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13. ABSTRACT *(Maximum 200*

Our long term goal of this proposal is to study the role of elk-1 in the progression of breast cancer. Previously, we have developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1 proteins. We have shown that serum deprivation or calcium ionophore treatment of BRCA1 transfectants resulted in programmed cell death, suggesting BRCA1 to function as an inducer of apoptosis. We have found significant higher levels of expression of elk-1 protein by western blot analysis in the $BRCA1^{S}$ transfectants, suggesting elk-1 proteins to be potential targets for BRCA1. To prove this hypothesis, we have developed rat fibroblast and human breast cancer cell lines expressing elk-1 proteins. Our results indicate that constitutive expression of elk-1 proteins induce apoptosis in both rat fibroblasts and human breast cancer cells. These results suggest that BRCA1 and elk-1 genes may play a critical role in the regulation of apoptosis of human breast cancer cells. Thus a wide variety of human malignancies like breast cancers have a decreased ability to undergo apoptosis. This could be due to lack/decreased levels of functional BRCA1 proteins. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies.

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Veena R 11/17/97

PI - Signature Date

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INTRODUCTION

Majority of the ets-oncogene superfamily members, which we and others have previously isolated, cloned and characterized are involved in leukemias, lymphomas and solid tumors (1-8). One of the members elk-1 (4) was shown to form a SRF (serum response factor) dependent ternary complex with the SRE (serum response element) similar to P62TCF (ternary complex factor) (9). Elk-1 was previously shown to be involved in the growth factor mediated signal transduction pathway involving activation of MAP kinase (mitogen activated protein kinase), leading to the transcriptional activation of c-fos proto oncogene both of which are activated in tumor cells. Since the fos regulatory function of elk-1 is dependent on the presence of a growth factor regulated transcriptional activation domain whose activity is dependent on phosphorylation by MAP kinase (10-13) and JNK kinases (Jun amino terminal kinase) (14) *in vivo* both of which are activated in tumor cells, we speculated that elk-1 proteins may be obligatory intermediates in the estrogen and growth factor mediated signal transduction pathway leading to the progression of breast cancer. We tested this hypothesis by studying the levels of expression of elk-1 protein in breast tumor samples as well as in several breast cancer derived tumor cell lines and compared expression with histologically normal breast tumor samples by western blot analysis using the elk-1 polyclonal antibody to correlate expression with stages of the disease. We observed high levels of expression of elk-1 protein (~3-8 fold higher) in samples derived from invasive

breast cancer than normal breast. These results suggested that higher levels of elk-1 proteins may be involved in the progression of breast cancer.

Breast cancer has been described in patients overtime to change from an estrogen dependent in initial stages of the disease to a hormonally independent tumor. We therefore studied the expression of elk-1 in MCF-7 cells that have been made quiescent by depletion of steroids and growth factors and stimulated to enter the cell cycle by the addition of E2 by RT-PCR analysis. We found a rapid induction of elk-1 and Δelk-1 RNA following growth stimulation by E2 in MCF-7 cells, indicating the involvement of elk-1 in the E2 induced signal transduction pathway in human breast cancer cells. Furthermore we observed no significant difference in the expression of elk-1 protein on immunoprecipitation analysis of ^{35}S methionine labeled MCF-7 cells exposed to estrogen for different time intervals, which was a little surprising. One explanation for this could be that MCF-7 cells have been maintained *in vitro* in tissue culture for a long time and hence may have drifted far from their origins *in vivo*, which could explain the results found in breast tumor samples. As suggested by one of the reviewers, MCF-7 cells are E2 responsive and non-metastatic, whereas MDA-MB-231 cells are estrogen independent, highly invasive and metastatic. We plan to compare the expression of elk-1 in these two lines which would indicate the role of elk-1 in early i.e., E2 dependent and late (invasive) breast cancer cell growth.

We have previously investigated and found that high level expression of elk-1 or ∆elk-1 protein transforms mouse fibroblasts *in vitro* and induces tumors in nude mice indicating that elk-1 and ∆elk-1 proteins are potentially tumorigenic. These results correlated well with the results obtained using breast tumor samples. One should bear in mind that breast cells are epithelial in origin unlike mouse/rat fibroblasts which are mesenchymal.

Recently a familial breast and ovarian cancer susceptibility gene BRCA1 was identified (15) and shown to be either lost or mutated in families with breast and ovarian cancer (16-20). Earlier, results on the expression of BRCA1 mRNA in sporadic breast cancer indicated decrease in the level of BRCA1 mRNA levels during the transition from carcinoma in situ to invasive cancer (21). But recent results of us and others have indicated increase in the expression of BRCA1 in tumor compared to normal samples, similar to elk-1 protein and BRCA1 expression has been correlated with the proliferation state of the cell (27, 28). At this juncture, we were tempted to look for any relation between BRCA1 expression and elk-1 expression or vice versa. So, in order to understand the role of BRCA1 in cell transformation, we obtained stable NIH3T3 cell lines expressing BRCA1 antisense RNA. The antisense BRCA1 expressing NIH3T3 cells showed accelerated growth rate, anchorage independent growth and tumorigenicity in nude mice unlike the parental transfectants (25, reprint enclosed). These results provide direct biological evidence for the possible function of BRCA1 as a tumor suppressor gene. These results suggested that inhibition of expression

of BRCA1 protein is sufficient to achieve transformation and BRCA1 functions as a growth regulator in normal cells. Similar results were obtained by others using BRCA1 antisense oligonucleotides in MCF-7 cells (21). Previously, we have performed the SRE TK CAT (Serum Response Element, Thymidine Kinase, Chloramphenicol acetyl transference reporter vector) functional assay for checking the levels of elk-1 protein in cells that have been transfected with antisense RNA to BRCA1 ($BRCA^{AS}$) that have been developed by us. We observed high levels of CAT activity which made us to speculate that BRCA1 could be a regulator of elk-1 gene. But we know that there are many ets-1 related proteins which would also respond to this reporter like SAP-1, SAP-2, Fli-1, etc. and hence, these results need to be confirmed by western blot analysis.

In order to study whether any relationship exists between BRCA1 and elk-1 we have developed mouse fibroblasts cell lines and human breast cancer cell lines expressing BRCA1 (26, see enclosed reprint). We have found that serum deprivation or calcium ionophore treatment of $BRCA1^{S}$ transfectants resulted in programmed cell death (26, see enclosed reprint). These results indicate that BRCA1 genes may play a critical role in the regulation of apoptosis of human breast cancer cells. We have examined the expression of elk-1 protein in the MCF-7 $BRCA1^{S}$ cells by western blot analysis and have observed higher levels of expression of elk-1 compared to vector transfected MCF-7 cells. These results suggested that elk-1 could be a target for BRCA1. In order to test the hypothesis whether elk-1 lies

downstream of BRCA1 in the apoptotic pathway and to investigate the role of elk-1 in apoptosis of breast cancer cells, we have developed rat fibroblast cell lines and human breast cancer cell lines expressing elk-1 proteins. We have found that constitutive expression of elk-1 and ▵elk-1 proteins induce apoptosis when cells are treated with calcium ionophore. These results suggest that elk-1 proteins may be downstream targets and inducers of apoptosis in human breast cancer cells. We also plan to study the growth/tumor suppressor function of elk-1 proteins both *in vitro* and *in vivo* in nude mice. Recent evidence suggests that hormone dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis. It may be possible that inability to undergo apoptosis in these cancers may be due to the decrease levels of functional BRCA1 proteins and BRCA1 downstream signals like elk-1 proteins. These experiments will give us a clue as to the possible regulation of elk-1 function in breast cancer. Future experiments will be directed towards studying the role of elk-1 in apoptosis/growth and tumor suppression. It may be possible that treatments that are aimed at increasing the apoptotic threshold by BRCA1/elk-1 gene therapy may have the potential to prevent the progression of these malignancies.

BODY

Task 1 **COMPLETED PREVIOUSLY**

Task 2 **IN PROGRESS**

Task 2 (a)

Previously we have examined the expression of elk-1 RNA and protein in MCF-7 cells that have been stimulated to proliferate in the presence of E2 by RT-PCR and immunoprecipitation analysis. We observed increase in the expression of elk-1 RNA but not protein following growth stimulation by E2 in MCF-7 cells. **WITH THESE RESULTS WE HAVE COMPLETED TASK 2 (a).**

Task 2 (b) **COMPLETED PREVIOUSLY**

Task 2 (c) **COMPLETED PREVIOUSLY**

Task 2 (g) **IS IN PROGRESS**

Since our preliminary results suggested a link between elk-1 and BRCA1 proteins, we studied the subcellular localization, phosphorylation and protein-protein interactions of BRCA1 proteins. Recently, we have characterized two new variant BRCA1 proteins (BRCA1a/p110 and BRCA1b/p100) which are phosphoproteins containing phosphotyrosine (33, reprint enclosed). Immunofluorescence and western blotting analysis indicated cytoplasmic and nuclear localization of BRCA1a and BRCA1b proteins (33, reprint enclosed). In an attempt to isolate proteins that interact with BRCA1, we have detected two cellular proteins (p65 BIP, p32 BIP) that specifically interact with BRCA1 (33, reprint enclosed). Western blot analysis of BIP indicated association with E2F, cyclins, and CDK's

and *in vitro* translated BRCA1a and BRCA1b proteins interacted directly with transcription factors E2F-1, E2F-4, cyclins A, B1, D1 and cyclin dependent kinases cdc2 and cdk2 suggesting a role for BRCA1 proteins in cell cycle regulation (33, reprint enclosed).

Task 2 (h) **COMPLETED**

Task 2 (I) **COMPLETED**

Task 2 (j) **COMPLETED** (Figures in reference 26, reprint enclosed in appendix)

Task 2 (k)

Previously we have observed high levels of expression of elk-1 protein in MCF-7 cells that have been transfected with BRCA1 compared to vector transfected MCF-7 cells by western blot analysis using elk-1 polyclonal antibody (Figure 1). These results suggested that BRCA1 gene may regulate the expression of elk-1 or elk-1 could be a downstream target for BRCA1 in the apoptotic pathway. Since BRCA1 is an inducer of apoptosis we speculated whether elk-1 could also be involved in apoptosis. Recently, mitogen activated/extracellular response kinase kinase kinase (MEKK), a serine-threonine kinase that regulates phosphorylation and activation of mitogen-activated protein kinases (MAPK) was shown to induce cell death (29). Inducible expression of activated MEKK (which induces apoptosis) stimulated the transactivation of c-Myc and elk-1 (29). c-Myc and c-jun transcription factors which are regulated by MAPK phosphorylation also induce apoptosis (30). Since elk-1

protein regulates c-Fos oncogene and is a target for MAPK and JNK both of which are activated by MEKK, we speculated whether it could play a similar role in inducing an apoptotic response. To test this hypothesis, we have transfected Rat-1 fibroblasts with pcDNA expression vector or pcDNA expression vectors containing human Elk-1 and ΔElk-1 cDNA and obtained stable G418 resistant cell lines expressing Elk-1 and ΔElk-1 proteins as described previously (26).

Morphology of Rat-1 Elk-1 and Rat-1 ΔElk-1 transfectants

The morphology of the Elk-1 transfectant was different from that of the parental Rat-1 fibroblast cells. The Elk-1 transfectants appeared to be shorter and flatter compared to the parental Rat-1 cells. The morphology of the ΔElk-1 transfectants were similar to that of the parental Rat-1 cells (Figure 2).

Analysis of expression of Elk-1 and ΔElk-1 proteins in Rat-1 Elk-1 and Rat-1 ΔElk-1 transfectants

We analyzed the expression of Elk-1 and ΔElk-1 proteins in Elk-1 and ΔElk-1 transfectants by immunoperoxidase staining (Figure 3A), indirect immunofluorescence analysis (Figure 3B) and western blot analysis (Figure 3C) using Elk-1 polyclonal antibody as described previously (26 and 33). The nuclear and cytoplasmic staining was brighter and stronger in both Elk-1 and ΔElk-1 transfected cells compared to parental Rat-1 fibroblast

cells suggesting higher level of expression of Elk-1 and ΔElk-1 proteins than Rat-1 cells. Western blot analysis revealed a significant increase in the levels of expression of Elk-1 and ΔElk-1 proteins when compared to the parental Rat-1 cells (Figure 3C).

Constitutive Elk-1 and ΔElk-1 expression induces cell death in Rat-1 cells after calcium ionophore treatment

Previously, we have shown calcium ionophore A23187 to induce apoptosis in BRCA1a transfected NIH3T3 and MCF-7 cells (26). This led us to examine apoptosis in Rat-1 Elk-1 and Rat-1 ΔElk-1 transfectants after A23187 treatment. Rat-1 and Rat-1 Elk-1 and Rat-1 ΔElk-1 were treated with calcium ionophore A23187 for 48 hours and the cell cycle distribution was determined by flow cytometry with propidium iodine staining method (31). Histogram of the DNA content and the percentage of cells in G1, S, and G2 plus M phase of the cell cycle were evaluated using EPICS profile analyzer. The Elk-1 and ΔElk-1 transfected cells showed accelerated rates of apoptosis (Ap value 49% for Rat-1 Elk-1 and 42% for Rat-1 ΔElk-1 cells) in the presence of calcium ionophore (Figure 4A) whereas the control Rat-1 fibroblasts cells showed lower levels of apoptosis under identical conditions (Ap value 16%).

Apoptosis in the Rat-1 Elk-1 and Rat-1 ΔElk-1 cells confirmed by cell viability staining

Measurement of apoptosis through the sub G1 peak in the DNA histogram gives no

distribution between viable and dead cells since all the cells are fixed. We, therefore, studied the viability of Rat-1 cells, Rat-1 Elk-1 and Rat-1 ΔElk-1 cells cultured in the presence of calcium ionophore by crystal violet staining. Cell viability staining showed that most of the Elk-1 and ΔElk-1 cells treated with calcium ionophore A23187 were dead whereas most of the control Rat-1 cells survived (Figure 4B). These results suggest that constitutive expression of Elk-1 and ΔElk-1 proteins induce death in Rat-1 fibroblast cells.

Apoptosis in the Rat-1 Elk-1 and Rat-1 ΔElk-1 cells confirmed by chromatin condensation

Elk-1 and ΔElk-1 transfected Rat-1 cells were cultured in the presence of calcium ionophore to induce apoptosis and the incidence of cell death was determined by phase contrast microscopy after staining the cultures with Hochest 33258 (32). Majority of the nuclei of Rat-1 Elk-1 and Rat-1 ΔElk-1 cells showed strong chromatin condensation and nuclear degradation into small, spherical nuclear particles of condensed chromatin characteristic of apoptosis. Whereas the parental Rat-1 cells did not show any significant change in the staining pattern (Figure 4C).

Apoptosis in the Elk-1 and ΔElk-1 transfectants confirmed by DNA fragmentation

The induction of apoptosis in the Elk-1 and ΔElk-1 transfectants upon treatment with calcium ionophore was further confirmed by analysis of DNA fragmentation. The DNA of

Elk-1 and ΔElk-1 cells treated with calcium ionophore was broken into oligo nucleosomal DNA ladder typical of apoptosis whereas the parental Rat-1 cells had no significant DNA degradation (Figure 4D). These results suggested that calcium ionophore induces apoptosis more readily in Rat-1 Elk-1 and Rat-1 ΔElk-1 cells than Rat-1 cells. All the above results shown for a single clone of Rat-1 Elk-1 or Rat-1 ΔElk-1 cells have been reproducibly obtained with several other independent clonal isolates of Rat-1-Elk-1 and Rat-1-ΔElk-1 cells. **WITH THESE RESULTS WE HAVE COMPLETED TASK 2(K) OF THE GRANT PROPOSAL**

Task 3 **IN PROGRESS**

Task 3(a)

In an attempt to study the role of Elk-1 gene in the regulation of apoptosis of human breast cancer cells, we have transfected MCF-7 cells with pcDNA expression vector or pcDNA expression vector containing human Elk-1 and ΔElk-1 cDNA's and obtained stable G418 resistant cell lines expressing Elk-1 and ΔElk-1 proteins.

Morphology of MCF-7 Elk-1 and MCF-7 ΔElk-1 transfectants

The morphology of the Elk-1 transfectants were different than that of the parental Rat-1 fibroblast cells. The Elk-1 transfectants were slow growing and appeared to be flatter and larger compared to the parental MCF-7 cells. The morphology of the ΔElk-1 transfectants

were similar to that of the parental MCF-7 cells (Figure 5). These MCF-7 Elk-1 and MCF-7 ΔElk-1 cell lines were analyzed for Elk-1 and ΔElk-1 protein expression by immunoperoxidase staining and western blot analysis and found to express Elk-1 and ΔElk-1 proteins.

Constitutive expression of Elk-1 and ΔElk-1 proteins induces apoptosis in MCF-7 cells treated with calcium ionophore

Apoptosis in the MCF-7 Elk-1/ΔElk-1 transfectants were analyzed after treatment with calcium ionophore A23187. MCF-7 and MCF-7 Elk-1/ΔElk-1 cells were treated with calcium ionophore A23187 for 24 hours and the cell cycle distribution was analyzed by flow cytometry with propidium iodide staining. The Elk-1 transfected MCF-7 cells showed accelerated rates of apoptosis (Ap value 72%) in the presence of calcium ionophore (Figure 6A), unlike the parental MCF-7 cells which showed very low levels of apoptosis (Ap value 4%). The MCF-7 ΔElk-1 cells showed low levels of apoptosis in presence of calcium ionophore (Ap value 14%). The viability of MCF-7 cells, MCF-7 Elk-1 and MCF-7 ΔElk-1 cells cultured in the presence of calcium ionophore were tested by crystal violet staining. Cell viability staining showed that a vast majority of the MCF-7 Elk-1 cells treated with calcium ionophore A23187 were dead whereas most of the control MCF-7 cells survived (Figure 6B). These results suggest that Elk-1 induces death in MCF-7 cells. The induction of apoptosis in the MCF-7 Elk-1 and MCF-7 ΔElk-1 transfectants was further confirmed by

DNA fragmentation analysis upon treatment with calcium ionophore. The DNA of MCF-7 Elk-1 cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder unlike the parental MCF-7 cells (Figure 6C). The DNA fragmentation observed in MCF-7 ΔElk-1 cells was much less. These results suggest that overexpression of Elk-1 in breast cancer cells accelerates apoptosis. We plan to identify the role of DNA binding, transcriptional activation and protein protein interactions on the apoptosis inducing function of elk-1 proteins in breast cancer cells.

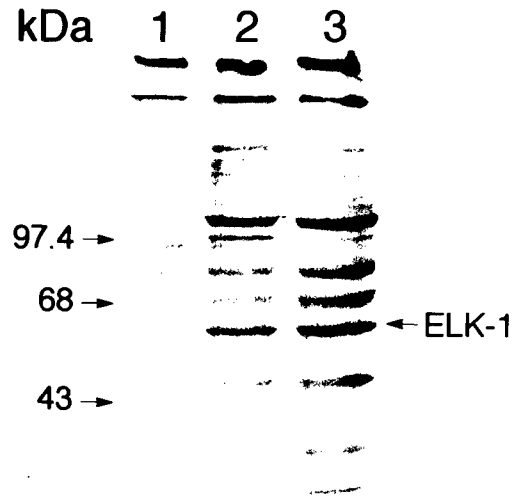
CONCLUSION

Our studies are designed to investigate the role of elk-1 in the progression of breast cancer. In summary, our results suggest that BRCA1, which is a tumor suppressor and elk-1 gene product function as inducers of apoptosis in breast cancer cells. This study demonstrates for the first time a role for elk-1 proteins in mediating apoptosis.

Recent evidence suggests that hormone-dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis. Our results suggest that lack or decreased levels of expression of functional BRCA1 or elk-1 gene products in breast cancer may be responsible for the increased resistance of these cells to undergo apoptosis. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 or elk-1 gene therapy may have

the potential to prevent the progression of these malignancies. Alternatively, one can use therapeutic agents that can activate BRCA1 downstream signals involved in apoptosis like elk-1 for the treatment of breast cancers. Results from this work would be utilized in the early detection, diagnosis and also treatment of breast cancer. We also plan to make deletion mutants of elk-1 proteins, introduce them into breast tumor cells and look for growth/tumor suppression and reversion from the transformed phenotype.

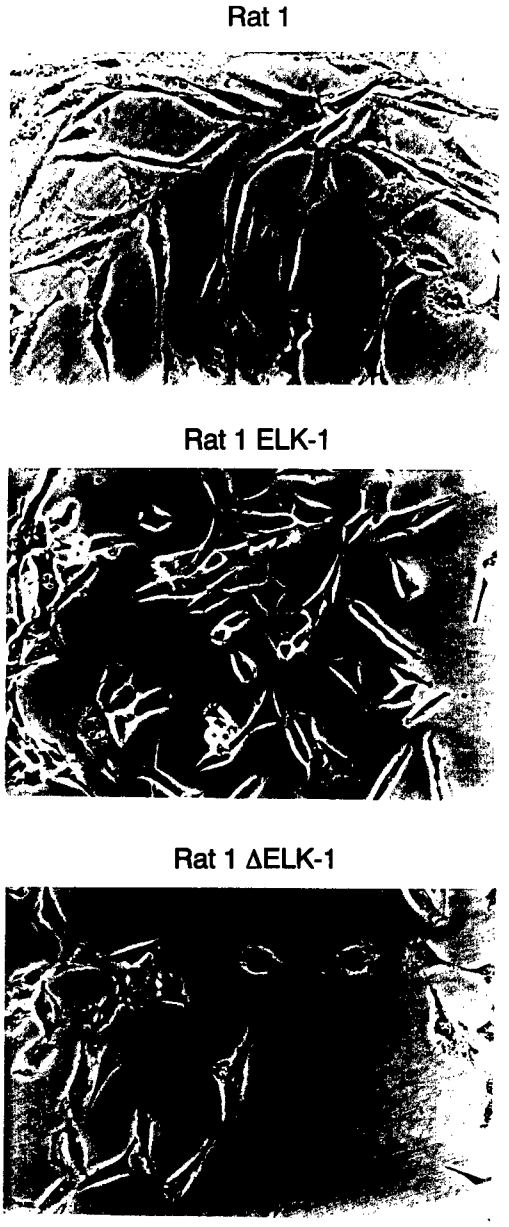
Figure 1 **Veena N. Rao, Ph.D.**



Western blot analysis of total cellular elk-1 protein levels in MCF-7 and MCF-7 cells transfected with BRCA1. Total cellular extracts from MCF-7 cells (lane 1), MCF-7 $BRCA1^S$ cell line no. 4 (lane 2), MCF-7 $BRCA1^S$ cell line no. 5 (lane 3) were electrophoretically separated on a 10% SDSPAGE, transferred to PVDF membrane and subjected to immunoblot analysis using a elk-1 antipeptide antibody (Santa Cruz Biotechnology, Inc.). The apparent molecular weight of the prestained protein standards is shown. The molecular weight of the elk-1 protein band (62KD) is represented by an arrow.

Limit the report

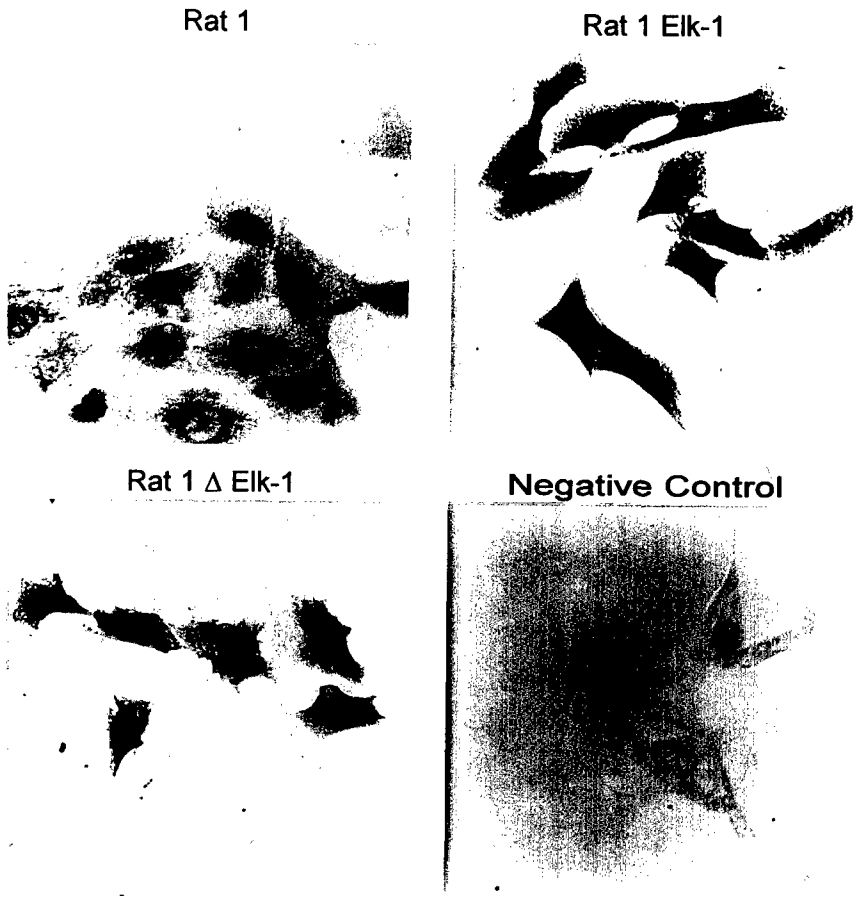
Figure 2 **Veena N. Rao, Ph.D.**



(A) Morphology of Rat-1 Elk-1 and Rat-1 ΔElk-1 transfectants. Phase-contrast photomicrographs of Rat-1, Rat-1 Elk-1 and Rat-1 ΔElk-1 cell lines cultured in normal media (10% CS/DMEM).

Limit the report

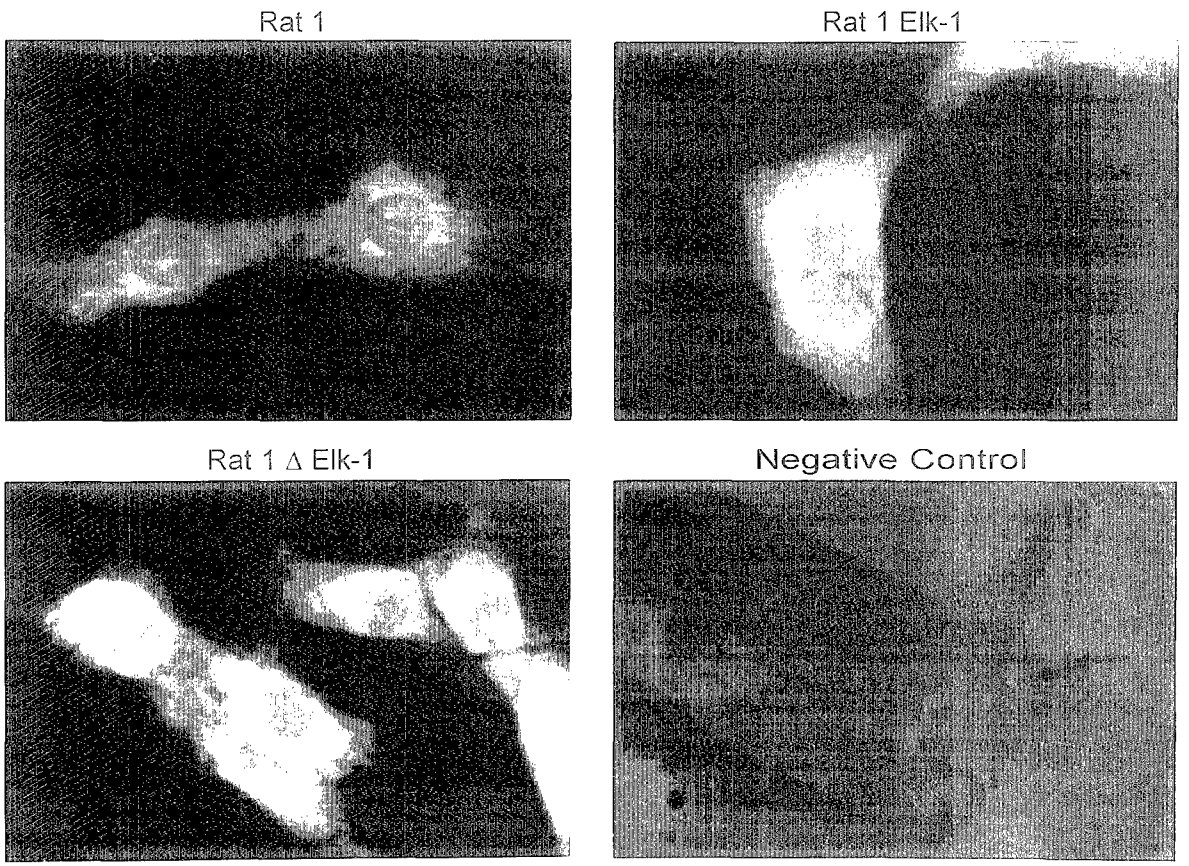
Figure 3A **Veena N. Rao, Ph.D.**



Detection of Elk-1 and ΔElk-1 proteins in Rat-1, Rat-1 Elk-1 and Rat-1 ΔElk-1 cells by immunohistochemistry, immunofluorescence and western blot analysis **(A) Immunoperoxidase** (B) Immunofluorescence (C) Immunoblot analysis.

Limit the report

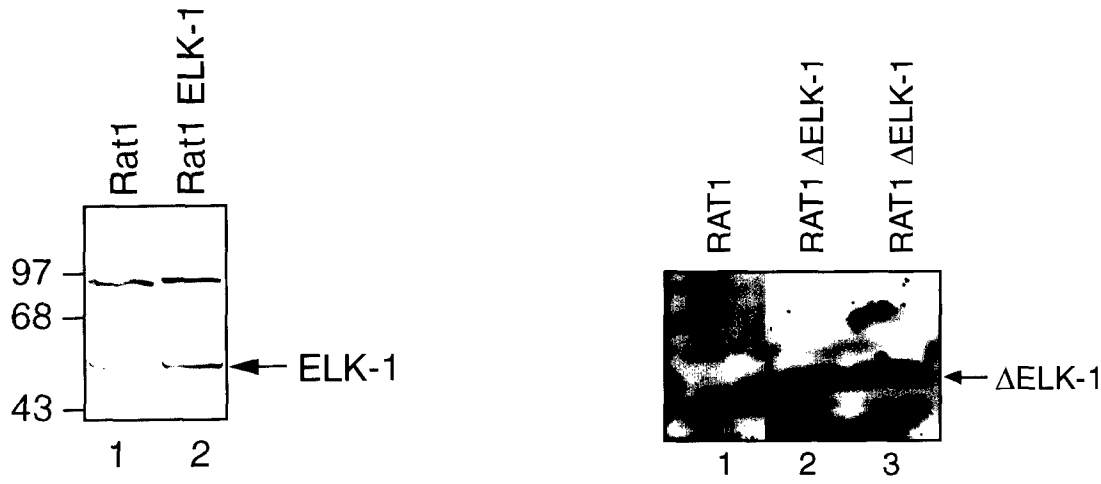
Figure 3B **Veena N. Rao, Ph.D.**



Detection of Elk-1 and ΔElk-1 proteins in Rat-1, Rat-1 Elk-1 and Rat-1 ΔElk-1 cells by immunohistochemistry, immunofluorescence and western blot analysis (A) Immunoperoxidase **(B) Immunofluorescence** (C) Immunoblot analysis.

Limit the report

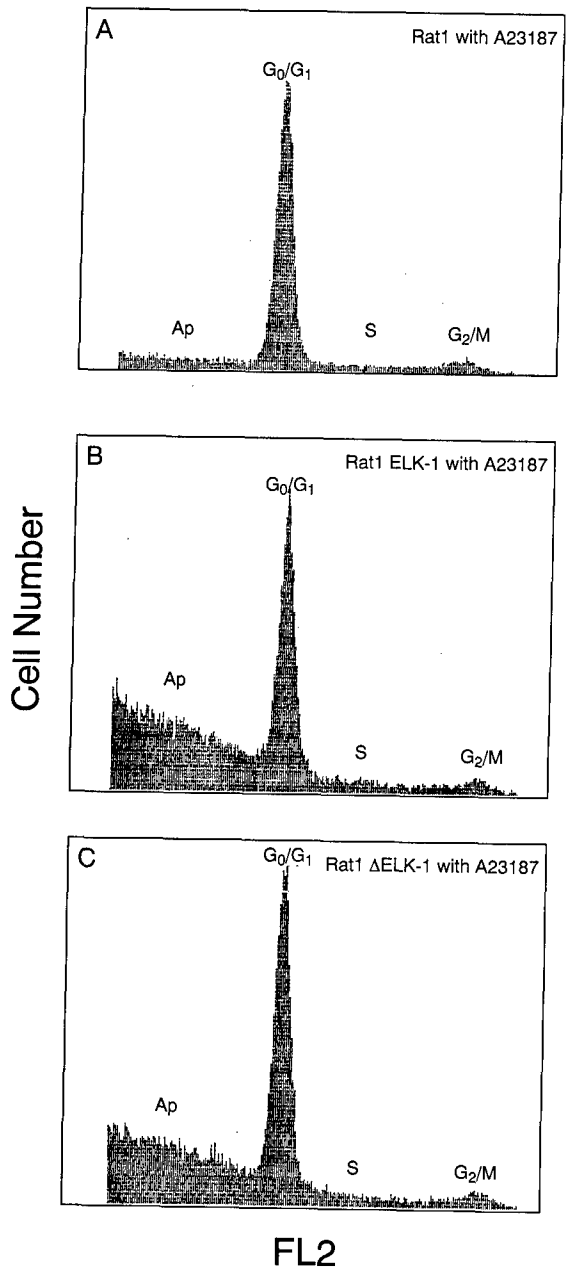
Figure 3C **Veena N. Rao, Ph.D.**



Detection of Elk-1 and ΔElk-1 proteins in Rat-1, Rat-1 Elk-1 and Rat-1 ΔElk-1 cells by immunohistochemistry, immunofluorescence and western blot analysis (A) Immunoperoxidase (B) Immunofluorescence **(C) Immunoblot analysis.**

Limit the report

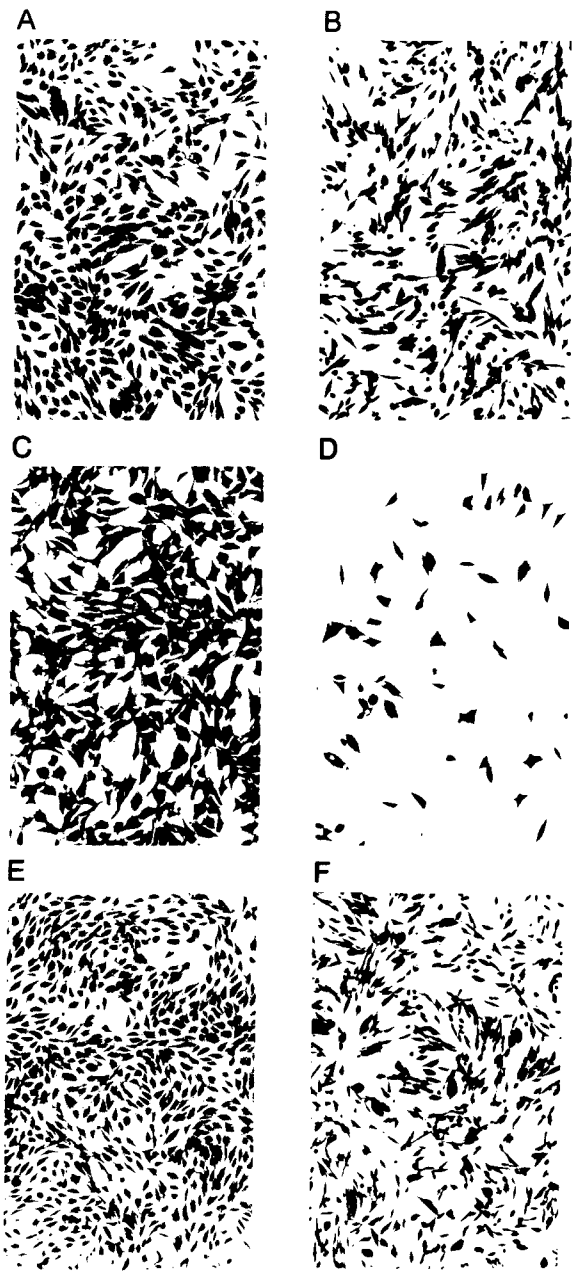
Figure 4A **Veena N. Rao, Ph.D.**



Elk-1 and ΔElk-1 proteins induce apoptosis in Rat-1 cells after calcium ionophore treatment. **(A) Flow cytometric analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 48 h.**

Limit the report

Figure 4B **Veena N. Rao, Ph.D.**

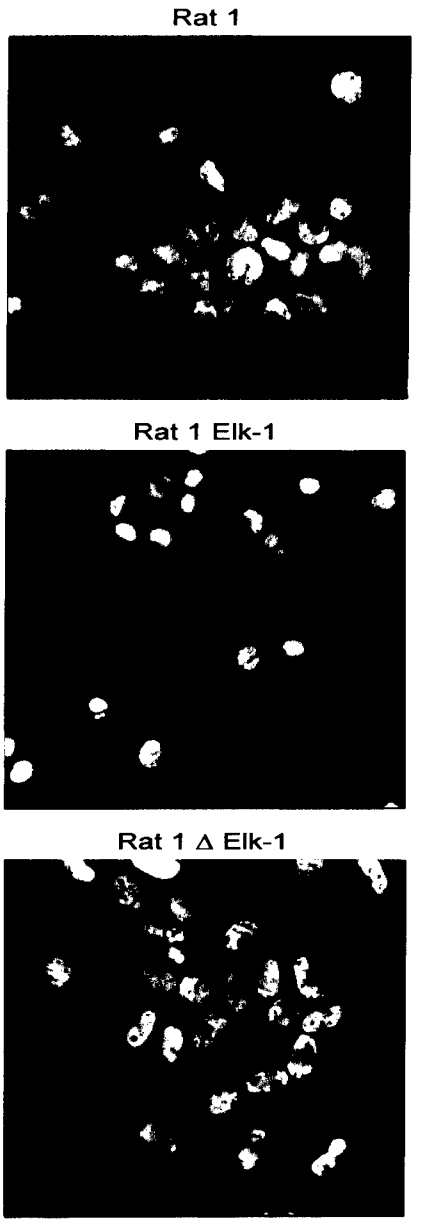


Elk-1 and ΔElk-1 proteins induce apoptosis in Rat-1 cells after calcium ionophore treatment. **(B) Viability of Rat-1, Rat-1 Elk-1 and Rat-1 ΔElk-1 cells treated with calcium ionophore for 48 h. A, Rat-1; B, Rat-1 cells treated with calcium ionophore; C, Rat-1Elk-1; D, Rat-1Elk-1 treated with calcium ionophore; E, Rat-1ΔElk-1; F, Rat-1ΔElk-1 treated with calcium ionophore.**

Limit the report

Figure 4C

Veena N. Rao, Ph.D.

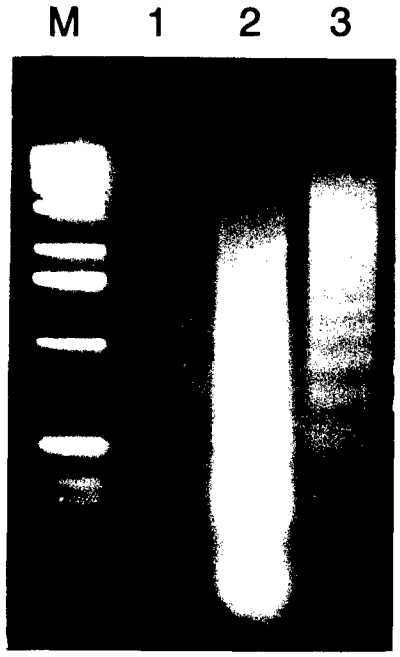


Elk-1 and ΔElk-1 proteins induce apoptosis in Rat-1 cells after calcium ionophore treatment. **(C) Chromatin condensation shown morphologically by Hoeschst staining of the Elk-1 and ΔElk-1 transfected cell lines induced to undergo cell death by calcium ionophore treatment for 48 h.**

Limit the report

Figure 4D

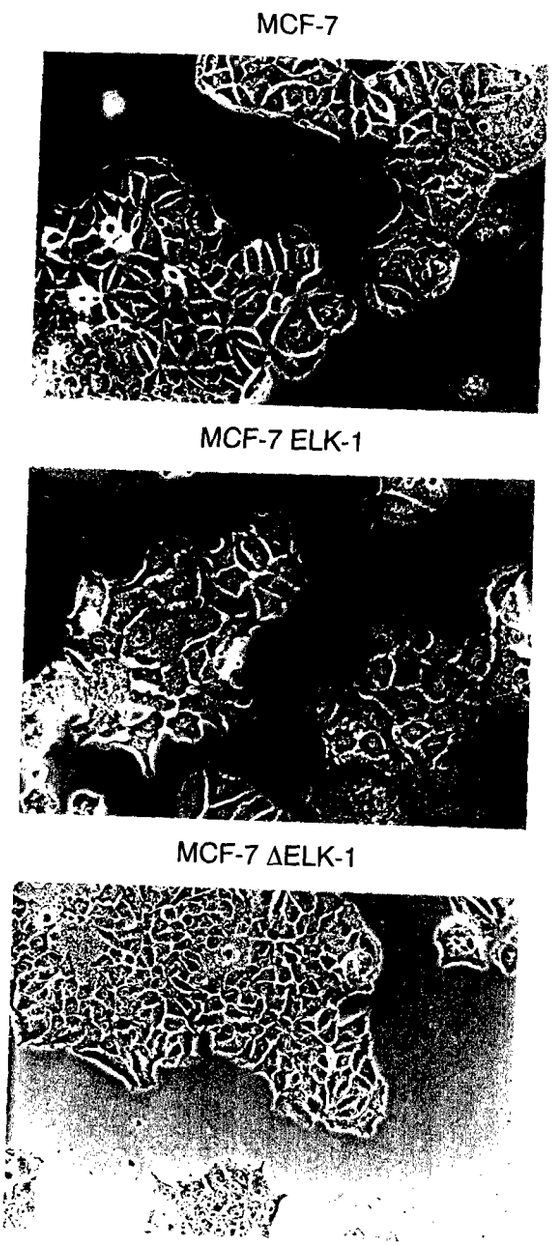
Veena N. Rao, Ph.D.



Elk-1 and ΔElk-1 proteins induce apoptosis in Rat-1 cells after calcium ionophore treatment. **(D) DNA fragmentation induced by Elk-1 and ΔElk-1 overexpression in Rat-1 cells. 1, Rat-1 cells treated with calcium ionophore; 2, Rat-1 Elk-1 cells treated with calcium ionophore; 3, Rat-1 ΔElk-1 cells treated with calcium ionophore**.

Limit the report

Figure 5 **Veena N. Rao, Ph.D.**

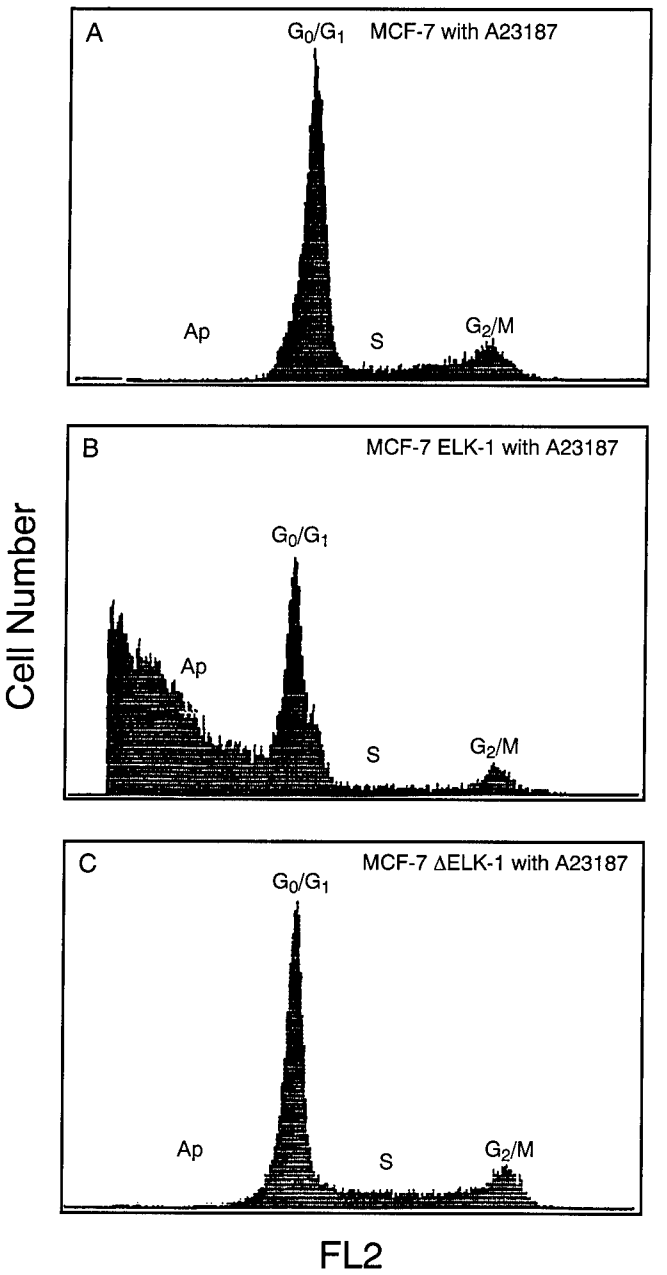


Morphology of the Elk-1 and ΔElk-1 transfected MCF-7 cells. Phase-contrast photomicrographs of MCF-7 cells, MCF-7 Elk-1 and MCF-7 ΔElk-1 cell lines cultured in normal media (10% FBS/DMEM).

Limit the report

Figure 6A

Veena N. Rao, Ph.D.



Elk-1 and ΔElk-1 overexpression induces apoptosis in MCF-7 cells after treatment with calcium ionophore. **(A) Flow cytometry analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 24 h**.

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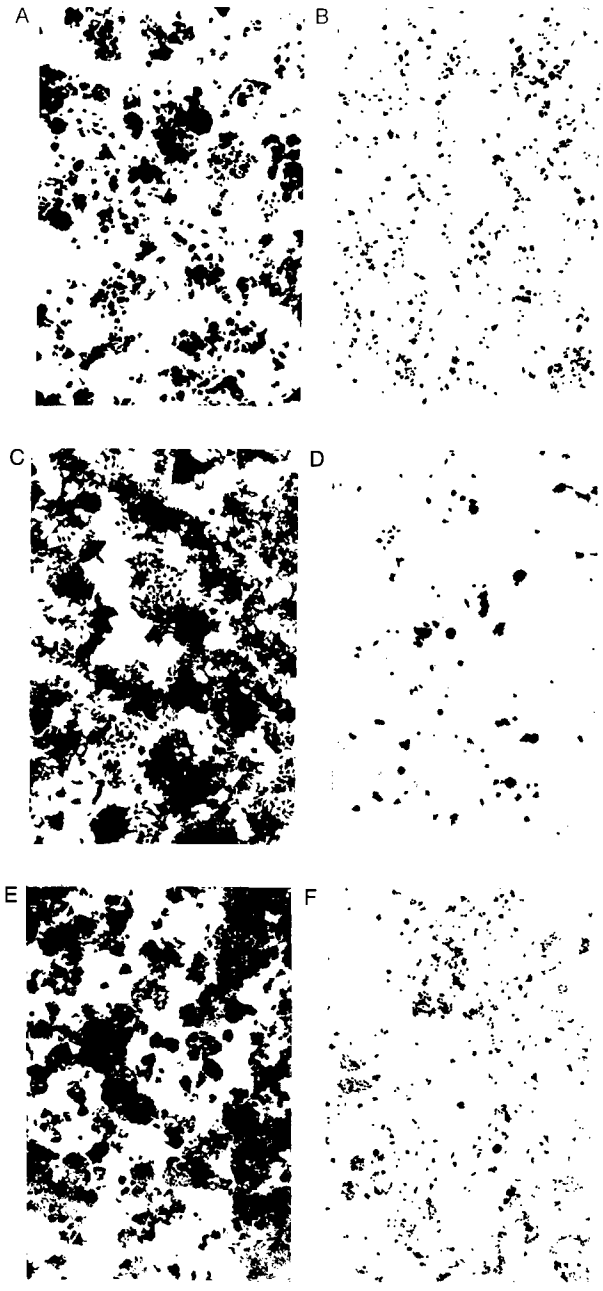
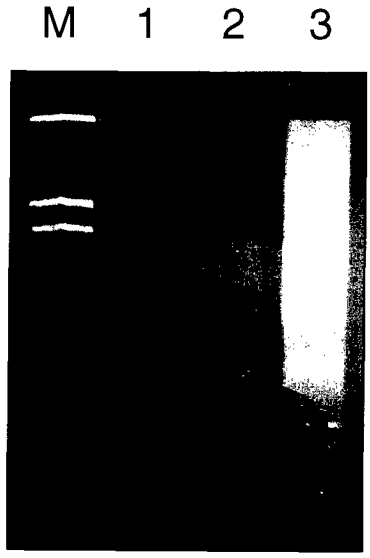
Figure 6B **Veena N. Rao, Ph.D.**



Elk-1 and ΔElk-1 overexpression induces apoptosis in MCF-7 cells after treatment with calcium ionophore. **(B) Cell viability of MCF-7, MCF-7 Elk-1 and MCF-7 ΔElk-1 cells treated with and without calcium ionophore for 24 h. A, MCF-7; B, MCF-7 cells treated with calcium ionophore; C, MCF-7 Elk-1 cells, D, MCF-7 Elk-1 cells treated with calcium ionophore for 24 h; E, MCF-7 ΔElk-1 cells; F, MCF-7 ΔElk-1 cells treated with calcium ionophore for 24 h.**

Limit the report



Elk-1 and ΔElk-1 overexpression induces apoptosis in MCF-7 cells after treatment with calcium ionophore. **(C) DNA fragmentation induced by Elk-1 and ΔElk-1 overexpression in MCF-7 cells. Lane 1, MCF-7 cells treated with calcium ionophore, 2, MCF-7ΔElk-1 cells treated with calcium ionophore; 3, MCF-7 Elk-1 cells treated with calcium ionophore**.

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Antisense RNA to the putative tumor suppressor gene BRCA1 transforms mouse fibroblasts

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Recently, BRCA1, a familial breast and ovarian cancer susceptible gene has been cloned and shown to be either lost or mutated in families with breast and ovarian cancers. BRCA1 has been postulated to encode a tumor suppressor, a protein that acts as a negative regulator of tumor growth. We have characterized the BRCA1 gene products by Western blot and immunoprecipitation analysis in mouse and tumor cells. Multiple BRCA1 polypeptides of approximately 225, 185, 160, 145, 100, 52 and 38 kD were identified in these cells. BRCA1 proteins were found to be localized mainly in the nucleus of normal Rat1 cells and human breast cancer cells. In order to understand the role of BRCA1 in cell transformation, we have established a stable NIH3T3 cell line expressing BRCA1 antisense RNA. The inhibition of expression of endogenous BRCA1 protein was detected in NIH3T3 transfectants by Western blot analysis. The antisense BRCA1 expressing NIH3T3 cells showed accelerated growth rate, anchorage independent growth and tumorigenicity in nude mice unlike the parental and sense transfectants. These results provide the first direct biological evidence for the possible function of BRCA1 as a tumor suppressor gene.

Keywords: BRCA1 proteins; antisense RNA; tumor suppressor; breast and ovarian cancers

Introduction

Breast cancer is one of the most frequent cancers affecting women. Although majority of cases are thought to be sporadic, about 5% of cases are estimated to be familial (Claus *et al.*, 1991). The clinical progression of human breast cancer reflects accumulated molecular defects in specific genes that are important in regulating the growth of normal breast tissue. The breast cancer susceptible gene BRCA1 gene was shown to be lost or mutated in families with hereditary breast and ovarian cancers (Miki *et al.*, 1994; Takanashi *et al.*, 1995; Easton *et al.*, 1993). Some recent reports have also implicated a role for BRCA1 directly in sporadic cancers (Merajver *et al.*, 1995; Hosking *et al.*, 1995; Futreal *et al.*, 1994). Studies of allele loss in tumors from breast and ovarian cancer affected families suggesting that BRCA1 is a tumor suppressor gene (Smith *et al.*, 1992). Thus the inherited mutation results in inactivation of one copy of the gene by mutation and the loss of the second wild type allele (Smith *et al.*, 1993; Kelsell *et al.*, 1993). These results implicate a key role for tumor suppressor genes like BRCA1 in the genesis and progression of breast cancers. The BRCA1 gene is composed of 22 coding exons stretching roughly 100 Kb of genomic DNA (Miki *et al.*, 1994). The gene codes for a 1863 amino-acid protein with an amino terminal zinc finger domain and a carboxy terminal acidic region typical of several transcriptional factors (Miki *et al.*, 1994). The first insight into the potential role of BRCA1 in breast tumor progression came from the work described by Thompson *et al.* (1995) where they show inhibition of BRCA1 expression with antisense oligonucleotides resulted in accelerated growth of normal and malignant mammary cells but not non-mammary epithelial cells. These results suggested that BRCA1 negatively regulates the proliferation of mammary epithelial cells. Taking all these results into consideration, we reasoned that if BRCA1 functions as a tumor suppressor regulating cell growth and division, allelic loss or damage by mutation of BRCA1 as seen in patients with breast cancer could result in loss of function of BRCA1 protein and uncontrolled cell growth leading to cancers. To test the hypothesis whether BRCA1 is a tumor suppressor gene, we used antisense RNA methodolgy (Iszat and Weintraub, 1985). We reasoned that if BRCA1 functions as a growth regulator in normal cells, inhibiting its expression should result in transformation. Our results demonstrate that antisense RNA to BRCA1 transforms mouse fibroblasts, providing the first direct biological evidence for the possible function of BRCA1, as a tumor suppressor gene.

Results and discussion

The experimental strategy that we have used to test the hypothesis whether BRCA1 is a tumor suppressor gene is shown in Figure 1. NIH3T3 cells express significant levels of a major $\approx$100 kD and a minor $\approx$145 kD BRCA1 protein as analysed by Western blot analysis (Figure 2a, lane 1). Similar sized BRCA1 proteins were also observed in several human cell lines (Figure 2c). In addition to these bands, we have also observed both higher $\approx$225 kD (using nuclear extracts, data not shown), 185 kD (Figure 2c, lane 3), $\approx$160 kD (data not given) and lower ($\approx$52 and $\approx$38 kD) molecular weight BRCA1 polypeptides (Figure 2c). Interestingly, we have detected high level of expression of $\approx$185–200 kD and $\approx$38kD BRCA1 proteins in HL 60 cells by immunoprecipitation analysis (Figure 2e). These results suggest that Western blot and immunoprecipitation analysis detect different size and level of expression of BRCA1 proteins. Detection of

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different size BRCA1 proteins is consistent with the presence of alternatively spliced transcripts of BRCA1 in different cells (Miki *et al.*, 1994; Rao, unpublished results). Our results suggest that these BRCA1 proteins undergo phosphorylation (Rao, unpublished results). We have isolated and characterized a BRCA1 cDNA corresponding to $\approx$100 kD BRCA1 protein in human cells (Rao, unpublished results). It remains to be seen whether these multiple protein bands represent other isoforms of BRCA1 or its related proteins. We have studied the subcellular distribution of BRCA1 proteins in normal (Rat 1 cells, Figure 2d) and in transformed cells (MCF7 cells, Figure 2d) using immunohistochemical methods (Figure 2d) and indirect immunofluorescense staining (data not given). Our results suggest that BRCA1 proteins are localized mainly in the nucleus of Rat 1 and MCF7 cells. However we have also observed weak cytoplasmic staining in the case of MCF7 cells.

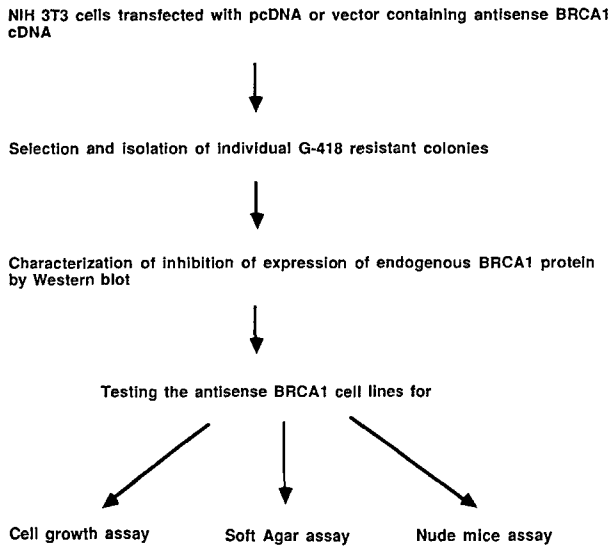
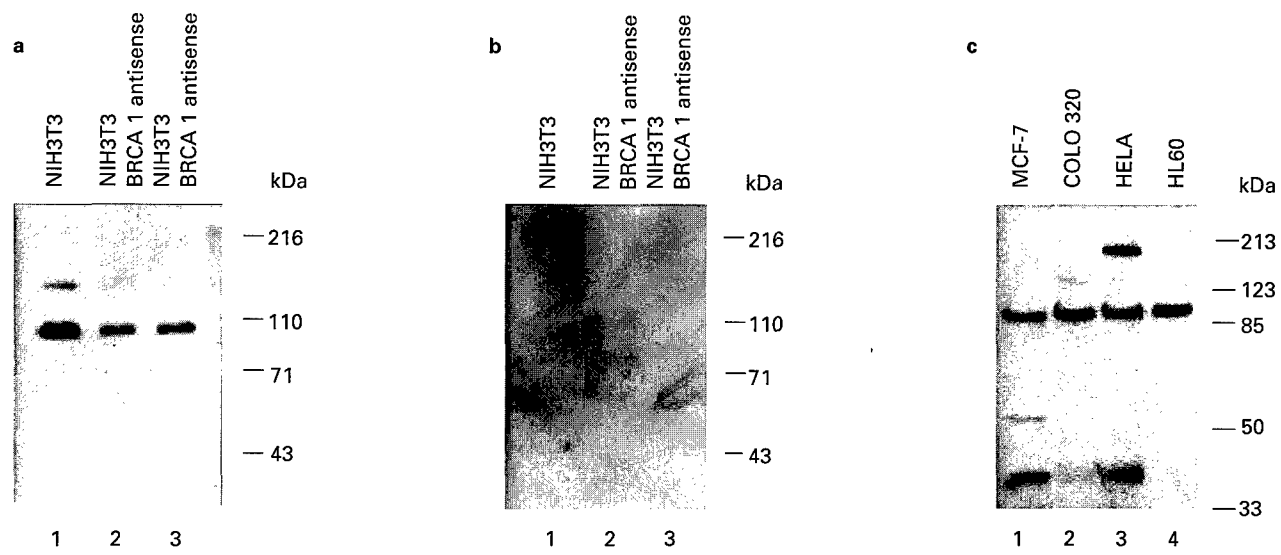
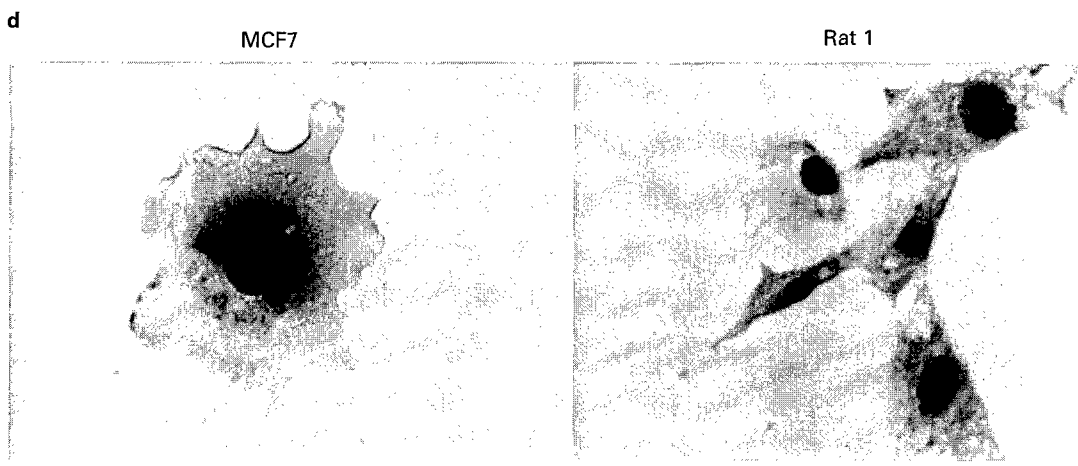


Figure 1 Experimental strategy used for obtaining the antisense BRCA1 transfectants

We have transfected NIH3T3 cells with pcDNA expression vector or pcDNA expression vectors containing antisense BRCA1 cDNA and obtained stable G418 resistant cell lines expressing antisense RNA to BRCA1. Initially we have screened several BRCA^{AS} cell lines for BRCA1 protein expression by Western blot analysis using BRCA1 polyclonal peptide antibody. We selected two cell lines which showed a significant decrease in the expression of BRCA1 protein (Figure 2a, lanes 2 and 3). These lines showed roughly 3–5-fold decrease in the expression of BRCA1





protein when compared to the parental NIH3T3 cells. We have performed BRCA1 peptide competition to show that the protein band(s) seen on the immunoblot are specific for the BRCA1 protein(s) (Figure 2b). We did not find total inhibition of expression with BRCA^{AS} transfectants because in theory, a high concentration of antisense RNA is necessary to completely inhibit any target gene, however, our results (discussed below) suggests that total inhibition may not be necessary to observed a biological change, since mRNA molecule can synthesize several copies of protein.

The BRCA^{AS} transfectants showed no major morphological alterations except for a slightly more flattened phenotype when compared to the parental NIH3T3 cells (compare Figure 3a and d–f). Transformed cells unlike normal cells can proliferate faster and grow in serum-free or low serum culture medium as they become independent of growth factors present in the serum. Thus we investigated the growth of BRCA1^{AS} transfectants in different serum culture conditions (10%, 0.1% and serum free). The BRCA1^{AS} transfectants proliferated at a much faster rate than NIH3T3 cells and also the BRCA1^{AS} cells proliferated in low serum media (Figure 3e and 4a and b) and serum free media (Figure 3f), whereas the parental NIH3T3 cells were unable to proliferate under these conditions (Figures 3b, c and 4b). These data indicate that BRCA1^{AS} transfectants behave like transformed cells in that they become independent of growth factors present in fetal bovine serum.

The accelerated growth rate and growth in serum free media of the BRCA1^{AS} cells raised the possibility that they might have become transformed, hence we tested their ability to grow in soft agar. Interestingly the BRCA1^{AS} cells were anchorage independent (Figure 5a) unlike the parental NIH3T3 cells and cells transfected with the BRCA1 sense constructs (Figure 5a). BRCA1^{AS} transfectant cell line #6 showed high clonogenic affinity (average 150 colonies, Figure 5b) in soft agar assay whereas NIH3T3 cells, NIH3T3/pcDNA and NIH3T3/BRCA1^{S} transfectants cells showed no colonies (Figure 5b). Another BRCA1^{AS} cell line number 3 also showed accelerated growth rate and growth in soft agar but was less tumorigenic than BRCA1^{AS} cell line no. 6 (data not given). Our results clearly suggests that a certain threshold level of BRCA1 protein is required for the regulation of cell growth in both mammary epithelial cells (Thompson *et al.*, 1995) and mouse fibroblasts (this study). Mere down regulation of expression of BRCA1 protein may result in deregulation of BRCA1 function leading to the progression from a normal to a transformed state. These results demonstrate that inhibition of expression of BRCA1 protein in BRCA1^{AS} cells might be sufficient to achieve transformation.

We next tested the tumorigenicity *in vivo* of NIH3T3 cells transfected with either pcDNA vector to BRCA1 antisense cDNA in nude mice. Our results show that subcutaneous injection of BRCA1^{AS} cells into nude mice consistently resulted in the development of tumors at the site of injection with a latency of 3 to 4 weeks (six out of six animals). None of the mice injected with the vector transfectants induced tumors at least up to 6 weeks.

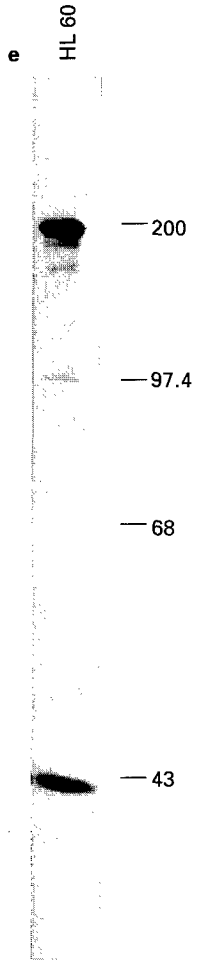


Figure 2 Characterization and subcellular localization of BRCA1 proteins. (**a**) Western blot analysis of total cellular BRCA1 protein levels in NIH3T3 and BRCA^{AS} transfectants. Total cellular extracts from NIH3T3 cells (lane 1), BRCA1 AS cell line no 3 (lane 2) and BRCA^{AS} cell line no 6 (lane 3) were electrophoretically separated, transferred to PVDF membrane and subjected to immunoblot analysis using a BRCA1 antipeptide antibody (Santacruz Biotechnology, Inc.). The apparent molecular weight of the prestained protein standards is shown. The molecular weight of the major BRCA1 protein band corresponds to $\approx$100 kD. (**b**) The same blot as in (**a**) was stripped and reprobed with the BRCA1 peptide antibody but in the presence of excess BRCA1 antigen. (**c**) Total cellular extract ($\approx$70 μg) from MCF-7 cells (human breast cancer cells) (lane 1); Colo 320 (human colon adenocarcinoma) (lane 2); HeLa cell (human epitheloid carcinoma) (lane 3); HL-60 (human promyelocytic leukemia) ($\approx$35 μg) (lane 4) were subjected to Western blot analysis as described in (**a**). (**d**) Immunohistochemical detection of BRCA1 proteins in normal and breast cancer cells. In Rat1 cells BRCA1 is localized only to nucleus and in MCF7 cells BRCA1 is localized mostly in nucleus. Original magnification $\times$400. (**e**) Immunoprecipitation of BRCA1 proteins in HL60 cells. HL60 cells were labelled with [^{35}S] trans label and subjected to immunoprecipitation using a carboxy terminal BRCA1 peptide antibody

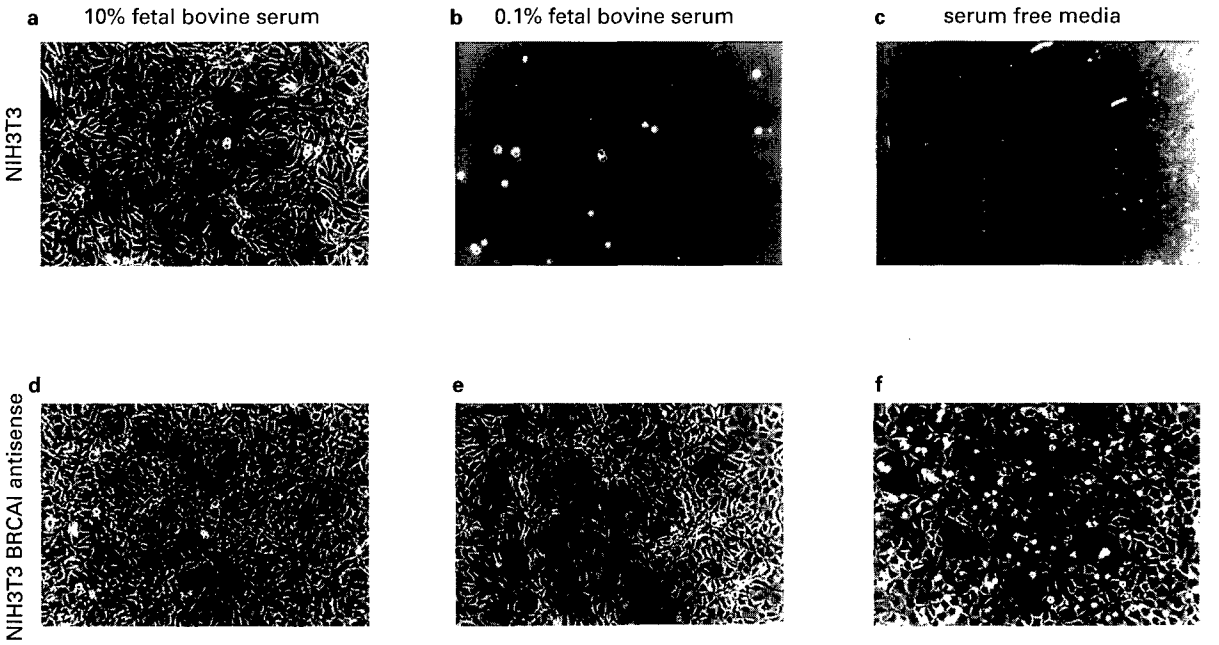


Figure 3 Growth properties and morphology of $BRCA^{AS}$ transfectants. Phase-contrast photomicrographs of NIH3T3 (**a-c**) and $BRCA^{AS}$ cell lines (**d-f**). Cells were cultured in normal media (10% FBS/DMEM) (**a** and **d**); low serum (0.1% FBS/DMEM) (**b** and **e**); serum free media (DMEM alone) (**c** and **f**)

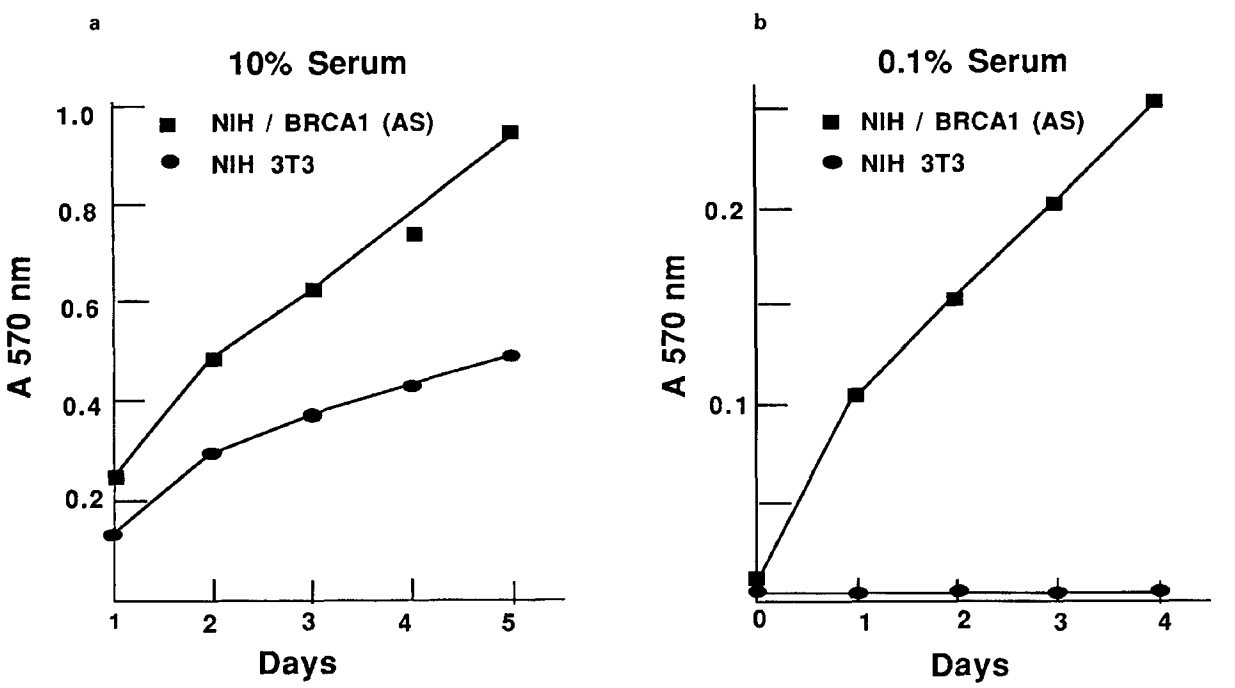


Figure 4 Inhibition of BRCA1 expression by antisense RNA accelerates NIH3T3 cell proliferation. Growth curves represent numbers of viable cells as measured by MTT dye assay (Loveland *et al.*, 1992) after different periods of cultivation in medium containing 10% FBS (**a**) or in 0.1% serum (**b**). The points represent mean of duplicates from a representative experiment

In summary, our results demonstrate that the BRCA1 gene product is a nuclear phosphoprotein which has tumor-suppressor function in mouse fibroblast cells since inhibition of endogenous BRCA1 expression by antisense RNA to BRCA1 results in neoplastic transformation. Our study provides the first direct biological evidence for the role of BRCA1 in transformation. The mechanism by which BRCA1 regulates cell proliferation is not known. The cell lines generated in this study should be useful to study the molecular mechanism involved in the function of BRCA1 gene. Knowledge of the pathways from inhibition of BRCA1 function leading to the progression to cancer will be important for the development of diagnostic kits and for designing targeted therapeutic strategies. Future efforts will be directed towards directly testing the BRCA1 gene product for growth inhibitory function.

Materials and methods

Plasmid construction and transfections

cDNA of BRCA1 (V Rao and ESP Reddy, unpublished results) containing the coding exons were subcloned into pcDNA vector by PCR using the published BRCA1 primers (Miki *et al.*, 1994). Purified DNA (15 μg) of pcDNA expression vector or vector containing the sense/antisense BRCA1 cDNAs were transfected into mouse NIH3T3 cells using the Strategene kit according to the manufacturers instructions. G418 resistant colonies were picked and propagated in the selective medium.

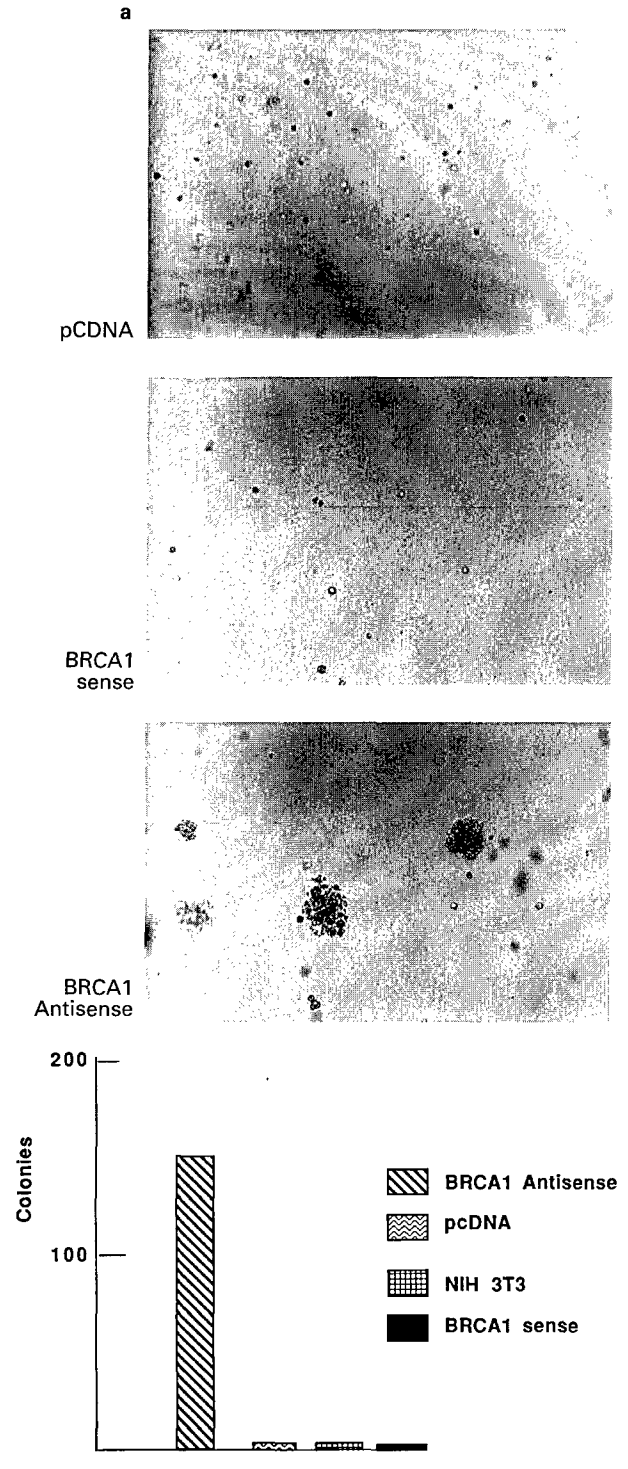


Figure 5 Growth of BRCA1^{AS} cells in soft agar (**a**) 2×10^3 cells per dish of pcDNA vector transfectant, BRCA1^{S} transfectant and BRCA1^{AS} transfectant were analysed for anchorage independent growth. (**b**) clonogenicity of BRCA1^{AS} cells; pcDNA vector cells; parental NIH3T3; and BRCA1^{S} cells

Western blot analysis

Cell extracts were prepared from exponentially growing cells, the protein concentration in the extracts were determined by Bradfords method (Bio Rad) and $\approx$18–87 μg of protein from each of the extracts were loaded on a 10% SDS–PAGE as described previously (Rao *et al.*, 1989). After electrotransfer onto PVDF membrane, the filter was probed with a polyclonal BRCA1 peptide antibody and visualized using a chemiluminiscent assay as described by the manufacturer (Clontech kit) and exposed to Kodak X-AR film. Duplicate SDS–PAGE gels were run for each experiment and stained with coomassie blue.

MTT dye assay

The MTT metabolic assay was performed as described (Loveland *et al.*, 1992). In brief, cells were seeded at a density of 1×10^4 cells into microtitre plates and incubated in 10% FBS or 0.1% FBS media. At five 24 h intervals viable cells were stained for 4 h with the MTT dye and absorbance was read at 570 nm. The experiments were performed in duplicates or triplicates and reproduced at least three times.

Anchorage independence assay

Soft agar growth assay was done in 0.3% agar/DMEM/10% FBS and plated on a base of 0.5% agar/DMEM/10% FBS. Cells were plated at a concentration of 2×10^3 cells per 35 mm plate in soft agar containing DMEM and 10% FBS. Colonies greater than 100 μm in diameter were scored after 3 weeks. Each soft agar assay was performed in triplicates.

Immunohistochemistry

MCF7 and Rat1 cells cultured in chamber slides were washed in PBS, fixed with 3.7% formaldehyde in PBS at room temperature for 30 min. This was followed by washes in PBS and 30 min block in blocking serum (VECTASTAIN, ABC system from Vector). The specimens were incubated with primary BRCA1 carboxyterminal peptide antibody diluted 1 : 100 at room temperature for 2 h. After washing with PBS, slides were incubated for an hour with diluted biotinylated secondary antibody solution. The slides were washed with PBS and incubated for 30 min with Vectastain ABC reagent. Slides were further washed and incubated for 5 min in 0.01% H_2O_2–0.05% DAB solution. Slides were washed for 5 min in water, mounted in cytoseal 60 (Stephens scientific) and photographed on a immunofluorescence microscope (Olympus).

Immunoprecipitation

Briefly, confluent 100 mm plates of HL60 cells were labelled with [^{35}S] trans label for 3 h. The cells were lysed in radio immunoprecipitation assay buffer. Following sedimentation the supernatants were subjected to immunoprecipitation using rabbit anti-BRCA1 peptide antibody or preimmune serum as described previously (Rao and Reddy, 1993). The samples were subjected to 12% SDS polyacrylamide gel electrophoresis, fluorography and autoradiography.

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BRCA1 proteins are transported to the nucleus in the absence of serum and splice variants BRCA1a, BRCA1b are tyrosine phosphoproteins that associate with E2F, cyclins and cyclin dependent kinases

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BRCA1, a familial breast and ovarian cancer susceptibility gene encodes nuclear phosphoproteins that function as tumor suppressors in human breast cancer cells. Previously, we have shown that overexpression of a BRCA1 splice variant BRCA1a accelerates apoptosis in human breast cancer cells. In an attempt to determine whether the subcellular localization of BRCA1 is cell cycle regulated, we have studied the subcellular distribution of BRCA1 in asynchronous and growth arrested normal, breast and ovarian cancer cells using different BRCA1 antibodies by immunofluorescence and immunohistochemical staining. Upon serum starvation of NIH3T3, some breast and ovarian cancer cells, most of the BRCA1 protein redistributed to the nucleus revealing a new type of regulation that may modulate the activity of BRCA1 gene. We have also characterized two new variant BRCA1 proteins (BRCA1a/p110 and BRCA1b/p100) which are phosphoproteins containing phosphotyrosine. Immunofluorescence and Western blotting analysis indicate cytoplasmic and nuclear localization of BRCA1a and BRCA1b proteins. To elucidate the biological function of BRCA1, we created a bacterial fusion protein of glutathione-transferase (GST) and BRCA1 zinc finger domain and detected two cellular proteins with molecular weights of approximately 32 and 65 kD, one of which contains phosphotyrosine designated p32 and p65 BRCA1 interacting proteins (BIP) that specifically interact with BRCA1. Western blot analysis of BIP with cyclins/CDKs and E2F antisera indicated association with cdc2, cdk2, cdk4, cyclin B, cyclin D, cyclin A and E2F-4 but not with cdk3, cdk5, cdk6, E2F-1, E2F-2, E2F-3, E2F-5 and cyclin E. Furthermore, we have also demonstrated a direct interaction of *in vitro* translated BRCA1a and BRCA1b proteins with recombinant cyclin A, cyclin B1, cyclin D1, cdc2, cdk2 and E2F fusion proteins *in vitro*. Taken together these results seem to suggest that BRCA1 could be an important negative regulator of cell cycle that functions through interaction with E2F transcriptional factors and phosphorylation by cyclins/cdk complexes with the zinc ring finger functioning as a major protein-protein interaction domain. If the interactions we observe *in vitro* is also seen *in vivo* then it may be possible that lack or impaired binding of the disrupted BRCA1 proteins to E2F, cyclins/CDKs in patients with mutations in the zinc finger domain could deprive the cell of an important mechanism for braking cell proliferation leading to the development of breast and ovarian cancers.

Keywords: BRCA1a; BRCA1b; zinc finger; cyclins; CDKs; E2F

Introduction

Mutations in the breast and ovarian cancer susceptibility gene BRCA1, accounts for half of the inherited breast and ovarian cancers (Miki *et al.*, 1994; Easton *et al.*, 1995; Ford *et al.*, 1995) and only 10% of the sporadic ovarian cancers (Futreal *et al.*, 1994; Hosking *et al.*, 1995; Merajver *et al.*, 1995). The BRCA1 cDNA codes for a 1863 amino acid protein with an amino terminal zinc ring finger domain and a carboxy terminal acidic region (Miki *et al.*, 1994) typical of several transcriptional factors. Recently, the C terminal region of BRCA1 was shown to activate transcription in a heterologous GAL-4 system (Chapman and Verma, 1996; Monteiro, 1996; Rao *et al.*, unpublished results). Several groups have cloned and studied the developmental patterns of expression of murine BRCA1 (Lane *et al.*, 1995; Marquis *et al.*, 1995; Abel *et al.*, 1995; Sharan *et al.*, 1995). Expression was found to be high in rapidly proliferating tissues (Lane *et al.*, 1995; Marquis *et al.*, 1995) particularly those undergoing differentiation suggesting a role for BRCA1 in cellular growth and differentiation. We and others have shown the BRCA1 gene product to be a nuclear phosphoprotein (Chen *et al.*, 1995; Rao *et al.*, 1996; Scully *et al.*, 1996), that when over expressed in breast and ovarian cancer cells results in growth inhibition *in vitro* and *in vivo* in breast cancer cells (Holt *et al.*, 1996; Rao *et al.*, unpublished results). Conversely, inhibition of BRCA1 expression by antisense RNA in mouse fibroblasts or by antisense oligonucleotides in breast cancer cells resulted in transformation of mouse fibroblasts as well as increased the rate of growth of breast cancer cells (Thompson *et al.*, 1995; Rao *et al.*, 1996). We have recently reported a new function for the BRCA1 splice variant BRCA1a in the regulation of apoptosis of human breast cancer cells (Shao *et al.*, 1996). Previously, the BRCA1 gene product was shown to be localized in the nucleus (Chen *et al.*, 1995; Rao *et al.*, 1996). Since then there have been several

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differences in the literature regarding the size and subcellular location of BRCA1 (Chen *et al.*, 1995, 1996; Jensen *et al.*, 1996; Scully *et al.*, 1996; Thakur *et al.*, 1997; Wilson *et al.*, 1997). Two proteins BARD1 and Rad51, a human homolog of bacterial Rec A were shown to interact both *in vitro* and *in vivo* with BRCA1 indicating a role for BARD1 in tumor suppression of BRCA1 and a role for BRCA1 in the control of recombination and genomic integrity (Wu *et al.*, 1996; Scully *et al.*, 1977). In the present study, we have further investigated the subcellular localization of BRCA1 protein using several BRCA1 specific antibodies in normal, breast and ovarian cancer cells grown under different serum culture conditions and conclude that the BRCA1 proteins are nuclear phosphoproteins that are transported to the nucleus in the absence of serum. Our results suggest that BRCA1a and BRCA1b, two BRCA1 splice variants that are localized mainly in the cytoplasm with a small fraction going into the nucleus, are phosphoproteins containing phosphotyrosine that associate via their amino-terminal zinc ring finger domain with E2F transcriptional factors, cyclins/cdk complexes suggesting a function for this domain in mediating protein-protein interaction and a role for BRCA1 in cell cycle regulation.

Results

Subcellular localization of BRCA1 proteins

In an attempt to understand the normal function of the BRCA1 protein, we have generated polyclonal antibodies against different regions of the human BRCA1 protein. Initially, we have studied the subcellular distribution of BRCA1 in normal human mammary epithelial cell Hs578 Bst and several human breast cancer cell lines HBL-100, CAL-51, MDA MB-453, T-47D, BT-474, CAMA-1 and ZR 75-1 by immunohistochemistry and immunofluorescence staining. BRCA1 was detected mainly in the cytoplasm with weak nuclear staining of normal human breast epithelial cell Hs578 Bst and several breast tumor cell lines HBL-100, MDA MB-453, T-47D, BT474, CAMA-1 and ZR 75-1 (Figure 1a). One cell line CAL-51, which was originally obtained fro a patient with invasive adenocarcinoma with extensive intraductal involvement (Gioanni *et al.*, 1990), contained three distinct populations of cells, some in which BRCA1 was localized in the cytoplasm, some in the perinucleus and some in the nucleus (Figure 1a). We next studied the subcellular distribution of BRCA1 in two ovarian carcinoma cell lines NIH:OVCAR-3 and SK-OV-3. In NIH:OVCAR-3 cells BRCA1 was localized mainly to the cytoplasm (Figure 1b) and in SK-OV-3 BRCA1 was localized mainly in the nucleus (Figure 1b). We also studied the subcellular distribution of BRCA1 in several normal and tumor cells. BRCA1 was found to be distributed mainly in the cytoplasm of NIH3T3 mouse fibroblast cells and Saos-2 cells (Figure 1c), both in the cytoplasm and nucleus of BRACA1a transfected NIH3T3 cells, HeLa, Colo 320, A431 and PC12 cells (Figure 1c) and mainly in the nucleus with weak cytoplasmic staining of BALB/3T3 cells (Figure 1c). All these results suggested variable, subcellular distribution of BRCA1 proteins. These results were obtained using different BRCA1 antibodies.

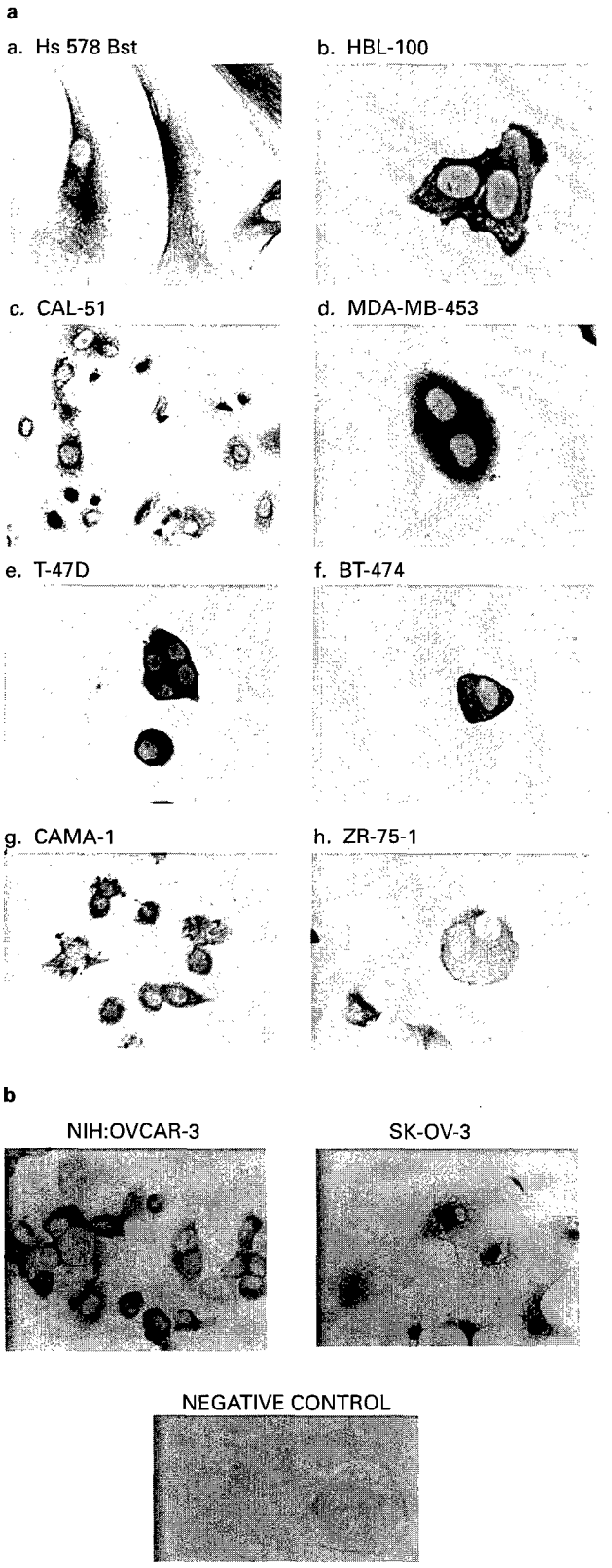
Subcellular localization and tyrosine-phosphorylation of BRCA1a and BRCA1b proteins

We have next studied the *in vivo* phosphorylation of BRCA1 proteins in HL60 cells. Immunoprecipitation of lysates from HL60 cells labeled with ^{32}P-phosphoric acid using BRCA1 polyclonal antibody revealed three major bands with molecular weights of $\approx$175, 125 and 110 kD (Figure 2a). All these results suggest BRCA1 to be a phosphoprotein. In order to explain the differences in the subcellular localization of BRCA1, we speculated whether this could be due to the presence of multiple splice variants wherein some could be preferentially sequestered in the cytoplasm and some could translocate to the nucleus as observed recently by other investigators (Thakur *et al.*, 1977; Wilson *et al.*, 1977). So, we subcloned the differentially spliced human BRCA1 cDNA, BRCA1a into pFLAG-CMV-2 expression vector (Eastman Kodak Company) which contains a FLAG epitope-tag sequence at the N-terminal and this tag allows detection of BRCA1a protein with the use of FLAG antibody. When transfected into COS cells the FLAG BRCA1a protein was found to be localized mainly in the cytoplasm with weak nuclear staining (Figure 2b). Subcellular fractionation of the transfected COS cells into total and nuclear fractions followed by Western blot analysis using the same FLAG antibody revealed two polypeptides migrating at 105–110 kD mainly in the cytoplasm with a small fraction of the total BRCA1a protein in the nuclear fractions, which agrees with our immunofluorescence data. These results suggest BRCA1a p110 to be a localized both in the cytoplasm and nucleus, the doublet protein bands observed could represent hyper and hypophosphorylated forms of BRCA1a proteins, similar to the 220 kDa BRCA1 protein (Chen *et al.*, 1996a). Western blot analysis of FLAG immunoprecipitates obtained from p-FLAG-CMV-2-BRCA1a transfected COS cells using phosphotyrosine antibodies revealed BRCA1a to be a phosphoprotein containing tyrosine (Figure 2c lane 3) which migrated with mobility similar to an endogenous BRCA1 tyrosine phosphorylated band seen in HL60 cells (Figure 2c). All these results suggest that BRCA1a is an $\approx$110 kD phosphoprotein which contains tyrosine. Similarly, BRCA1b was also found to be a phosphoprotein phosphorylated on tyrosine migrating with a molecular weight of $\approx$100 kD (data not shown). We next studied the subcellular distribution of FLAG-BRCA1a in breast cancer cell line CAL-51 after transfection of pFLAG-CMV-2-BRCA1a plasmid into these cells followed by immunofluorescence analysis. Here, we observed BRCA1a protein to be distributed both in the cytoplasm as well as in the nucleus of serum fed cells, the cytoplasmic staining appeared to be much stronger compared to the weak nuclear staining (Figure 2d).

Intracellular localization of BRCA1 proteins in serum-grown and serum deprived mouse fibroblasts

Our results suggested that the interesting differences in the subcellular localization of BRCA1 observed by us

and others may not be due to using different BRCA1 antibodies or aberrant localization of BRCA1 in breast tumor cells. We therefore speculated whether this could be due to culturing the cells in different serum culture conditions. In order to test the hypothesis whether the nuclear localization of BRCA1 is dependent on the proliferation state of the cell, we studied the subcellular distribution of BRCA1 in asynchronous and serum deprived NIH3T3 cells using different BRCA1-specific antibodies developed by us by immunofluorescence and immunohistochemical methods. In asynchronous serum fed NIH3T3 cells BRCA1 was found to be localized predominantly in the cytoplasm with weak nuclear staining (Figure 3a). In contrast, in serum starved quiescent cells most of the BRCA1 was found to be localized in the nucleus with little cytoplasmic staining.



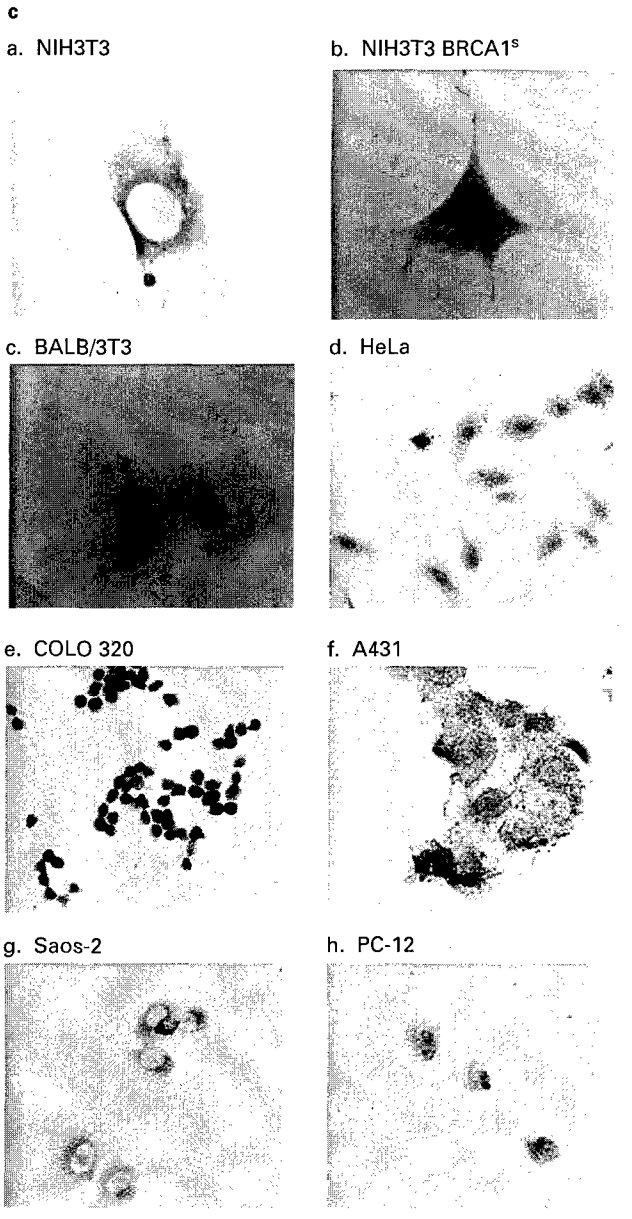


Figure 1 Subcellular localization of BRCA1 in normal human mammary epithelial cells, breast and non breast cancer cells and ovarian carcinoma cells by immunohistochemical staining using carboxy terminal BRCA1 peptide antibody. (**a**) Localization of BRCA1 in normal human mammary epithelial cells and human breast cancer cell lines. a, HS 578 Bst; b, HBL-100; c, CAL 51; d, MDA-MB-453; e, T-47D; f, BT-474; g, CAMA-1; h, ZR-75-1. (**b**) Localization of BRCA1 in human ovarian carcinoma cells by immunohistochemistry. NIH:OVCAR-3 cells; SK-OV-3 cells and negative control. (**c**) Localization of BRCA1 in several normal and cancer cells. a, NIH3T3; b, NIH3T3 BRCA1s; c, BALB/3T3; d, HeLa; e, COLO320; f, A431; g, Saos-2; h, PC-12 cells

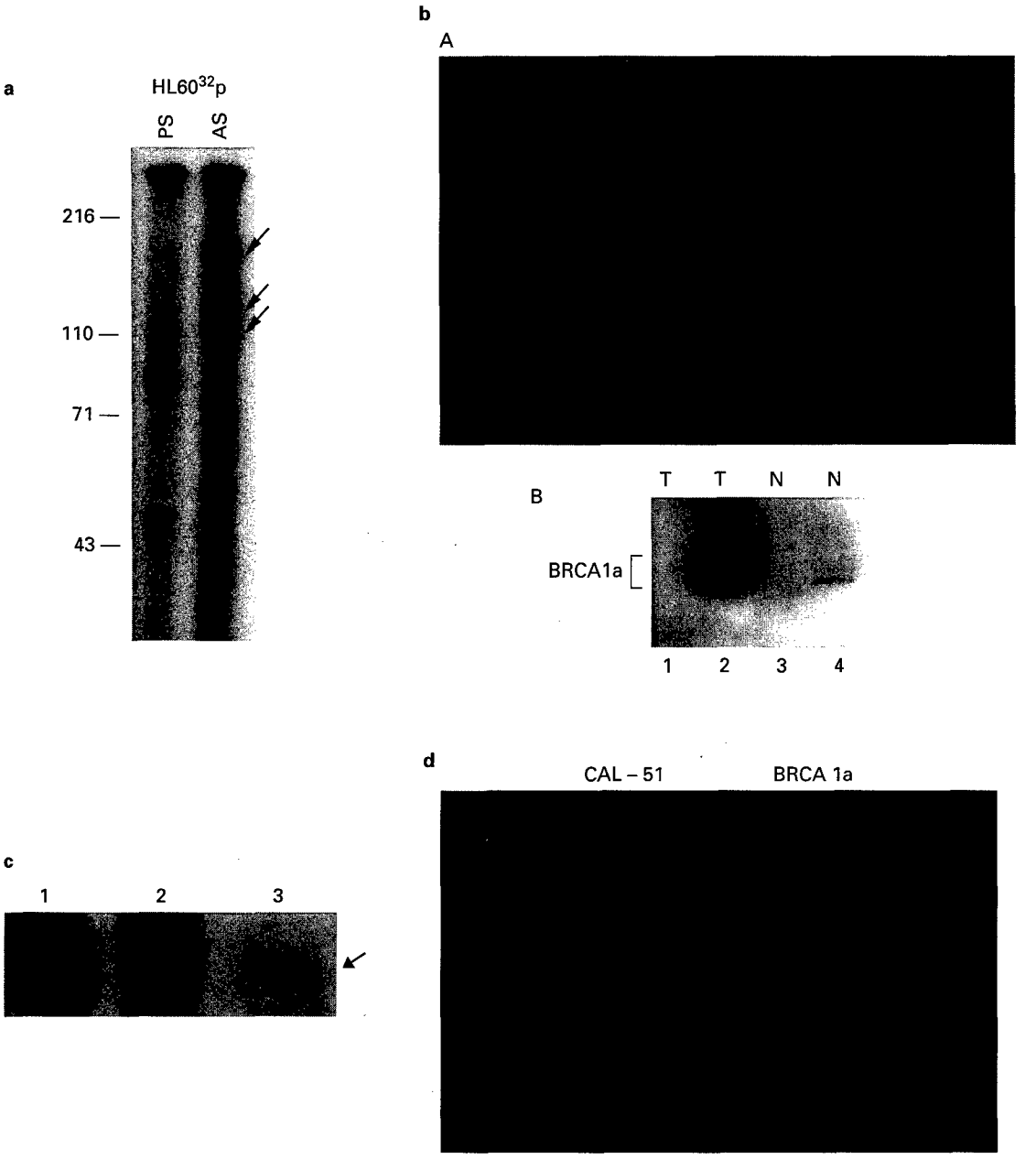


Figure 2 Subcellular localization and tyrosine phosphorylation of endogenous BRCA1 and FLAG-BRCA1a proteins in HL60, CAL-51 cells and COS cells by immunofluorescence and Western blot analysis. (**a**) Immunoprecipitation of ^{32}P-labeled HL60 cells with carboxy terminal BRCA1 peptide antibody. The three arrows represent the phosphorylated BRCA1 protein bands. (**b**) A, Immunostaining of COS cells transfected with FLAG-BRCA1a with FLAG antibody showing mostly cytoplasmic and weak nuclear staining; B, Western blot analysis of COS cells transfected with FLAG-BRCA1a with FLAG antibody shows BRCA1a to be localized both in the total (nucleus and cytoplasm) and nuclear fractions of COS cells. (**c**) Western blot analysis of FLAG-BRCA1a transfected COS cells shows BRCA1a to be a phosphoprotein containing phosphotyrosine. Lane 1 represents HL60 cells immunoprecipitated with pre-immune serum and Western blotted using phosphotyrosine antibody; lane 2 represents HL60 cells immunoprecipitated with carboxyterminal BRCA1 peptide antibody and then Western blotted using phosphotyrosine antibody; lane 3 represents COS cells transfected with CMV2 FLAG-BRCA1a, immunoprecipitated with FLAG antibody and Western blotted using phosphotyrosine antibody. The arrow represents the 110 kD BRCA1a protein. (**d**) Immunostaining of CAL-51 cells transfected with CMV2-FLAG-BRCA1a using FLAG antibody, shows BRCA1a to be localized mainly in the cytoplasm with weak nuclear staining

On prolonged incubation in serum free media for 48–72 h the staining was mostly nuclear with typical nuclear dot like pattern (data not shown). When the cells were refed with 10% serum for 24 h the BRCA1 protein was found to relocate back to the cytoplasm with weak nuclear staining similar to the situation seen in asynchronous serum fed NIH3T3 cells (Figure 3a). The same results were obtained using four different antibodies specific to different regions of BRCA1 protein, (data not given) thus ruling our the possibility that nuclear BRCA1 immunofluorescence might be an artifact due to starvation. Simultaneously, we have also determined the DNA content of these cells in the presence and absence of serum by FACS analysis to give a measure of the relative percentage of G1, S, G2/M cells in each sample. All these results seem to suggest that nuclear transport of BRCA1 is not cell cycle dependent and irrespective of the cell cycle state,

BRCA1 protein accumulates in the nucleus in the absence of serum and in the cytoplasm in the presence of serum, in NIH3T3 cells. We have also arrested the cells in G1 phase of the cell cycle by using drugs such as aphidicolin, mimosine and double thymidine block and studied the subcellular localization of BRCA1 protein. Our results suggest that the redistribution of the BRCA1 proteins to the nucleus in drug-treated cells is not as dramatic as seen with serum starved cells (Shao and Rao, unpublished results). It may be possible that accumulation of BRCA1 proteins in the nucleus of serum depleted NIH3T3 cells may be responsible for the induction of apoptosis seen previously in BRCA1a transfected NIH3T3 cells (Shao *et al.*, 1996). Two splice variants BRCA1 Δ672-4095 (Thakur *et al.*, 1997) and BRCA1 Δ11b which codes for a 110 kD protein (Wilson *et al.*, 1997) were both shown to be localized to the cytoplasm by immunostaining. Surprisingly, the authors found substantial levels of BRCA1 Δ11b to be in the nuclear fraction on immunoblotting (Wilson *et al.*, 1997), similar to what we have observed with BRCA1a and BRCA1b in COS cells. The molecular weight of the p110 kDa BRCA1a polypeptide reported in this and earlier studies (Shao *et al.*, 1996) is comparable to the 110 kDa BRCA1 Δ11b. Thus even though the reported nuclear localization signal (Thakur *et al.*, 1997; Wilson *et al.*, 1997) for BRCA1 is missing in BRCA1a and BRCA1b proteins, some of which still gets transported to the nucleus suggesting that both BRCA1a and BRCA1b may carry potential nuclear localization signals. It may be possible that there can be a serum-responsive regulatory protein that could be responsible for retention of BRCA1 protein within the cytoplasm of serum fed cells similar to NF-kB (Baeuerle and Baltimore, 1988) and the levels of which could determine the subcellular localization of BRCA1 protein. We are presently investigating these possibilities.

Subcellular localization of BRCA1 proteins in breast and ovarian cancer cells grown in the presence and absence of serum

Since all these experiments were done in mouse fibroblasts, which may not be physiologically relevant to BRCA1 function we studied the subcellular distribution of BRCA1 in asynchronous and growth arrested serum deprived normal human breast epithelial cells Hs578 Bst, breast cancer cells HBL-100, ZR-75-1, CAMA-1 and ovarian carcinoma cells NIHOVCAR-3, using different BRCA1 specific polyclonal antibodies by indirect immunofluorescence and immunohistochemical methods. We have used only those cell lines in which BRCA1 was found to be localized to the cytoplasm in asynchronous conditions. Our results suggest BRCA1 to be localized mainly in the cytoplasm of serum fed asynchronous normal breast epithelial cells Hs578 Bst, breast tumor cells HBL-100, ZR-75-1, CAMA-1 and ovarian carcinoma cell NIHOVCAR-3 (Figure 3b–f) and predominantly in the nucleus of growth arrested serum deprived Hs578 Bst, HBL-100, ZR-75-1 CAMA-1 and ovarian carcinoma cell line NIHOVCAR-3 (Figure 3b–f). All these results suggest that the nuclear or cytoplasmic transport of BRCA1 is not spontaneous but is controlled by the extracellular environment with serum growth factors inhibiting the nuclear transport of the BRCA1 protein. It remains to be seen whether posttranslational modification (such as phosphorylation, etc.) of BRCA1 proteins plays a role in the subcellular localization of these proteins. The scenario we observe with BRCA1 is different to that seen for the c-Fos protein whose nuclear translocation appears to depend on the continuous stimulation of cells by serum factors (Roux *et al.*, 1990).

Detection of cellular proteins that interact with GST-BRCA1 fusion protein

Since the subcellular localization of BRCA1 is dependent on the presence of serum factors, and to investigate whether BRCA1 protein-protein interactions are of biological significance in the growth inhibitory, tumor suppressor and death inducing functions of BRCA1 protein, we studied the interaction of BRCA1 with cellular proteins. The amino terminal region of BRCA1 contains a zinc ring finger domain (Miki *et al.*, 1994) which are known to interact with DNA/RNA either through direct binding or indirectly by mediating protein-protein interactions. We have expressed a fusion protein that contains GST and the zinc finger domain of BRCA1 (residues 1–76) in bacteria using the Gex 2T expression vector system. In order to detect cellular proteins that interact with GST-BRCA1 fusion protein, whole cell lysates of human breast cancer cells ZR-75-1 or CA1-51 metabolically labeled with ^{35}S-methionine were incubated with either GST or the GST-BRCA1 fusion protein immobilized on glutathione-agarose beads (GSH-beads). The beads were washed, lysed in SDS sample buffer and subjected to SDS–PAGE. SDS–PAGE analysis of the bound complex revealed bands with relative molecular weight of $\approx$32 kD and $\approx$65 kD which bound exclusively to the GST-BRCA1 fusion protein and not to GST (Figure 4a). These bands were consistently detected in ZR-75-1, CAL-51 and HL 60 cell lysates and were designated as p65 BIP and p32 BIP respectively (data not shown).

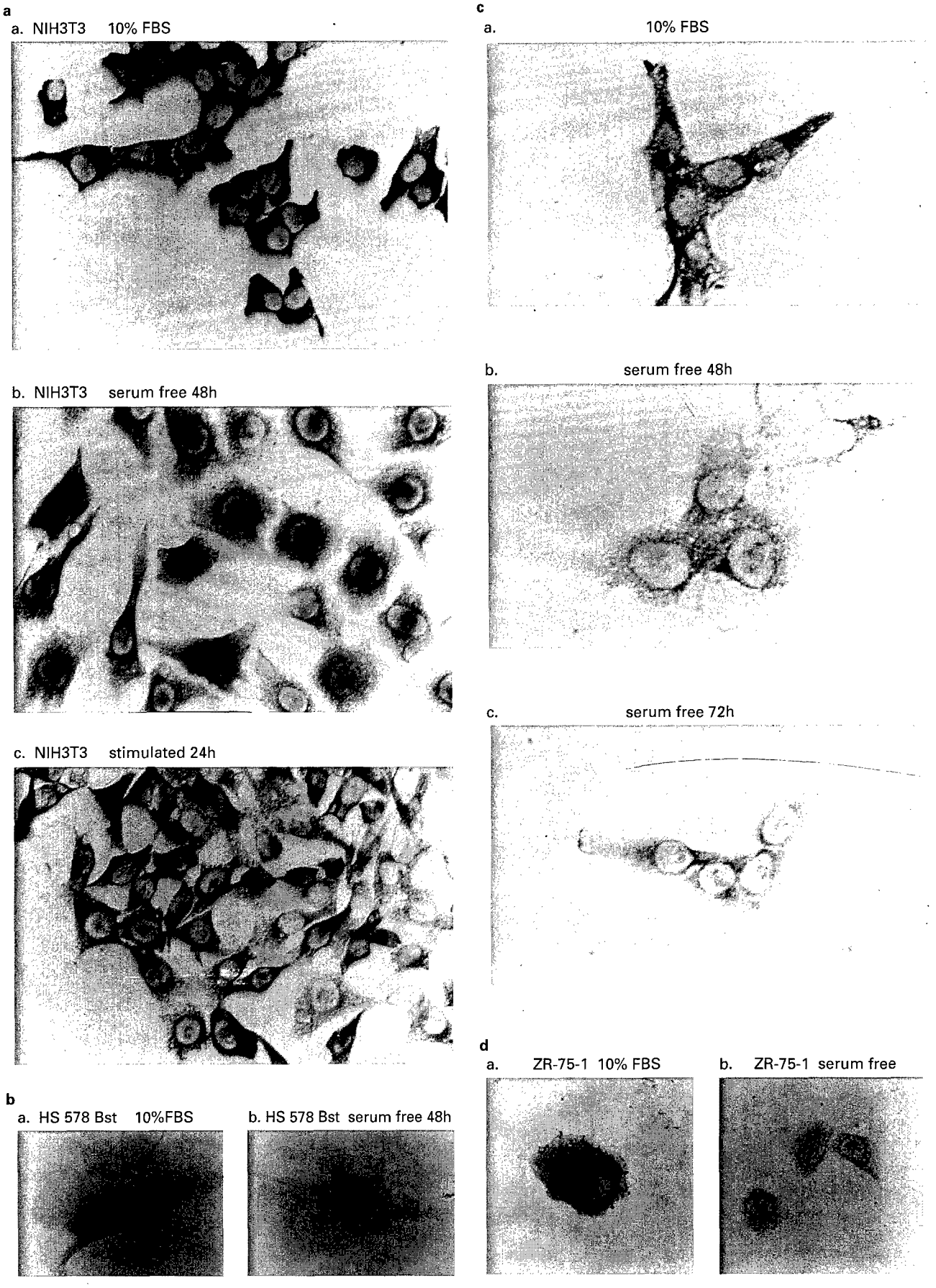
Direct association of p65 BIP and p32 BIP with BRCA1

To determine whether p65 BIP and p32 BIP directly interact with BRCA1, we performed a far Western blot analysis. The BIP complexes prepared from CAL-51 cell extract were separated on SDS–PAGE, transferred onto a nitrocellulose membrane, and probed with ^{32}P-labeled GST-TK-BRCA1 fusion protein. Since GST-TK-BRCA1 contains consensus phosphorylation site for protein kinase C at the amino terminal end, the purified protein can be ^{32}P-labeled by an *in vitro* protein kinase reaction. GST-BRCA1 bound to both p65 BIP and p32 BIP unlike GST protein (Figure 4b). These results suggest that both p65 BIP and p32 BIP bind strongly to BRCA1 even under stringent conditions without any mediating proteins (Figure 4b).

Expression of BRCA1 binding proteins in different cell lines

To determine the distribution of BRCA1-binding protein in various cell lines, we metabolically labeled

promyelocytic cell line HL60, breast cancer cell lines ZR-75-1 and CAL-51 cells and performed the GST-pull down assay as described previously. Both p65 BIP and p32 BIP were detected in all cell lysates examined (data not given) although at variable levels suggesting the ubiquitous expression of p65 BIP and p32 BIP.



Association of BRCA1 proteins with E2F, cyclins/CDKs

Recently, cdk-2 and other kinases associated with cyclins D and A were shown to phosphorylate BRCA1, suggesting a role for cdk's in regulating the activity of BRCA1 proteins (Chen, 1996a) and the growth inhibitory function of BRCA1 also suggested a role in cell cycle control (Holt *et al*., Rao *et al*.,

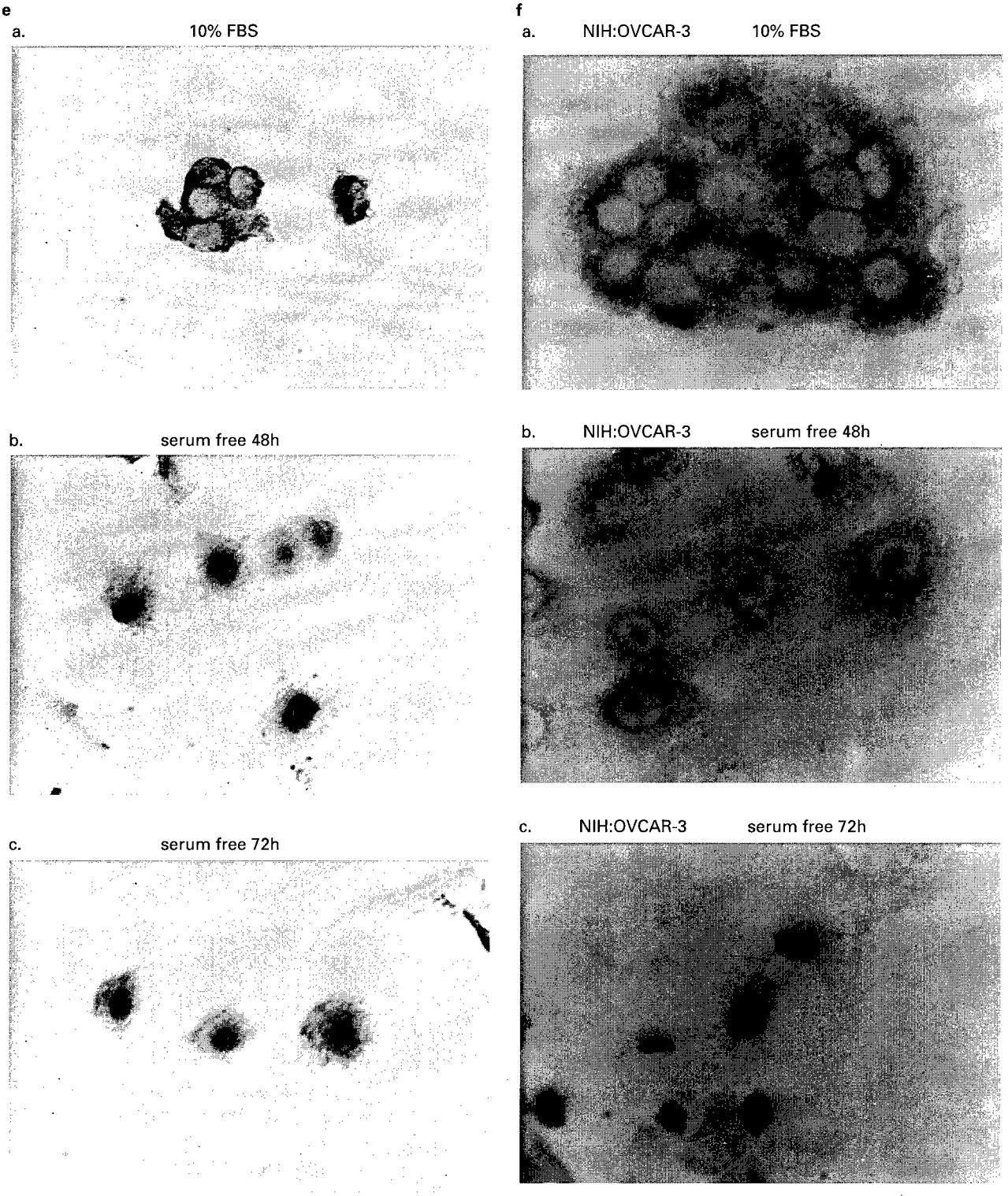
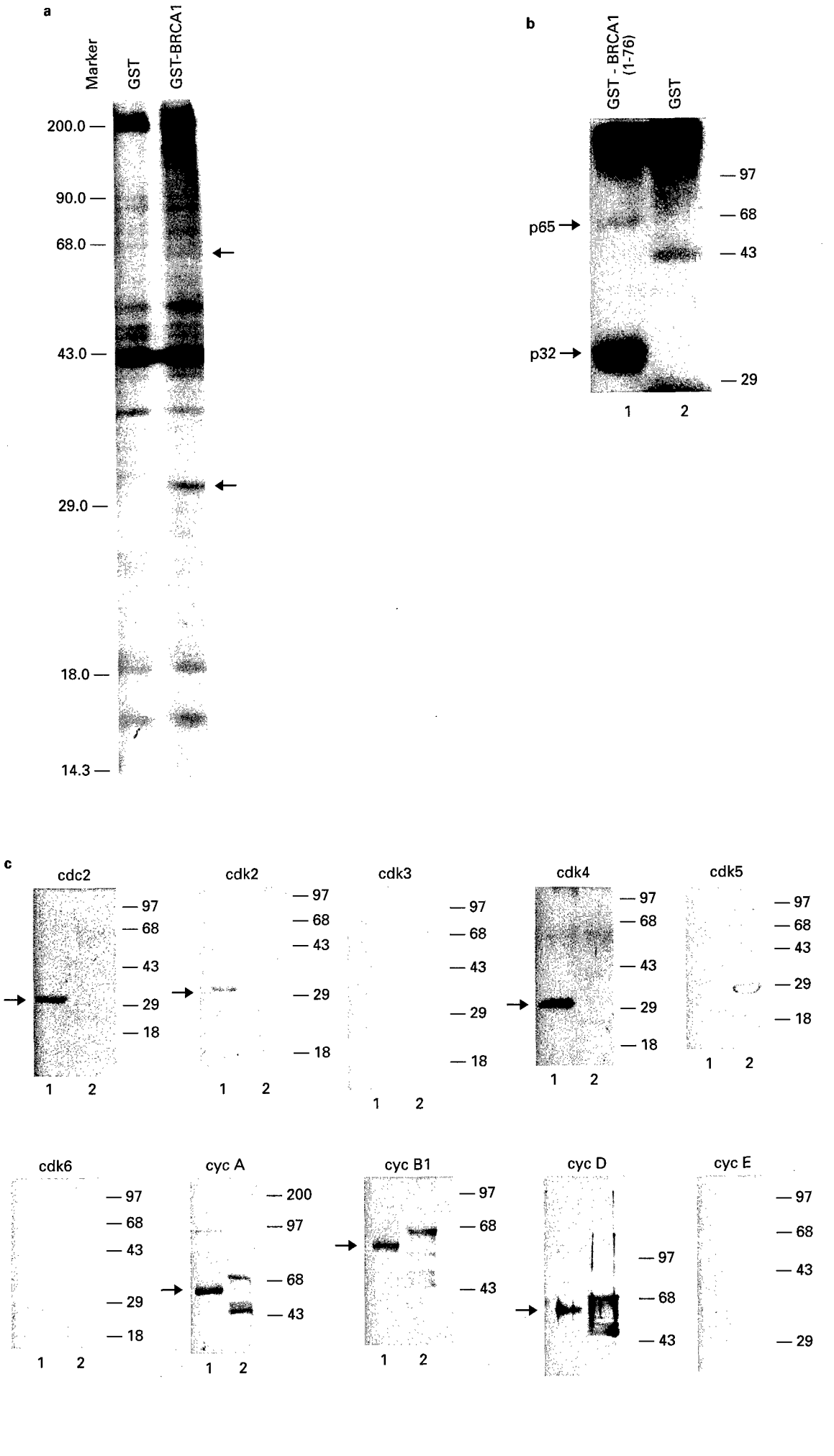


Figure 3 BRCA1 localizes to the nucleus in serum deprived NIH3T3, normal mammary epithelial cells, breast and ovarian cancer cells by immunohistochemical analysis. Intracellular localization of BRCA1 protein in serum-starved and serum fed NIH3T3 cells. (**a**) using amino-terminal BRCA1 peptide antibody. The above results were also confirmed using carboxy terminal BRCA1 peptide antibody, and an antibody raised against recombinant BRCA1 protein (aa 1–331), ethyl green was used as a positive control for nuclear staining (data not given). (**b**) BRCA1 redistributed to the nucleus in serum deprived normal human mammary epithelial cells HS578 Bst using immunohistochemical analysis. (**c**) BRCA1 translocated to the nucleus in serum deprived human breast cancer cells HBL-100. (**d**) ZR-75-1 cells, and (**e**) CAMA-1 cells using immunohistochemical analysis. (**f**) BRCA1 was found to translocate to the nucleus of serum deprived human ovarian carcinoma cell line NIHOVCAR-3. The above results were repeated using three different BRCA1 specific antibodies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Marker
GST
GST-BRCA1
200.0
90.0
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b
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cyc A
cyc B1
cyc D
cyc E
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unpublished results). Since tumor suppressor genes like Rb regulate cell cycle by interaction with transcription factor E2F, cyclins/cdk's (Lees *et al.*, 1992; Kato *et al.*, 1993; Ewen *et al.*, 1993; Weinberg, 1995) and the sizes of BIPs are close to E2F, cyclins/CDKs, we speculated whether BRCA1 could similarly associate with E2F, cyclins/CDKs. We therefore incubated cell lysates obtained from CAL-51 cells with GST-immobilized on GSH-beads and BRCA1 fusion protein conjugated GSH-beads. The beads were then washed and heated in SDS sample buffer. The BIP complexes were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membrane and probed with antibodies specific to cdc2, cdk2, cdk3, cdk4, cdk5, cdk6, cyclin A, cyclin B1, cyclin D1, cyclin E, E2F-1, E2F-2, E2F-3, E2F-4 and E2F-5 as described previously (Rao *et al.*, 1996). The BIP complexes were recognized by antibodies specific to cdc2, cdk-2, cdk-4, cyclin D1, cyclin A, cyclin B1 (Figure 4c) and E2F-4 (Figure 4d), but not to cdk3, cdk-5, cdk-6, cyclin E, E2F-1, E2F-2, E2F-3 and E2F-5. All these results suggest association of BRCA1 with cyclin A, D1 and B1, cdc2, cdk-2, cdk-4 and E2F-4, but not to cdk-3, cdk-5, cdk-6, cyclin E, E2F-1, E2F-2 and E2F-5.

Tyrosine phosphorylation and kinase activity of BRCA1 interacting proteins

We next wanted to examine whether p32 BIP and p65 BIP contain phosphotyrosine. The BIP complexes obtained from CAL-51 cells were immunoblotted with a phosphotyrosine antibody (Figure 4d). Band corresponding to p32 was detected (Figure 4d) indicating that p32 BIP contains phosphotyrosine, a characteristic specific to cyclin associated protein kinases. We have also tested the BRCA1 immunoprecipitates from CAL-51 cells for kinase activity. Our

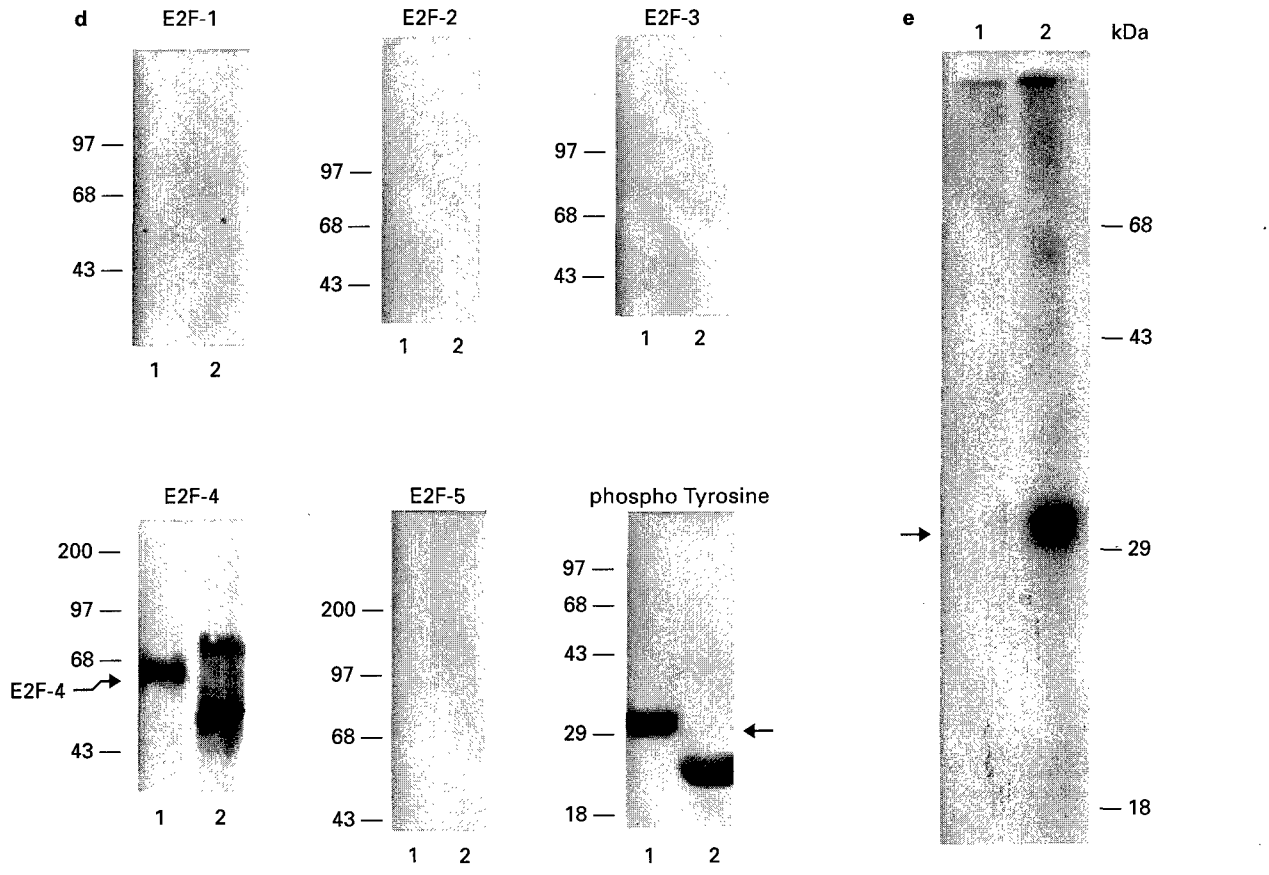
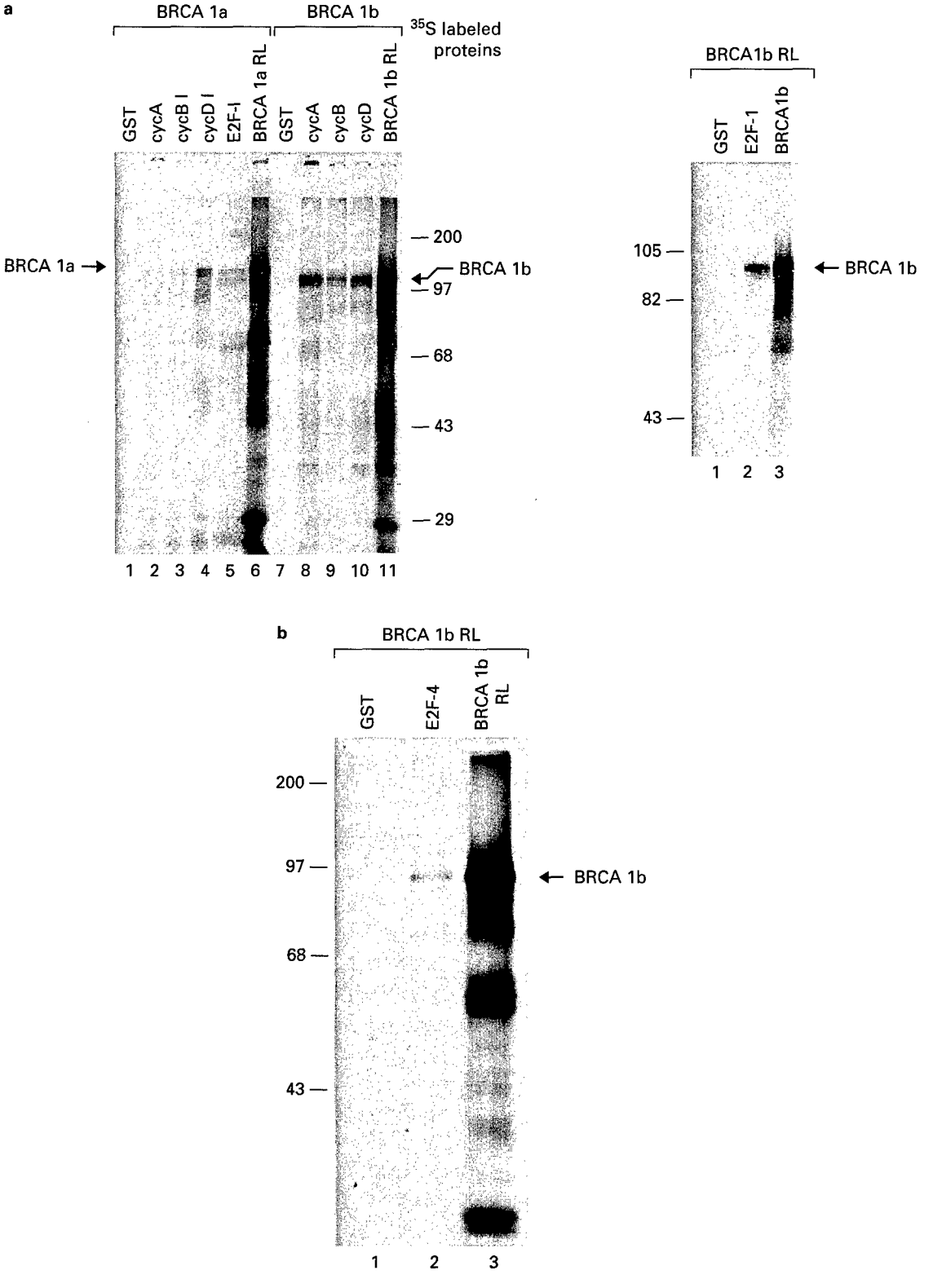


Figure 4 Identification of BRCA1 interacting proteins (BIP) that associate with E2F's cyclins/CDKs in breast cancer cells. (**a**) Detection of BRCA1-binding proteins. Lysates of ^{35}S-methionine-labeled CAL-51 cells were incubated with either GST or the GST-BRCA1 fusion protein immobilized on GSH beads. The bound proteins were washed, subjected to 10% SDS–PAGE and visualized by fluorography. The position of the major ≈p32 BIP complex is indicated on the right with a solid arrow and the minor ≈p65 BIP complex is represented by a small arrow on the right. The positions of the ^{14}C-labeled protein standards and their sizes are shown on the left. (**b**) Direct binding of BRCA1 with p32 BIP and p65 BIP by far Western blot analysis. CAL-51 cell extracts were passed through GST and GST-BRCA1 immobilized on glutathione beads. The protein complexes were separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes. After blocking, the filters were incubated with the ^{32}P-labeled GST-BRCA1 fusion protein. The arrows represent the p32 and p65 BIP proteins. (**c**) Association of BRCA1 with cyclin/cdk's. Unlabeled cell extract from CAL-51 cells were incubated with GST conjugated GSH-BRCA1 beads (lane 1) or with GST-conjugated GSH-beads (lane 2). The beads were then washed and subjected to 10% SDS–PAGE. The proteins were transferred onto nitrocellulose membrane and subjected to Western blot analysis using antibodies specific to cdc2, cdk2, cdk3, cdk4, cdk5, cdk6, cyclin A, cyclin B, cyclin D and cyclin E obtained from Santa Cruz Biotechnology. (**d**) Association of BRCA1 with transcriptional factor E2F-4 and the p32 BIP complex are tyrosine phosphoproteins. CAL-51 cell extracts were passed through GST-BRCA1 GSH beads (lane 1) or GST-GSH beads (lane 2) and the bound proteins were subjected to SDS–PAGE and Western blot analysis using antibodies specific to E2F-1, E2F-2, E2F-3, E2F-4, E2F-5, and phosphotyrosine obtained from Santa Cruz Biotechnology. The arrows represent E2F-4 and tyrosine phosphorylated p32 BIP proteins respectively. (**e**) Kinase activity of BRCA1 immunocomplexes on histone H1. Lane 1, buffer control; lane 2, histone H1. The arrow represents phosphorylated histone H1

results demonstrate that these immunocomplexes show histone H1 kinase activity confirming the association of BRCA1 with cyclins/cdk kinase complexes (Figure 4e).

In vitro *interactions of BRCA1a, BRCA1b with cyclins, E2F and CDKs*

To examine the binding of cyclin A, cyclin B1, cyclin D1, E2F-1 and E2F-4 with BRCA1a and BRCA1b *in vitro*, we inserted the cDNA sequences encoding BRCA1a and BRCA1b into pcDNA3 expression vectors (Rao *et al*., unpublished results). *In vitro* transcription and translation of BRCA1a and BRCA1b in the presence of [^{35}S]methionine generated radiolabeled BRCA1a and BRCA1b polypeptides of ~110 and ~100 kD respectively (Figure 5a lane 6 and 11). These radiolabeled proteins were passed through GST-cyclin A, GST-cyclin B1, GST-cyclin D1, GST-



E2F-1, GST-E2F-4 and GST respectively. Both *in vitro* translated BRCA1a and BRCA1b specifically bind to GST-cyclin A, GST-cyclin B1, GST-cyclin D1, GST-E2F-1 and GST E2F-4 unlike GST alone (Figure 5a, b). The BRCA1a splice variant bound at a reduced level compared to BRCA1b to all these different proteins. It may be possible that phosphorylation may regulate the binding of BRCA1a and BRCA1b proteins to cyclins, cdk's and E2F transcriptional factors. To confirm our results, further GST and GST E2F-1 fusion proteins were subjected to far Western blot analysis using ^{32}P-labeled amino-terminal BRCA1 (GST-BRCA1a amino acids 1–76, numbering from first ATG codon) fusion protein. The GST E2F-1 fusion protein band hybridized specifically to BRCA1 (Figure 5c). To further confirm the results, a fragment of BRCA1 encoding the amino-terminal 182 amino acids (amino acids 1–182) was *in vitro* translated and assayed for binding to GST-E2F-1 protein. The BRCA1 polypeptides bound specifically to GST-E2F-1 (Figure 5c). In a reciprocal assay, we *in vitro* translated the full length human E2F-1 cDNA and assayed it for binding to GST-BRCA1 zinc finger fusion protein (amino acids 1–76). The E2F-1 polypeptide bound very weakly to GST BRCA1 (Figure 5c). These results suggest that the amino-terminal 76 amino acids of BRCA1 were sufficient to provide specific association with E2F-1. The amount of E2F-1 and E2F-4 that got bound to BRCA1 appeared to be relatively low. It may be possible that E2Fs may need to associate with other proteins in order to bind efficiently to BRCA1. Similarly, we have *in vitro* translated human cdc2, cdk2, cdk4 and cdk5. The proteins thus generated were assayed separately for binding to GST and GST-BRCA1 (amino acids 1–76) fusion proteins. GST-BRCA1 specifically bound to cdc2 and cdk2 (Figure 5d) but not to cdk4 and cdk5 (data not given). All these results suggest that BRCA1 zinc finger domain can interact directly with cdc2 and cdk2.

Discussion

In the present report, we have presented data regarding the subcellular localization; phosphorylation and protein-protein interactions of BRCA1 and two BRCA1 isoforms, BRCA1a and BRCA1b. Initially, we have generated polyclonal antibodies against different regions of the human BRCA1 protein and used them to analyse BRCA1 expression in several normal, breast and ovarian cancer cells grown under different serum culture conditions. Our results suggest that BRCA1 proteins accumulate in the nucleus in the

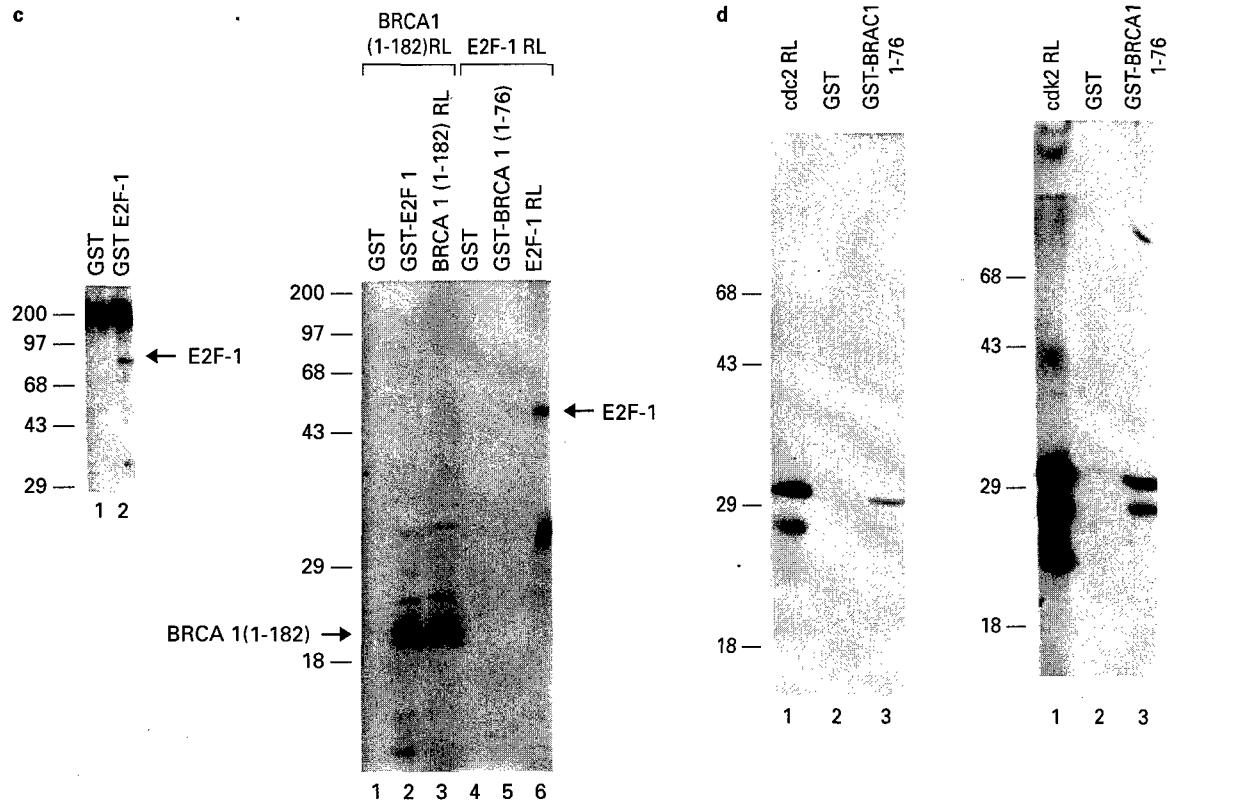


Figure 5 Direct physical interaction *in vitro* of BRCA1a and BRCA1b with cyclins, CDKs, E2F-1 and E2F-4 transcriptional factors. (**a**) *In vitro* translated BRCA1a and BRCA1b bind to GST-cyclin A, GST-Cyclin B1, GST-Cyclin D1, GST-E2F-1, 2 μl of the *in vitro* translated BRCA1a and BRCA1b were run as controls. Arrows represent *in vitro* translated BRCA1a (p110) and BRCA1b (p100). (**b**) *in vitro* translated BRCA1b binds to GST-E2F-4. Arrow represents *in vitro* translated BRCA1b (p100). (**c**) The panel on the left represents far Western blot analysis of BRCA1 (amino acids 1–76) binding directly to E2F-1. The arrow represents the position of GST-E2F-1 fusion protein. The panel on the right represents *in vitro* translated BRCA1 (amino acids 1–182) and *in vitro* translated full length E2F-1 binding to GST-E2F1 and GST-BRCA1 (amino acids 1–76) respectively. The arrows represent *in vitro* translated BRCA1 (amino acids 1–182) and E2F-1. (**d**) *In vitro* translated full length cdc2 and cdk2 bind to GST-BRCA1 (amino acids 1–76). 2 μl of *in vitro* translated cdc2 (left panel) and cdk2 (right panel) were loaded as controls

absence of serum and in the cytoplasm in the presence of serum in NIH3T3 cells and some breast and ovarian cancer cells. It may be possible that the nuclear or cytoplasmic transport of BRCA1 is not spontaneous but is controlled by the extracellular environment with serum inhibiting the nuclear transport of the BRCA1 protein. Alternatively, it may be possible that a protein factor present in the cytoplasm of serum fed cells could be responsible for retention of BRCA1 protein within the cytoplasm, similar to NF-KB (Baeuerle and Baltimore, 1988) and the levels of which could determine the subcellular localization of BRCA1 protein. We are presently investigating these possibilities.

The BRCA1 cDNA codes for a 1863 amino acid protein (Miki *et al.*, 1994) with an amino terminal zinc ring finger motif and two putative nuclear localization signals, suggesting that it might be a nuclear protein (Miki *et al.*, 1994; Lane *et al.*, 1995). There have been several discrepancies in the literature regarding the size and subcellular localization of BRCA1 (Chen *et al.*, 1995, 1996; Rao *et al.*, 1996; Wilson *et al.*, 1997; Scully *et al.*, 1996; Jensen *et al.*, 1996). Previously, we have reported BRCA1 proteins to be localized mainly in the nucleus and some in the cytoplasm of Rat-1 and MCF7 cells (Rao *et al.*, 1996). Subsequently, we have isolated two new alternately spliced BRCA1 transcripts referred to as BRCA1a (p110) and BRCA1b (p100) (Rao *et al.*, unpublished results) and developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1a proteins (Shao *et al.*, 1996). Our results indicate that over expression of BRCA1a induces apoptosis in NIH3T3 and MCF-7 cells after calcium ionophore treatment indicating that BRCA1 genes may play a critical role in the regulation of apoptosis (Shao *et al.*, 1996). In this report, we have studied the subcellular localization of BRCA1a and BRCA1b proteins using FLAG epitope tagged CMV promoter vectors. Our data using immunofluorescence and immunoblotting analysis indicate cytoplasmic and nuclear localization of FLAG BRCA1a and FLAG BRCA1b proteins. Recently, two other groups (Thakur *et al.*, 1997; Wilson *et al.*, 1997) have identified two BRCA1 splice variants BRCA1Δ672-4092 (which lacks exon 11) and BRCA1Δ11b (which lacks majority of exon 11) both of which were found to localize to the cytoplasm by immunostaining. These investigators have mapped a nuclear localization signal in exon 11 of BRCA1 which is missing in BRCA1Δ672-4095 and BRCA1Δ11b suggesting that splicing may regulate the function of BRCA1 by altering the subcellular localization of these proteins (Thakur *et al.*, 1997; Wilson *et al.*, 1997). Interestingly, BRCA1Δ11b was also found to be present in significant quantities in the nuclear fractions on immunoblotting analysis (Wilson *et al.*, 1997), similar to what we have observed with BRCA1a and BRCA1b proteins (this study). We have also found the BRCA1 proteins to accumulate in the cytoplasm in the presence of serum and in the nucleus in the absence of serum. All these results suggest that the nuclear localization of BRCA1 may be regulated by external stimuli, phosphorylation or protein-protein interactions. BRCA1 expression was shown to be high in tissues that are undergoing rapid growth and differentiation (Lane *et al.*, 1995; Marquis *et al.*, 1995). Recently the BRCA1 protein was also shown to be expressed and phosphorylated in a cell cycle dependent manner (Chen *et al.*, 1996). The BRCA1 m-RNA levels were found to be high in exponentially growing cells and in cells just prior to entry into S-phase but decreased upon growth factor withdrawal or after treatment with transforming growth factor β-1 (Gudas *et al.*, 1996) suggesting cell cycle regulation of BRCA1 expression. In an attempt to isolate proteins that interact with BRCA1, we have detected two cellular proteins (p65 BIP, p32 BIP) that specifically interact with BRCA1. Western blot analysis of BIP indicated association with E2F, cyclins and CDKs and *in vitro* translated BRCA1a and BRCA1b proteins interacted directly with transcription factor E2F, cyclins and CDK's suggesting a role for these proteins in regulating the biological activity of BRCA1 proteins.

In summary our results indicate BRCA1 gene products to be nuclear tyrosine phosphoproteins (Rao *et al.*, 1996; Chen *et al.*, 1995; this study) that translocate to the nucleus in the absence of serum, function as tumor/growth suppressors (Rao *et al.*, 1996; Rao *et al.*, unpublished results); Holt *et al.*, 1996), inducers of apoptosis (Shao *et al.*, 1996) and associate *in vitro* with E2F transcriptional factors, cyclins and cdk complex, suggesting a role for CDKs in regulating the biological activity of BRCA1. Several critical growth regulators like the product of the pRB, a tumor suppressor protein have been shown to associate with E2F both *in vivo* and *in vitro* (Weinberg, 1995) resulting in net inhibition of E2F-mediated transactivation and E2F reslease from pRB is an important event in the activation of genes required for S-phase entry (Weinberg, 1995). It may be possible that tumor suppressor proteins like BRCA1 may similarly interact with E2Fs and regulate cell proliferation. The interaction of BRCA1 zinc ring finger domain to E2Fs, cyclins/cdk complexes assigns a function for this domain in mediating protein-protein interaction. The function of BRCA1 zinc ring domain becomes important since it is the lcoation of some of the most frequently occurring mutations linked to breast and ovarian cancers. One of the BRCA1 mutations contains a frame shift in exon 2 (188 del 111; Miki *et al.*, 1994) which removes the zinc finger domain from the protein. The 185 del AG mutation, which is the most common BRCA1 mutation seen to date that occurs 1 in 100 Ashkenazi Jewish individuals (Shattuck-Eiden *et al.*, 1995) disrupts the BRCA1 gene product at the first residue of the C_3HC_4 domain. These frequent missense mutations Cys 61, Gly, Cys 64, Gly, and Cys 64 Tyr also disrupt the zinc finger domain structure. It may be possible that lack or impaired binding of the disrupted BRCA1 protein to E2F, cyclins/CDKs in patients with mutations in the zinc ring domain could deprive the cell of an important mechanism for regulating cell proliferation leading to the development of breast cancer. Future efforts will be directed towards showing the identity of p32 BIP and p65 BIP and identifying the cyclins/CDKs and E2Fs family members which specifically interact with BRCA1 *in vivo*. It remains to be seen whether the interactions we observe *in vitro* can be seen *in vivo* and if they are physiologically relevant.

Materials and methods

Cell lines

NIH3T3 cells and their derivatives, MCF-7, MDA-MB-453 and A431 cells were grown at 37°C in DMEM supplemented with 10% fetal bovein serum (FBS), 1% penicillin-streptomycin (PS); HS578 Bst cells were grown in DMEM supplemented with 10% FBS, 1% PS and 30 ng/ml EGF, CAL-51 cells were grown in DMEM containing 10% FBS, 1% PS, 0.6 μg/ml bovine insulin, 5×10^{-3} μg/ml transferrin and 146 mg/Liter glutamine; ZR 75-1 and COLO 320 were grown in RPMI 1640 supplemented with 10% FBS, 1% PS and 10 μg/ml bovine insulin; NIH:OVCAR-3 cells were cultured in RPMI 1640 supplememted with 20% FBS, 1% PS and 10 μg/ml bovine insulin; PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum and 5% FBS, 1% PS; SK-OV-3 and HBL 100 cells were grown in McCoy's 5a medium supplemented with 10% FBS and 1% PS; Saos-2 cells were grown in McCoy's 5a medium supplemented with 15% FBS and 1% PS; CAMA-1 cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% FBS and 1% PS. All the cell lines except CAL-51 cells were obtained from American type culture collection (Rockville, MD).

Immunochemistry

The different cell lines in the logarithmic stage of growth cultured in chamber slides were made quiescent in the presence of DMEM only for a period of 24–72 h. Cells were then washed in PBS and subjected to immunohistochemistry analysis using rabbit anti BRCA1 carboxyterminal or an amino terminal peptide antibody (Santa Cruz Biotechnology Inc.), rabbit polyclonal antibody generated against GST fusion proteins containing amino acids 1 to 76 and amino acids 1 to 331 of the BRCA1 protein as described previously (Rao *et al.*, 1996).

Plasmid construction and transfections

BRCA1a cDNA and BRCA1b cDNA (Rao, unpublished results) were subcloned into pFLAG-CMV vector (Eastman Kodak Company) by PCR. Purified DNA 5 μg for chamber slides and 20 μg for 100 mm petri dishes of pFLAG-CMV-2 expression vector or pFLAG-CMV-2 expression vector containing the BRCA1a cDNA were transfected into COS and CAL-51 cells using the Invitrogen kit according to the manufacturer's recommendations. After 48–72 h post transfection cells were processed for immunofluorescence analysis or the cell extracts were subjected to Western blot analysis using FLAG M2 antibody (Eastman Kodak Company) as described previously (Rao *et al.*, 1996).

Immunofluorescence

Immunofluorescence analysis was done as described previously (Rao 1996; Shao *et al.*, 1996) except with slight modifications. In brief, pFLAG-CMV-2 BRCA1a transfected COS and CAL-51 cells cultured in chamber slides were fixed in 3.7% formaldehyde at room temperature, followed by washing in PBS and PBS with 0.05% Triton X-100 and blocking in blocking solution (4% normal goat serum, 0.05% Triton X-100 in PBS) for 10 min at room temperature. The cells were incubated with primary anti FLAG M2 antibody diluted 1 : 100 for COS and CAL-51 cells, then washed three times with PBS, blocked with blocking solution for an additional 10 min at room temperature followed by incubation with the secondary antibody (FITC conjugated goat anti mouse IgG). After rinsing in PBS, the slides were mounted with mounting media and photographed on a confocal microscope.

Preparation of total nuclear extract and Western blot analysis

COS cells were harvested 48 h post transfection by washing in PBS and treating with trypsin. For preparing total cell extracts the cells were lysed in RIPA buffer and the lysate was centrifuged at 14 000 r.p.m. for 30 min at 4°C. The supernatant was assayed for protein concentration by Bradford's method (Bio-Rad) and $\approx 50-100$ μg of protein was subjected to Western blot analysis as described previously (Rao *et al.*, 1996). The nuclear extract was prepared as described (Hurst *et al.*, 1990). In brief, cells were lysed in nuclear extract buffer 1 (Hurst *et al.*, 1990), centrifuged at high speed for 1 min at room temperature. The crude nucleus was suspended in nuclear extract buffer II (Hurst *et al.*, 1990). Nuclear debris was removed by centrifugation for 1 min at room temperature. The supernatant was diluted by the addition of 20 mM HEPES (pH 7.4). The protein concentrations were determined by Bradford's method (Bio-Rad) and $\approx 50-100$ μg of protein was subjected to Western blot analysis.

For Western blotting analysis $\approx 50-100$ μg of cell/nuclear extract in SDS sample buffer were loaded on a 10% SDS–PAGE in Bio-Rad mini-protean II cell as described previously (Rao *et al.*, 1996). After electro transfer onto PVDF membrane, the FLAG-BRCA1a fusion protein was detected with anti-FLAG M2 antibody diluted 1 : 100 using Western exposure chemiluminescent detection system from Clonetech or ECL as described previously (Rao *et al.*, 1996).

Metabolic labeling of cells

Briefly, confluent 100 mm plates of HL 60 cells were labeled with ^{32}P-orthophosphoric for 4 h. The cells were lysed in radioimmunoprecipitation assay buffer. Following sedimentation the supernatants were subjected to immunoprecipitation using rabbit anti BRCA1 peptide or recombinant protein antibody or preimmune serum as described previously (Rao *et al.*, 1996). The samples were subjected to 10% SDS polyacryladmide gel electrophoresis and autoradiography. In some cases the cold HL60 cell lysates were subjected to immunoprecipitation using carboxyterminal BRCA1 peptide antibody and then subjected to Western blot analysis using phosphotyrosine antibody (Santa Cruz Biotechnology).

Expression and purification of GST fusion protein

Expression and purification of GST fusion proteins were described previously (Rao and Reddy, 1993) except with slight modification (Frangioni and Neel, 1993). Briefly, log phase cultures of *E. coli* BL21 (DE3) LysS transformed with the pGEX 2TK-BRCA1 (aa 1–76), pGEX2T-BRCA1 (aa 1–76), pGEX 2T-E2F-1, pGex2T-CycA, pGEX2T-cycB1, pGEX2T-cycD1, pGEX2T-E2F-4 plasmids were incubated with IPGT for 3 h. The cells were pelleted in STE buffer (Frangioni and Neel, 1993) containing 100 μg/ml lysozyme, 5 mM DTT, 1 mM PMSF and 2% Sarkosyl, sonicated on ice and centrifuged at 10 000 *g* for 10 min. To the supernatant Triton X-100 was added and applied to a glutathione sepharose 4B column (Pharmacia) and the GST-BRCA1 or GST-cyclins or GST-E2F fusion proteins were either left immobilized or eluted with elution buffer containing glutathione (Rao and Reddy, 1993). The GST-BRCA1 fusion proteins were labeled with ^{32}P as described (Kaelin *et al.*, 1992). In brief, the GST-TK-fusion protein probes were labeled in a 100 μl final volume containing 20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$, 10 μCi of (γ-^{32}P) ATP, 1 μg GST-fusion protein and 100 units of cAMP dependent protein kinase (Sigma) on ice for 30 min.

GST pull down assay

CAL-51, ZR 75-1 or HL 60 cells were labeled with ^{35}S-methionine as described previously (Rao *et al.*, 1996). The cells were washed in cold phosphate buffered saline (PBS) and scrapped into 1 ml of TNN buffer (Takashima *et al.*, 1994) and lysed by rotating for 30 min at 4°C. The lysates were centrifuged at 14 000 *g* for 30 min and subjected to protein binding assay as described (Takashima *et al.*, 1994). For protein binding assay cell extracts were precleared overnight with GSH-beads and then incubated with either GST protein-conjugated GSH-beads or GST-BRCA1 (containing residue 1 to 76 of the BRCA1a protein). Proteins were incubated with GSH beads for 2 h at 4°C. The beads were then washed in TNN buffer and boiled in SDS sample buffer and loaded on a 10% SDS–PAGE. The gels were fixed, treated with enhance, dried and exposed to X-ray films. For *in vitro* binding experiments 10–20 μl of full length *in vitro* translated BRCA1a, BRCA1b, BRCA1 (amino acids 1–182), cdc2, cdk2, cdk4, cdk5 and E2F-1 were tested for binding to GST-E2F-1 or GST-E2F-4 as described previously for GST pull down assay.

Immunoprecipitation and in vitro *kinase assays*

CAL-51 cells were lysed in 1 ml TNN buffer and immunoprecipitated with recombinant BRCA1 polyclonal antibody (amino acid 1–76) as described previously (Rao *et al.*, 1996). The immunoprecipitates were washed in kinase buffer and measured for kinase activity toward histone H1 as described previously (Makela *et al.*, 1994).

Far Western blot analysis

Far Western blot analysis was done as described previously (Kaelin *et al.*, 1992; Singh *et al.*, 1989) except with slight modifications. In brief, after transfer the nitrocellulose membrane was washed in 1 × HBB buffer (Singh *et al.*, 1989) and treated sequentially with 1 × HBB buffer containing different concentrations of guanidine HCl ranging in concentration from 6 M to 0.19 M. The membrane was hybridized in Hyb 75 buffer (Kaelin *et al.*, 1992) containing 0.1 mM $ZnCl_2$ and ^{32}P-labeled GST-TK-BRCA1 protein (10^6 c.p.m./ml) overnight at 4°C. Subsequently the membrane was washed in Hyb 75 buffer, air dried and exposed to X-ray film.

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Induction of apoptosis by the tumor suppressor protein BRCA1

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The breast and ovarian cancer susceptibility gene BRCA1, is a nuclear phosphoprotein which functions as a tumor suppressor. To investigate the role of BRCA1 in apoptosis, we have developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1. The expression of BRCA1 protein in the BRCA1 transfectants were analysed by immunofluorescence and immunohistochemistry. The BRCA1 transfectants showed a flattened morphology compared to the parental cells. We show that serum deprivation or calcium ionophore treatment of BRCA1 transfectants resulted in programmed cell death. These results indicate that BRCA1 genes may play a critical role in the regulation of apoptosis. Thus, since a wide variety of human malignancies like breast and ovarian cancers have a decreased ability to undergo apoptosis, this could be due to lack/decreased levels of functional BRCA1 proteins. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies.

Keywords: BRCA1 proteins; tumor suppressor; breast and ovarian cancers; apoptosis; therapy

Introduction

Breast cancer is one of the most common malignancy affecting women in the United States and ovarian cancer although less frequent than breast cancer is the fourth leading cause of cancer mortality among women. The breast and ovarian cancer susceptibility gene BRCA1, was recently isolated and the conceptual cDNA encodes a 1863 aminoacid protein with an amino-terminal Zinc finger domain and a carboxy terminal acidic region (Miki *et al.*, 1994). We and others have identified multiple BRCA1 proteins approximately 185-220, 160, 145, 100, 52 and 38 kD in both human and mouse cells (Rao *et al.*, 1996; Chen *et al.*, 1995). It remains to be seen whether these proteins represent isoforms of BRCA1 or its related proteins. Recently, we like others have identified alternatively spliced transcripts of the BRCA1 gene (Rao, unpublished results). A recent report indicated that the BRCA1 gene product is localized in the nucleus of several normal cell lines including breast and tumor cells other than breast and ovary (Chen *et al.*, 1995). They have detected BRCA1 mainly in the cytoplasm of almost all breast and ovarian cancer cell lines examined. These results suggested aberrant subcellular localization of BRCA1 in breast cancer (Chen *et al.*, 1995). Interestingly, our results show BRCA1 to be localized mainly in the nucleus (or perinuclear) or cytoplasm or both of several normal or cancer cells (Rao, unpublished results) indicating variable subcellular localization of the BRCA1 proteins. Our results suggest that the subcellular localization of BRCA1 may be determined by the cell cycle status of the cells (Rao, unpublished results). Our results show that BRCA1 interacts with cyclin dependent kinases suggesting a role for BRCA1 in cell cycle regulation (Rao, unpublished results). We have previously reported that the BRCA1 gene product to be a nuclear protein with tumor suppressor function in mouse fibroblast cells since inhibition of endogenous BRCA1 expression by antisense RNA to BRCA1 resulted in neoplastic transformation (Rao *et al.*, 1996). We have recently observed that introduction of variant BRCA1 gene into human cancer cells results in suppression of growth and neoplastic phenotype (Rao *et al.*, unpublished results) implicating a direct role for BRCA1 in growth and tumor suppression.

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Results and discussion

To study the function of BRCA1 genes in the regulation of apoptosis, we have transfected NIH3T3 cells with pcDNA expression vector or pcDNA expression vector containing human BRCA1 cDNA and obtained stable G418 resistant cell lines expressing BRCA1. These BRCA1^s cell lines were analysed for BRCA1 protein expression by indirect immunofluorescence analysis (Figure 1a) and immunoperoxidase staining (Figure 1a) using BRCA1 polyclonal antibody as described previously (Rao *et al.*, 1996). The nuclear and cytoplasmic staining was brighter and stronger in BRCA1^s transfectants compared to parental NIH3T3 cells (Figure 1a). The morphology of the BRCA1^s transfectants were different from that of the parental NIH3T3 cells. The BRCA1^s cells are shorter and flatter when compared to the parental NIH3T3 cells (Figure 1b) and with several weeks in continuous culture the BRCA1^s cells become spindle shaped with elongated processes leading to their detachment. These results suggest that constitutive high level expression of the BRCA1 gene product for a prolonged period of time may result in apoptosis.

One of the most efficient ways of triggering the apoptotic response in fibroblasts is the removal of serum (Jimenez *et al.*, 1995). Thus we next investigated the effect of serum withdrawal on the induction of apoptosis in the BRCA1^s transfectants and compared it with that of the NIH3T3 cells. Subconfluent NIH3T3 and BRCA1^s cells were grown in medium containing

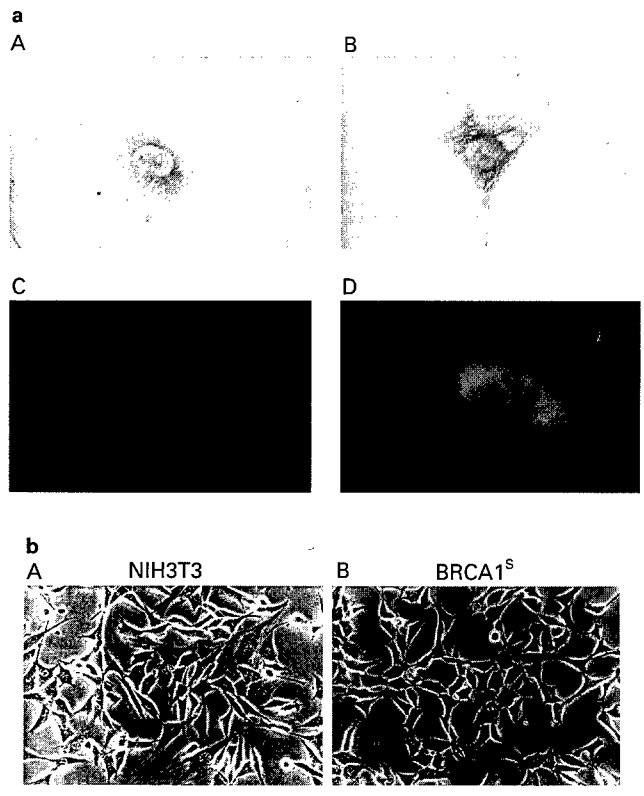


Figure 1 Detection of BRCA1 protein(s) in NIH3T3 and BRCA1^S cells by immunohistochemically and immunofluorescence analysis. (**a**) Immunoperoxidase (A, B) and immunofluorescence (C, D) analysis. A. NIH3T3; B, BRCA1^S; C, NIH3T3; D, BRCA1^S. (**b**), Morphology of the BRCA1^S transfectants. Phase-contrast photomicrographs of NIH3T3 (A) and BRCA1^S cell lines cultured in normal media (10% FBS/DMEM)

either 10% or 0% fetal bovine serum (FBS). After 24 h the cells were collected and subjected to flow cytometric analysis. The BRCA1^S transfected cells showed enhanced rates of apoptosis under serum depleted conditions, as measured by the appearance of an additional 'sub G1' peak on flow cytometry (Figure 2a). This peak is associated with high levels of DNA degradation. High levels of apoptotic cells in the sub G_0/G1 peak (A_p) population were measured in BRCA1^S cells (A_p value 75%) whereas the control cell line NIH3T3 showed lower levels of apoptosis under identical conditions (Figure 2a). BRCA1^S cells did not show any significant apoptosis levels when grown under normal conditions of DMEM supplemented with 10% FBS (Figure 2a).

The above results of apoptosis in BRCA1^S transfectants was further comfirmed by DNA fragmentation assay. Here NIH3T3 and BRCA1^S transfectant cells were cultured in serum free media for 24, 48 and 72 h and then analysed for DNA fragmentation in agarose gels. The BRCA1^S transfected cell line showed the production of a typical oligonucleosomal DNA ladder (Figure 2b; B, lanes 1–3) indicating activation of apoptosis. This effect was readily seen for BRCA1^S cell lines cultured in serum free media but not for the parental NIH3T3 cells grown under identical conditions (Figure 2b; A, lanes 1 to 3). These results indicate that over expression of BRCA1 accelerates apoptosis in serum depleted NIH3T3 cells.

Apoptosis can be induced by calcium ionophore, A23187, in thymocytes (McConkey *et al.*, 1989). This led us to examine apoptosis in BRCA1^S cells during A23187 treatment. NIH3T3 and BRCA1^S cells were treated with calcium ionophore A23187 for 24 h and the cell cycle distribution was determined by flow cytometry with propidium iodide staining method (Bendall *et al.*, 1994). Histogram of the DNA content and the percentage of cells in G1, S and G2 plus M of the cell cycle were evaluated by computer analysis using EPICS profile analyzer. The BRCA1^S transfected cells showed accelerated rates of apoptosis (A_p value 82%) in presence of calcium ionophore (Figure 3a). Measurement of apoptosis through the sub G1 peak in the DNA histogram has the advantage of simplicity. But it has the disadvantage that since all the cells are fixed there is no distribution between viable and dead cells. Therefore the viability of both NIH3T3 cells and BRCA1^S cells cultured in the presence of calcium ionophore was tested by crystal violet staining. Cell viability staining showed that majority of the BRCA1^S cells treated with calcium ionophore A23187 were dead whereas most of the control NIH3T3 cells survived (Figure 3). These results suggest that BRCA1 induces death in NIH3T3 cells.

Apoptosis in the BRCA1^S transfectants was further confirmed in two ways. First, NIH3T3 and BRCA1^S cells were cultured in the presence of calcium ionophore to induce apoptosis and the incidence of cell death was determined by phase contrast microscopy after staining the cultures with Hochest 33258 (Oberhammer *et al.*, 1994). Majority of the nuclei of BRCA1^S showed strong chromatin condensation and nuclear degradation into small, spherial nuclear particles of condensed chromatin characteristic of apoptosis (Figure 3c), whereas the parental NIH3T3 cells did not show any significant change in the staining pattern (Figure 3). Second, the analysis of DNA degradation upon treatment of BRCA1^S transfectants with calcium ionophore confirmed induction of apoptosis. Figure 3d shows that the DNA of BRCA1^S cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder whereas the parental NIH3T3 cells had no significant DNA degradation (Figure 3d). These results suggested that calcium ionophore induces apoptosis more readily in BRCA1^S cells than NIH3T3 cells. All the above results shown for one clone of NIH3T3–BRCA1^S cells, have been reproducibly obtained with several other independent clonal isolates of NIH3T3–BRCA1^S cell lines (data not shown).

In an attempt to understand the role of BRCA1 genes in the regulation of apoptosis of human breast cancer cells, we have transfected MCF7 cells with pcDNA expression vector or pcDNA expression vector containing human BRCA1 cDNA and obtained stable G418 resistant cell lines expressing BRCA1 (Rao *et al.*, unpublished results). These BRCA1^S cell lines were analysed for BRCA1 protein expression by indirect immunofluorescence analysis, immunoperoxidase staining and Western blot analysis (Rao *et al.*, unpublished results). Apoptosis in the MCF-7 BRCA1^S transfectants were analysed after treatment with calcium ionophore A23187. MCF-7 and BRCA1^S cells were treated with calcium ionophore A23187 for 24 h and the cell cycle distribution was determined by Flow cytometry with propidium iodide staining. The BRCA1^S transfected MCF-7 cells showed accelerated

rates of apoptosis (A_p value 75%) in the presence of calcium ionophore (Figure 4a). The viability of both MCF-7 cells and BRCA1^s cells cultured in the presence of calcium ionophore was tested by crystal violet staining. Cell viability staining showed that a vast majority of the MCF-7 BRCA1^s cells treated with calcium ionophore A23187 were dead whereas most of the control MCF-7 cells survived (Figure 4b). These results suggest that BRCA1 induces death in MCF-7 cells. The induction of apoptosis in the BRCA1^s transfectants was further confirmed by analysis of DNA fragmentation upon treatment of BRCA1^s transfectants with calcium ionophore. The DNA of BRCA1^s cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder unlike the parental MCF-7 cells (Figure 4c). These results suggest that over expression of BRCA1 in breast cancer cells results in apoptosis.

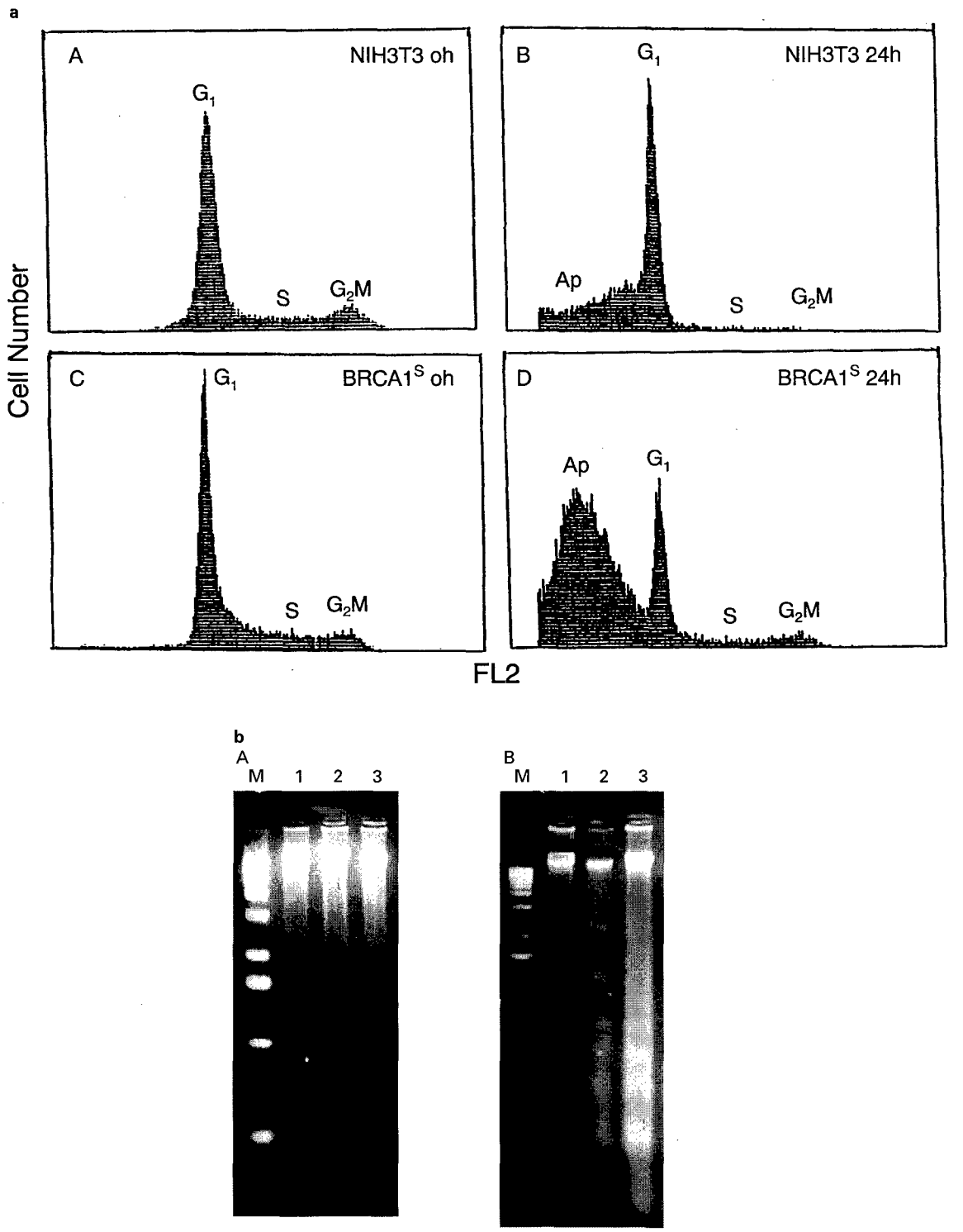


Figure 2 BRCA1 over expression induces apoptosis in serum depleted NIH3T3 cells. (**a**) Flow cytometric analysis of cells induced to undergo apoptosis by serum deprivation. A, NIH3T3, 0 h; B, NIH3T3, 24 h; C, BRCA1^s 0 h; D, BRCA1^s 24 h. (**b**) Over expression of BRCA1 gene induces DNA fragmentation typical of apoptosis in serum deprived cells. A, control NIH3T3 cells serum starved for 24 h (lane 1); 48 h (lane 2); 72 h (lane 3) and B, BRCA1^s cell serum starved for 24 h (lane 1); 48 h (lane 2); 72 h (lane 3)

In summary, our results demonstrate that the BRCA1 gene product which is a nuclear phosphoprotein (Rao *et al.*, 1996; Chen *et al.*, 1995) with tumor suppressor properties (Rao *et al.*, 1996; Rao *et al.*, unpublished results) functions as an inducer of apoptosis similar to rho, c-myc, p53, E1A and rel (Jimenez *et al.*, 1995; Fisher, 1994; Hoffman and Libermann, 1994; Yonish-Rouach *et al.*, 1991). The BRCA1 cDNA used in this study lacks majority of exon 11, suggesting that this region of exon 11 is dispensable for the apoptotic function of BRCA1. The precise mechanism by which BRCA1 triggers cell death remains to be investigated. It may be possible that BRCA1 gene products function as transcriptional regulators that may either activate death inducing genes or repress death inhibiting genes leading to apoptosis. Alternatively, BRCA1 may activate apoptosis inducing

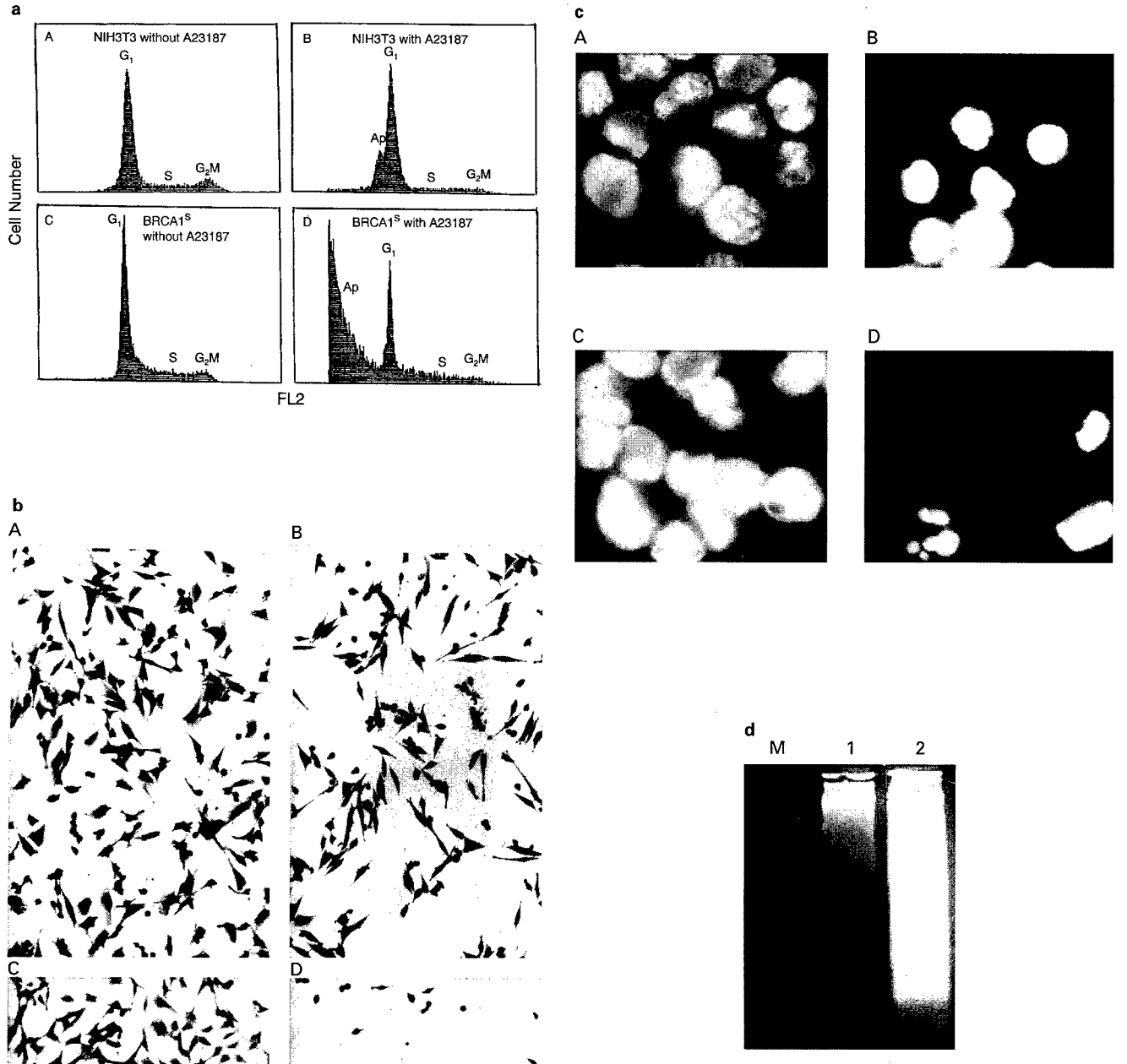
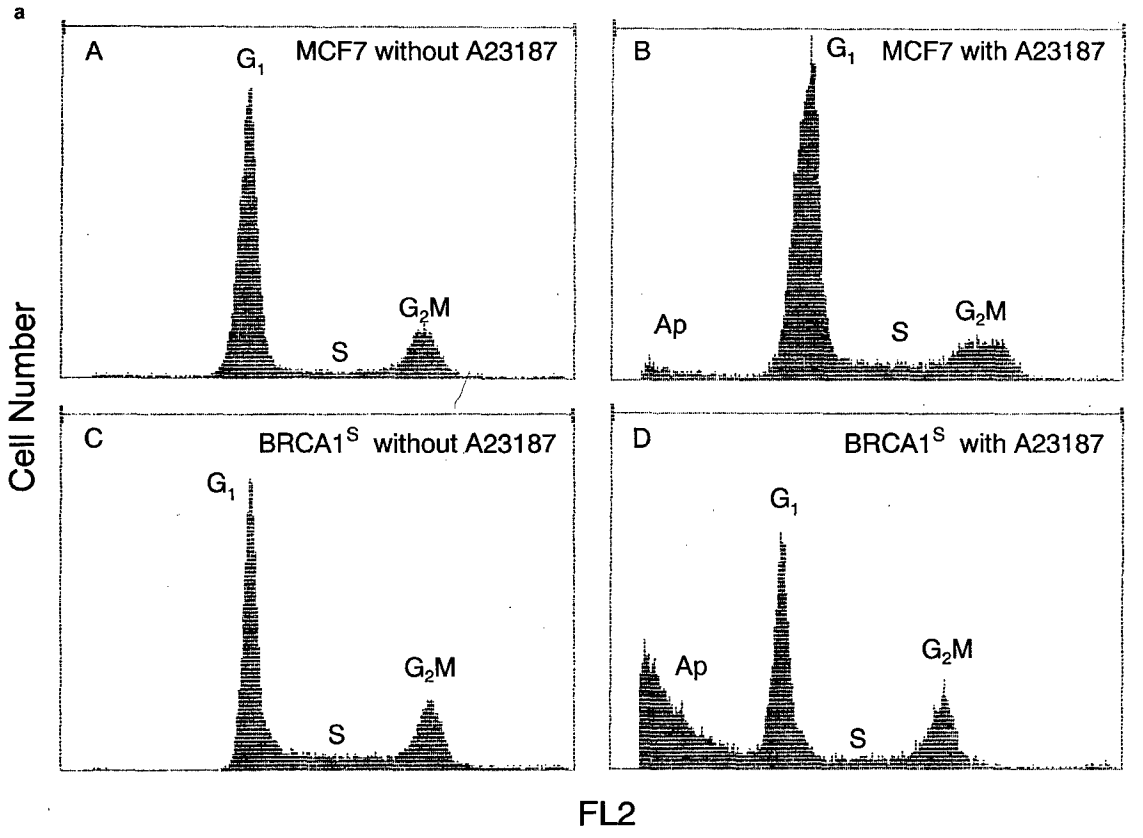
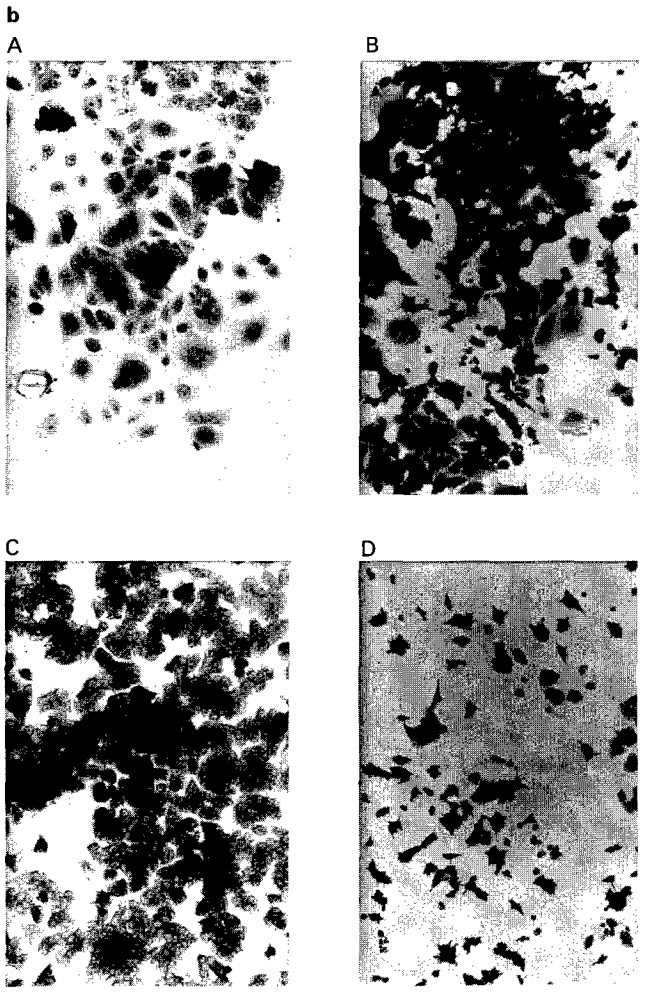


Figure 3 BRCA1 induces apoptosis in NIH3T3 cells after calcium ionophore treatment. (**a**) Flow cytometric analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 24 h. (**b**) Viability of NIH3T3 and BRCA1^s cells treated with and without calcium ionophore for 24 h. A, NIH3T3; B, NIH3T3 treated with calcium ionophore; C, BRCA1^s; D, BRCA1^s treated with calcium ionophore. (**c**) Chromatin condensation shown morphologically by Hoeschst staining of the BRCA1^s cell line induced to undergo apoptosis by calcium ionophore treatment for 24 h. A, NIH3T3 in the absence of calcium ionophore; B, NIH3T3 in the presence of calcium ionophore; C, BRCA1^s in the absence of calcium ionophore; D, BRCA^s in the presence of calcium ionophore. (**d**) DNA fragmentation induced by BRCA1 overexpression. 1, NIH3T3 treated with calcium ionophore; 2, BRCA1^s treated with calcium ionophore





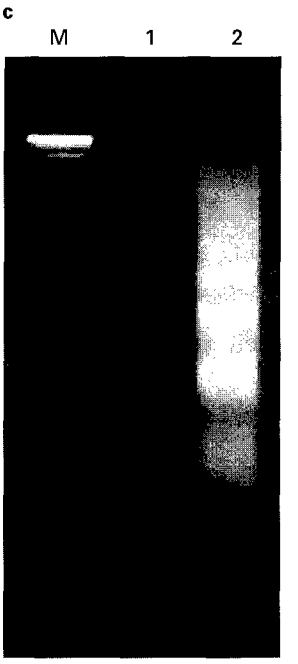


Figure 4 BRCA1 over expression induces apoptosis in MCF-7 cells after calcium ionophore treatment. (**a**) Flow cytometry analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 24 h. (**b**) Cell viability of MCF-7 and BRCA1^{s} cells treated with and without calcium ionophore for 24 h. A, MCF-7; B, MCF-7 cells treated with calcium ionophore; C, BRCA1^{s}; D, BRCA1^{s} treated with calcium ionophore. (**c**) DNA fragmentation induced by BRCA1 over-expression. Lane 1, MCF-7 cells treated with calcium ionophore; 2, BRCA1^{s} cells treated with calcium ionophore

proteins or target apoptosis inhibiting proteins through direct protein-protein interactions. In the mouse mammary gland BRCA1 expression was found to be elevated during pregnancy following treatment with ovarian hormones (Lane *et al.*, 1995; Marquis *et al.*, 1995) and in human breast cancer cells BRCA1 mRNA levels were found to be regulated by steroid hormone estrogen and progesterone (Gudas *et al.*, 1995). Recent evidence suggests that hormone-dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis (Thompson, 1995). Our results suggest that lack or decreased levels of expression of functional BRCA1 gene product in breast and ovarian cancers may be responsible for the increased resistance of these cells to undergo apoptosis. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies. Alternatively, one can use therapeutic agents that can activate BRCA1 downstream signals involved in apoptosis for the treatment of breast and ovarian cancers.

Materials and methods

Cell lines

NIH3T3 cells stably overexpressing the BRCA1 gene product has been previously described (Rao *et al.*, 1996). Cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FCS), 1% Penicillin-streptomycin and 200 μg/ml of G418 under 5% CO_2 atmosphere. MCF-7 cells were transfected with BRCA1 cDNA as described previsouly (Rao *et al.*, 1996; Rao *et al.*, unpublished results).

Immunohistochemistry

NIH3T3 cells stably overexpressing the BRCA1 gene product has been previously described (Rao *et al.*, 1996). Cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-streptomycin and 200 μg/ml of G418 under 5% CO_2 atmosphere. NIH3T3 and BRCA1^s transfectant cells were cultured in chamber slides and processed for immunohistochemistry using the BRCA1 peptide antibody as described previously (Rao *et al.*, 1996).

Immunofluorescence

Immunofluorescence analysis was done as described previously (Dyck *et al.*, 1994). In brief, NIH3T3 and BRCA^s cells cultured in chamber slides were fixed in 3.7% formaldehyde at room temperature, following by washing with PBS and blocking in blocking solution (3% bovine serum albumin-0.5% Triton X-100 in PBS) for 30 min at room temperature. The cells were incubated with primary BRCA1 peptide antibody diluted 1:100 for 2 h at room temperature, then washed three times with PBS and were incubated with the secondary antibody (FITC conjugated goat anti rabbit IgG (Cappel). After rinsing in PBS, the slides were mounted with fluoresecence mounting media (Vector) and photographed on an immunofluorescence microscope.

Flow cytometry analysis

Subconfluent to confluent NIH3T3, BRCA1^s cells were incubated in 10% FBS or 0% FBS media. After 24 h both adherent and nonadherent cells were pooled, washed in PBS and fixed in 80% cold ethanol at −18°C overnight. Cells were pooled, washed in PBS and stained with propidium iodide (20 μg/ml) and incubate with 20 μg/ml of RNAse A at 4°C in the dark overnight. Samples were analysed using a EPICs profile analyzer. Histograms showing the total DNA content at FL2 vs cell number are shown.

Flow cytometry analysis of NIH3T3, MCF7 and BRCA1^s cells treated with calcium ionophore A23187 was done similar to that described in Figure 2 legend except for the treatment with 20 μM calcium ionophore A 23187 (Sigma) for 24 h.

DNA fragmentation

DNA fragmentation assay was done as described (Kondo *et al.*, 1995). In brief, confluent NIH3T3 and BRCA1^s cells grown in DMEM supplemented with 10% FBS and penicillin streptomycin were changed into media containing 0% FBS for 24, 48 and 72 h. After the indicated incubation periods, both adherent and detached cells were collected ($2-5\times10^6$ cells), washed once in TBS buffer and lysed in 1 ml of 100 mM Tris-HCl, 0.1 M EDTA, 0.5% SDS and 20 μg/ml RNAse A (pH 8.0) and incubated at 37°C for 30 min. Proteinase K at a final concentration of 100 μg/ml was added and further incubated for 3 h at 55°C. After extraction with an equal volume of phenol:chloroform:isoamyl alcohol, followed by re-extraction with phenol:chloroform: isoamyl alcohol. The DNA was precipitated from the aqueous phase with sodium acetate and two volumes of ethanol, the DNA pellet was dissolved in TE buffer and analysed by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining.

DNA fragmentation analysis of NIH3T3, MCF7 and BRCA1^s cells treated with calcium ionophore A23187 was done as described in Figure 2 legend except for the treatment of A23187 (20 μM) for 24 h before analysis on a 2% agarose gel.

Cell viability

Subconfluent NIH3T3, MCF-7-BRCA1^s cells seeded into six well plates and grown in DMEM 10% FBS were treated with 20 μM calcium ionophore A23187 for 24 h. After which cells were washed with PBS and fixed in methanol:glacial acetic (3:1). Cells were stained with crystal violet. After washing in water, the cells were visualized and photographed under an Olympus microscope at 200× magnification.

Chromatin condensation

NIH3T3 and BRCA1 cells treated with or without 20 μM calcium ionophore for 24 h were cultured on glass cover slips and fixed in methanol:glacial acetic acid (3:1) at −18°C for 30 min. The cells were washed in PBS and stained with 8 μg/ml Hochest 33258 for 5 min in dark. The coverslips were rinsed in water and mounted with fluorescence mounting media. The cells were visualized and photographed under the fluorescence microscope.

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