in the T24 bladder carcinoma cell line (15). *BRCA1* protein levels are very low during the G₁ phase of the cell cycle, but rapidly rise to a maximum during S phase, before tailing off

slightly through mitosis. Cell cycle experiments using metabolically labeled T24 cells have revealed that BRCA1 is phosphorylated in a cell cycle-dependent manner that parallels its expression (Figure 1). Since BRCA1 is phosphorylated *in vivo* at all phases of its expression, it is reasonable to speculate that phosphorylation plays an important role in the cellular functions of this protein. The cell cycle-dependence of both the expression and phosphorylation of BRCA1 suggests that cell-cycle dependent protein kinases (cdks) may be responsible at least in part for its phosphorylation *in vivo*.

To address this possibility further, we immunoprecipitated a series of cdks and cyclins from detergent lysates of T24 cells. The immunoprecipitates were subjected to immune complex kinase assays in the presence of [γ - 32 P]-ATP, followed by dissociation of protein complexes and re-immunoprecipitation with BRCA1-specific antisera. Re-precipitated BRCA1 was separated by SDS-PAGE, and subjected to autoradiography (15). Phosphorylated BRCA1 was consistently 1) associated with and 2) an *in vitro* substrate for cdk2 as well as kinases associated with cyclin A and cyclin D1. In contrast, we could not detect BRCA1 in cdc2, cdk4, cyclin B or cyclin E immunoprecipitates. These results correlate well with the aforementioned *in vivo* phosphorylation data - i.e., those species that associated with and mediated *in vitro* phosphorylation of BRCA1 are known to be involved in active kinase complexes during those periods of the cell cycle in which we have shown BRCA1 to be phosphorylated. Specifically, cdk2 has been shown to be maximally active in mammalian cells during G1 and early S phase (16, 17), while cyclin A-associated kinase activity is present throughout S and G2, showing a slight peak in activity in G2 (18).

We hypothesize that the BRCA1 tumor suppressor protein is phosphorylated in breast tumor cells as well as normal breast cells by cyclin-dependent protein kinases such as cdk2. We believe that these modifications affect key biological properties of this protein, including subcellular localization and growth suppression. Our intent therefore, was to map the sites of *in vivo* BRCA1 phosphorylation by a combination of peptide mapping and site-directed mutagenesis. If the *in vivo* sites of cyclin/cdk-mediated BRCA1 phosphorylation are indeed important for the cancer-related functions of this protein, then mutation of these residues to alanine by site-directed mutagenesis might be expected to alter the known cancer-related properties of BRCA1, including subcellular localization and growth suppression. This could be tested experimentally by tetracycline-regulated ectopic expression of such mutants in both normal and transformed breast cell lines. Additionally, the contribution of these phosphorylation sites to BRCA1 subcellular localization and/or growth suppression should also be evident from their differential phosphorylation in normal versus tumorigenic breast cell lines.

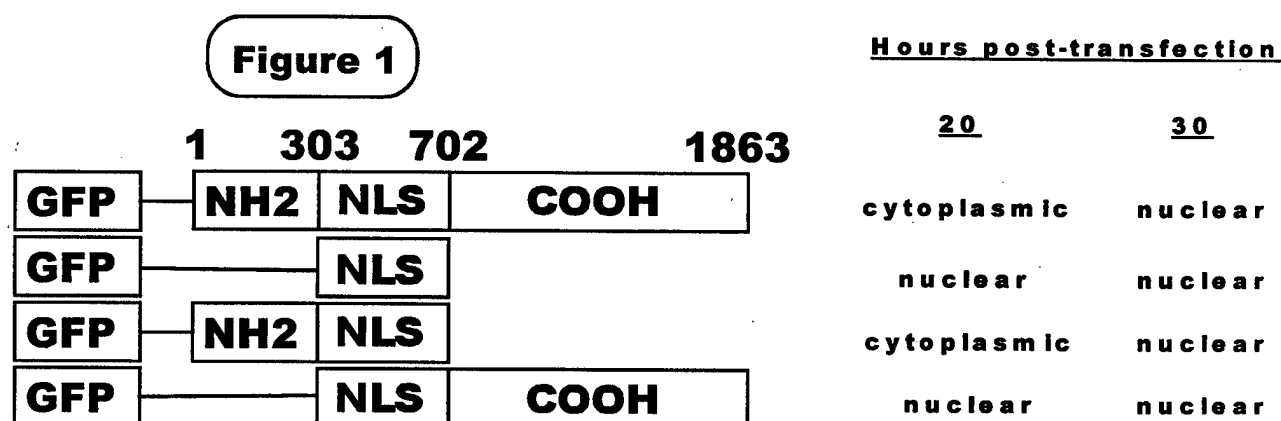
Body

Studies of the phosphorylation state of BRCA1. The first step in delineating the impact of BRCA1 phosphorylation on its cellular function is to identify the specific amino acid residues modified on BRCA1 in intact cells. This requires metabolic labeling of cells with [32 P]-inorganic phosphate (in order to label the intracellular ATP pools) followed by immunoprecipitation of metabolically phosphorylated BRCA1 for subsequent phosphopeptide analysis. This step has presented serious problems for us.

Numerous metabolic labeling experiments, involving both immortalized and transformed human breast cancer cell lines as well as a number of mono- and polyclonal antibodies have failed to generate sufficiently radiolabeled BRCA1 protein for subsequent phosphopeptide analysis. Indeed, Cherenkov counting of the immunoprecipitated BRCA1 protein band (following electrophoresis and transfer to PVDF membrane) gave only ~ 300 cpm at best.

We are not completely sure why these results have been so disappointing. It is possible that the metabolic labeling conditions themselves, which are quite toxic due to the serious depletion of phosphate pools, may cause the down-regulation of BRCA1 expression, thereby hindering our efforts to obtain sufficient labeled protein for subsequent analysis.

Studies of the mechanism of BRCA1 mislocalization in breast cancer. As mentioned in the introduction, our laboratory has previously shown that the BRCA1 protein is mislocalized in the cytoplasm of the majority of sporadic breast tumors and cell lines derived therefrom. To address the mechanism of this mislocalization, we have prepared a series of deletion mutants of the BRCA1 cDNA (Figure 1), and expressed them transiently as green fluorescent protein fusions in two human breast cell lines: HBL100, an immortalized, non-tumorigenic cell line; and T47D, a breast cancer cell line. The endogenous BRCA1 protein is nuclear in the former line and cytoplasmic in the latter. In these studies we were hoping to identify a portion of the BRCA1 protein that might be responsible for retaining the BRCA1 protein in the cytoplasm of breast cancer cells (T47D) as opposed to normal breast cells (HBL100). Note that all of the constructs possess the previously characterized nuclear localization signal (NLS) of BRCA1.

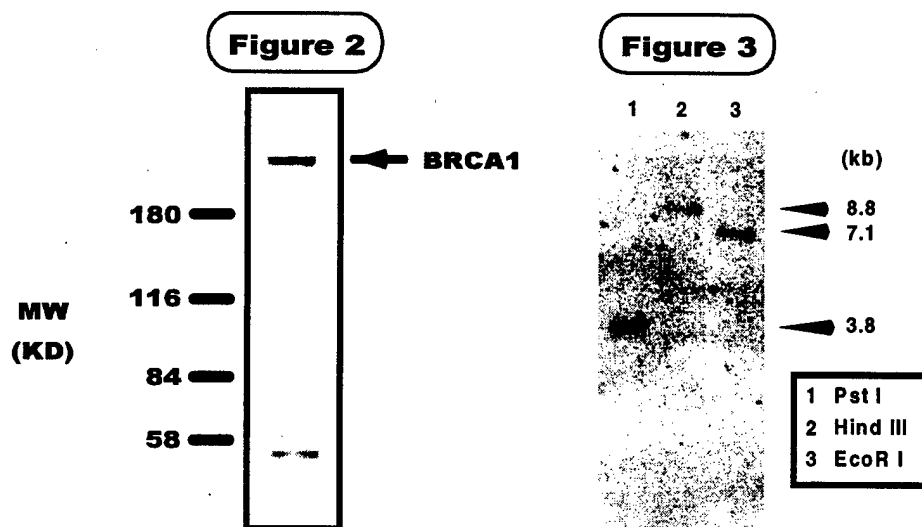


At 20 and 30 hours post-transfection, we examined the subcellular location of the expressed fusions by fluorescence microscopy.

As Figure 1 shows, all of the constructs entered the nucleus by approximately 30 hours post-transfection. It appears however, that the amino terminus of the protein does possess some type of motif that delays the entry of the protein into the nucleus. Based on our previous studies of the subcellular location the endogenous BRCA1 protein in normal and cancerous breast cells, we would

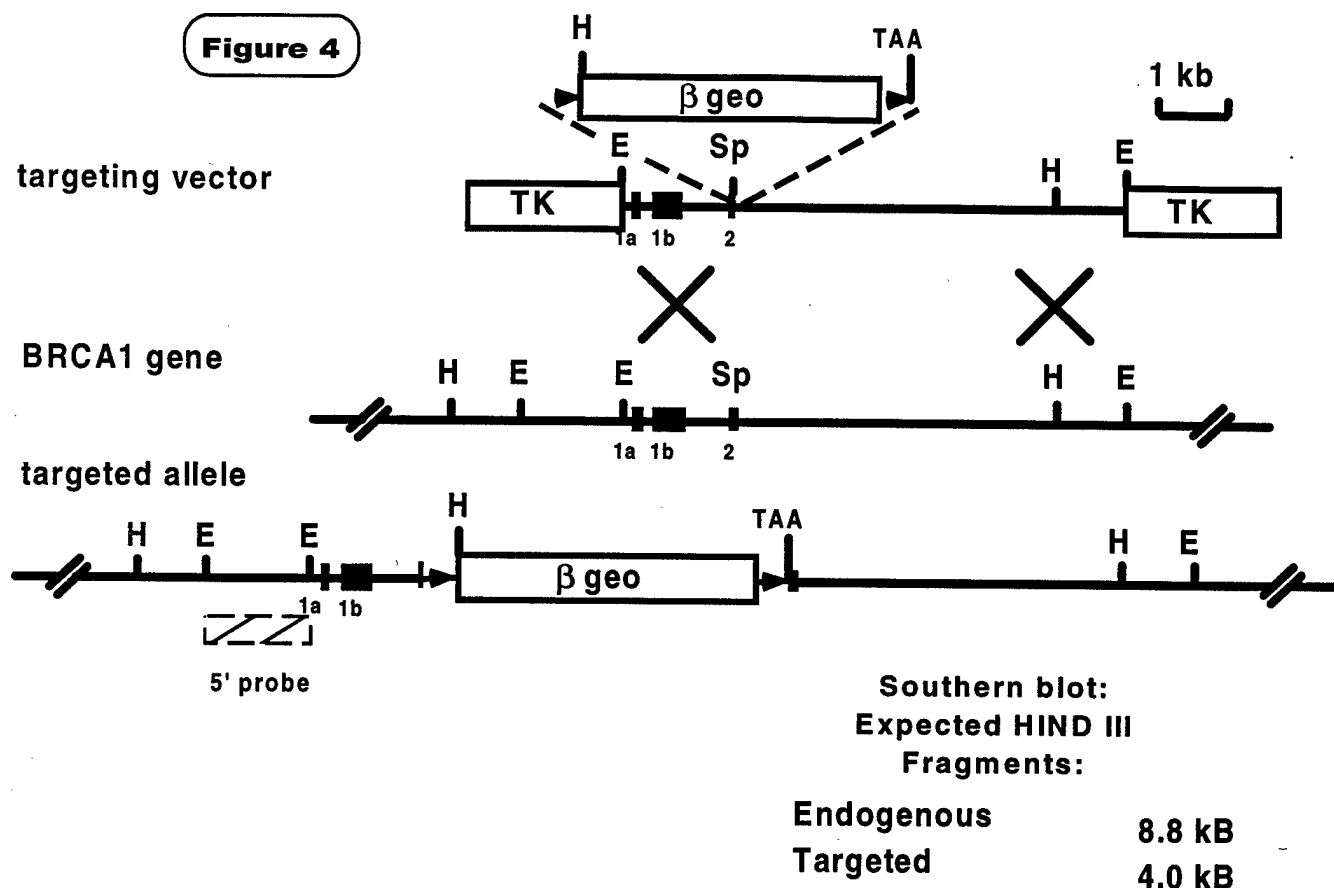
have expected this putative cytoplasmic retention domain to be operational in the T47D line only. However, as mentioned above, constructs containing the amino terminus of BRCA1 eventually localized to the nucleus. Perhaps, the amino terminus of BRCA1 is truly involved in some type of cellular retention mechanism, but this mechanism is being titrated out by the combination of the overexpression of the ectopic protein along with the endogenous protein. Future studies should involve inducible expression of these constructs to various levels. If the amino-terminus-containing constructs are cytoplasmic at lower expression levels, and nuclear at higher levels, than a titratable cytoplasmic retention mechanism is inferred. In addition, a cell line with no endogenous expression of BRCA1 would also be very useful, since endogenous protein complicates interpretation of the behavior of the ectopically-expressed proteins. Since no such line currently is available, we have begun, as described below to generate a BRCA1-deficient human breast cell line.

Production of a BRCA1-deficient somatic cell line. As mentioned above, a cell line devoid of BRCA1 expression would be of great utility in our efforts to understand the mechanism underlying the aberrant subcellular localization of this protein in breast cancer. Additionally, this line would be an extremely useful reagent in the study of the mechanism of tumor suppression by the BRCA1 protein. We have thus begun to derive, by targeted disruption, such a line from the human immortalized breast epithelial cell line MCF10A. This cell line is non-tumorigenic in nude mice (19), expresses full-length (220 kd) BRCA1 protein (Figure 2). Southern blot analysis using three restriction enzymes indicates that both alleles of the *BRCA1* gene are intact (Figure 3).

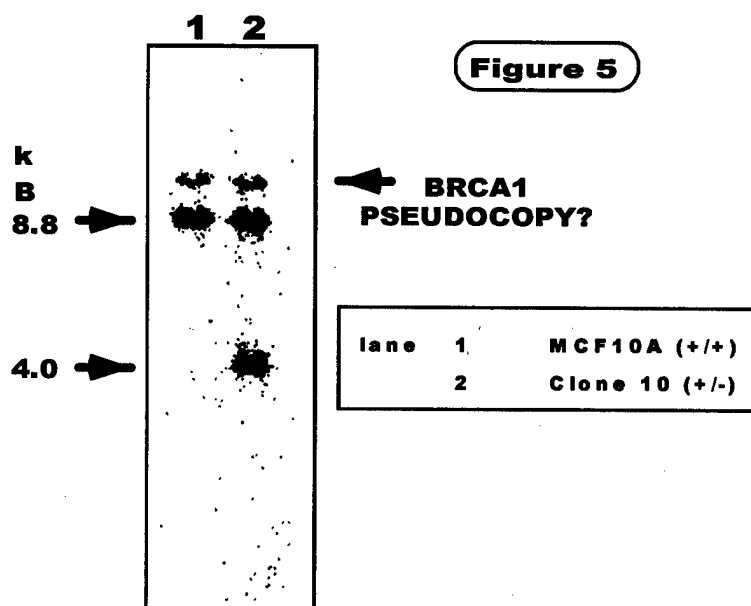


Our promoterless targeting vector is depicted in Figure 4. Electroporation of this vector into MCF10A cells yielded several hundred drug resistant colonies. Southern blot analysis of the first 100 clones revealed that one of these clones (#10) had undergone homologous recombination (Figure 5). In order to target the second allele, we constructed a targeting vector similar to that shown in Figure 5, except that the resistance marker was puromycin acetyl transferase gene. Unfortunately, analysis of over 300 hundred puromycin-resistant colonies (derived from electroporation of this vector into (+/-) clone #10) has not yielded a single homologous recombinant. We infer from these results that the *BRCA1* gene may indeed be required for survival and/or proliferation of this particular cell line, thus preventing us from isolating any stable (-/-) lines.

In the future, we believe that we can overcome these limitations by employing a slightly more circuitous approach to the problem. This would involve setting up a tetracycline-repressible BRCA1 cDNA expression system in the (+/-) cell line described above (20). Then ectopic allele would then

Figure 4

be de-repressed before carrying out the disruption of the second endogenous allele. Presumably, the ectopically expressed BRCA1 allele should rescue the cells from lethality caused by endogenous

**Figure 5**

BRCA1 deficiency. Once, both alleles have been targeted, the ectopic allele can be re-repressed with tetracycline and its effects on cell functions such as cell cycle progression could be evaluated. Hopefully, the nascent BRCA1-deficient cells will survive long enough to enable such analysis. This approach has been successfully used in the production of a RAD51 somatic cell knockout line (21).

Conclusions

In conclusion, the precise relationship between BRCA1 phosphorylation and its biological functions has been difficult to ascertain due to difficulties encountered in isolating the quantities of metabolically labeled protein necessary for phosphorylation site analysis.

The mechanism(s) underlying aberrant subcellular localization of BRCA1 protein in sporadic breast cancer have been investigated using a deletional analysis approach. This approach was intended to detect a cis-acting fragment in the BRCA1 protein that may be responsible for excluding the protein from its usual location in the cytoplasm. While this approach did not definitively define such a moiety in BRCA1, it did raise the possibility that there may be a region in the amino terminus that may tether the protein, albeit transiently, in the cytoplasm. This possibility can be further investigated by expressing the constructs in a tetracycline-regulated fashion, preferably in a BRCA1-deficient mammalian cell line.

Finally, we have initiated the production of a BRCA1-deficient human breast epithelial cell line using targeted disruption of both alleles of this gene. While disruption of the first allele was successful, analogous targeting experiments in the (+/-) cell line have not resulted in the isolation of the desired null cell line. This suggests that BRCA1-deficient cells are non-viable or at least seriously compromised in cell culture. We will overcome this barrier by introducing a tetracycline-regulated ectopic BRCA1 allele into the (+/-) line before disrupting the second endogenous BRCA1 allele. Repression of the ectopic allele will provide a BRCA1-deficient cell line which should be amenable to study for as long as the cells survive in culture.

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Personnel Receiving Pay For This Effort

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