BRCA1–Sp1 interactions in transcriptional regulation of the IGF-IR gene

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Abstract The insulin-like growth factor-I receptor (IGF-IR) plays a critical role in breast tumorigenesis and is overexpressed in most primary tumors. BRCA1 is a transcription factor involved in numerous cellular processes, including DNA damage repair, cell growth, and apoptosis. Consistent with its tumor suppressor role, we demonstrated that BRCA1 repressed the activity of co-transfected IGF-IR promoter reporter constructs in a number of breast cancer-derived cell lines. Results of electrophoretic mobility shift assay showed that BRCA1 did not exhibit any specific binding to the IGF-IR promoter, although it prevented binding of Sp1. Co-immunoprecipitation experiments demonstrated that BRCA1 action was associated with specific interaction with Sp1 protein. Furthermore, using a series of glutathione *S*-transferase-tagged BRCA1 fragments, we mapped the Sp1-binding domain to a segment located between aa 260 and 802. In summary, our data suggest that the IGF-IR gene is a novel downstream target for BRCA1 action.

Key words: Insulin-like growth factor; Insulin-like growth factor-I receptor; BRCA1; Sp1; Breast cancer

1. Introduction

The insulin-like growth factor-I receptor (IGF-IR) mediates the mitogenic, antiapoptotic, and transforming effects of the IGF ligands, IGF-I and IGF-II [1,2]. The IGF axis has a central role in cell cycle progression, as demonstrated by the fact that receptor overexpression in fibroblasts abrogates all requirements for additional growth factors [3]. IGFs, acting through the IGF-IR, have an important role in normal mammary gland growth and morphogenesis, as well as in mammary tumorigenesis [4,5]. Furthermore, epidemiological data demonstrated a positive correlation between circulating IGF-I concentration and risk of breast cancer [6]. The relative risk of developing breast cancer in premenopausal women less than 50 years of age was 4.6-fold higher in individuals in the upper tertile of IGF-I values (compared to women in the lower tertile). These studies, thus, suggest that ligand-dependent activation of the IGF-IR by endocrine IGF-I may be a predisposing factor in breast cancer etiology.

The level of expression of the IGF-IR gene during normal development, as well as in pathological states, is determined, to a large extent, at the transcriptional level. The IGF-IR promoter is extremely G-C-rich and contains multiple binding sites for transcription factor Sp1, a zinc-finger protein that strongly stimulates IGF-IR gene expression [7]. In addition, transcription of the IGF-IR gene is negatively regulated by a number of tumor suppressors, including p53 and the Wilms' tumor protein, WT1 [8,9].

The BRCA1 gene encodes a 220-kDa phosphorylated transcription factor whose mutation was correlated with the appearance of breast and ovarian cancer at very young ages [10]. Evidence in support of a tumor suppressor role for BRCA1 was provided by studies showing that transfer of BRCA1 arrested growth of breast and ovarian, but not colon or lung, cancer cell lines [11]. BRCA1 participates in multiple biological pathways, including DNA damage repair, cell growth and apoptosis, and gene transcription [12]. Consistent with its tumor suppressor role, we have previously shown that BRCA1 repressed the activity of co-transfected IGF-IR promoter–luciferase reporter constructs in a number of cell lines [13]. Furthermore, deletion analysis demonstrated that the promoter region responsible for this effect included a number of Sp1 sites.

In view of the central role of the IGF-IR in normal mammary gland growth and development, as well as in mammary tumorigenesis, and to extend our previous observations on transcriptional regulation of the IGF-IR gene by BRCA1 and Sp1, we have addressed the potential functional and physical interactions between these important proteins in controlling IGF-IR gene expression.

2. Materials and methods

2.1. Cell culture, plasmids, and DNA transfection

The human breast cancer-derived T47D, MCF-7, and MDA-MB-231, and the osteogenic sarcoma-derived Saos-2 cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 2 mM glutamine, and 50 μg/ml gentamicin sulfate. The BRCA1-null HCC1937 breast cancer cell line was maintained in RPMI 1640 medium with 10% FBS. HCC1937 cells were provided by Dr. L.C. Brody (National Center for Human Genome Research, NIH, Bethesda, MD, USA). *Drosophila* Schneider cells were grown in Schneider's Drosophila medium containing 10% FBS, 2 mM glutamine, and 20 μg/ml gentamicin sulfate.

For transient co-transfection experiments, a genomic DNA fragment extending from nucleotides −476 to +640 (nucleotide 1 corresponds to the transcription start site of the rat IGF-IR gene) was employed [14]. Wild-type and 185delAG mutant BRCA1 expression vectors (in pcDNA3) [15] were provided by Dr. L.C. Brody.

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T47D and MDA-MB-231 cells were transfected using Lipofectamine-2000 (Life Technologies), HCC1937 cells were transfected using Fugene-6 (Roche), MCF-7 cells were transfected using jetPEI™ (Polyplus), and Schneider cells were transfected with calcium phosphate (Life Technologies). Cells were harvested 40 h after transfection, and luciferase and β-galactosidase (β-gal) activities were measured as previously described [8]. Promoter activities were expressed as luciferase values normalized for β-gal.

2.2. In vitro transcription and translation reactions

Coupled in vitro transcription/translation of BRCA1 was performed using the TnT® T7 Quick Coupled Transcription/Translation System (Promega). T7 RNA polymerase-driven in vitro transcription reactions were followed by in vitro translations in the presence of [^{35}S]methionine, or unlabeled methionine for electrophoretic mobility shift assay (EMSA), using rabbit reticulocyte lysates. In vitro translation products were electrophoresed through 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiographed for 20 h.

2.3. Co-immunoprecipitation studies

Cells were collected and processed as described [16]. Cellular extracts were immunoprecipitated with antibodies against the C- or N-terminal domains of BRCA1 (C-20 and D-20 respectively, Santa Cruz Biotechnology), or with anti-Sp1 (Pep-2). Immunoprecipitates were electrophoresed through 8% SDS–PAGE, followed by transfer of the proteins to nitrocellulose membranes. Membranes were blocked with 3% bovine serum albumin and then incubated with either anti-Sp1 or anti-BRCA1 (C-terminal) antibodies, washed and incubated with horseradish peroxidase-conjugated secondary antibody. BRCA1 and Sp1 were detected using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce).

2.4. EMSA

A double-stranded oligonucleotide with the sequence AGCCCG-CCCGCCCTGCCGCCGCCC, which corresponds to nt −380/−357 of the IGF-IR 5′-flanking region and includes two overlapping Sp1 sites (underlined) [7], was used in EMSA. In addition, a DNA fragment extending from nt −40 to +115 (encompassing the IGF-IR initiator element) was employed. Binding reactions included 1 μl of in vitro translated BRCA1 protein and/or 0.375 footprinting units (f.p.u.) of recombinant Sp1 protein (Promega). EMSA was performed as previously described [16].

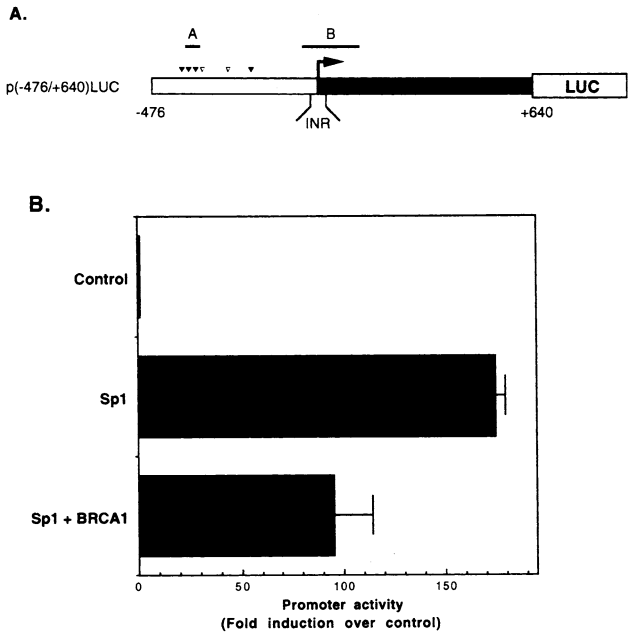

Fig. 1. Functional interactions between BRCA1 and Sp1 in control of IGF-IR promoter activity. A: Scheme of the IGF-IR promoter–luciferase construct, p(−476/+640)LUC, used in transient transfections. The reporter plasmid contains 476 bp of 5′-flanking (open bar) and 640 bp of 5′-untranslated (closed bar) sequences. The arrow denotes the initiator element (INR). Closed triangles represent GC boxes footprinted by Sp1 [7]. Open triangles are Sp1 sites (GGGCGG) that did not generate detectable footprints. Bars A and B denote probes used in EMSA. B: The p(−476/+640)LUC plasmid (5 μg) was co-transfected into Schneider cells, together with 300 ng of an Sp1 expression vector (pP_{ac}Sp1, or empty pP_{ac}0), in the absence or presence of a BRCA1 vector (1 μg). Luciferase activity was measured after 72 h. A value of 1 was given to the activity in the absence of expression vectors. Results are mean ± S.E.M. of four experiments.

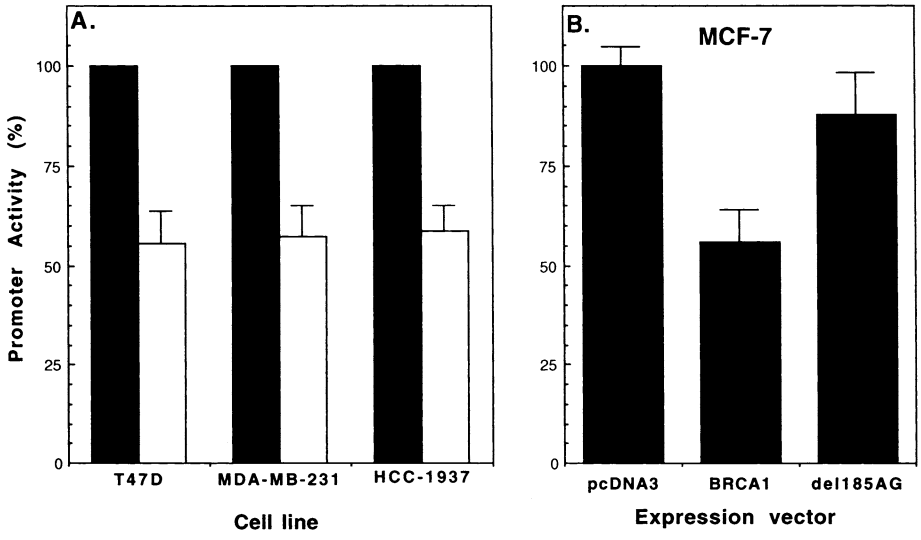

Fig. 2. Regulation of IGF-IR promoter activity by BRCA1 in breast cancer-derived cell lines. A: T47D, MDA-MB-231, and HCC1937 cells were cotransfected with a BRCA1 vector, together with the p(−476/+640)LUC reporter and pCMVβ. After 40 h, cells were harvested and the levels of luciferase and β-gal activities were measured. Luciferase values, normalized for β-gal, are expressed as a percentage of the luciferase activity of the empty pcDNA3 vector (black columns). Experiments were performed four to six times, each in duplicate. Bars are mean ± S.E.M. B: MCF-7 cells were co-transfected with the p(−476/+640)LUC reporter, along with wild-type or 185delAG mutant BRCA1. The figure shows the results of a typical experiment, performed in duplicate dishes and repeated six times.

2.5. GST-affinity purification and association assays

Six glutathione *S*-transferase (GST)–BRCA1 fusion proteins (aa 1–324; 260–553; 502–802; 758–1064; 1005–1313; and 1314–1863) were provided by Dr. D.M. Livingston (Harvard Medical School) [17]. Recombinant proteins were produced in *Escherichia coli* using standard procedures. For GST affinity purification, bacterial lysates were incubated with glutathione beads (Pharmacia) for 1 h, after which beads were washed with phosphate-buffered saline and incubated for 18 h with whole cell extracts of Schneider cells that were transfected with 1.5 μg of an Sp1 expression vector under the control of an actin promoter, $pP_{ac}Sp1$, or empty $pP_{ac}0$. Following incubation, beads were washed, boiled for 10 min in sample buffer, electrophoresed through 10% SDS–PAGE, transferred to nitrocellulose membranes, and blotted with GST or Sp1 antibodies.

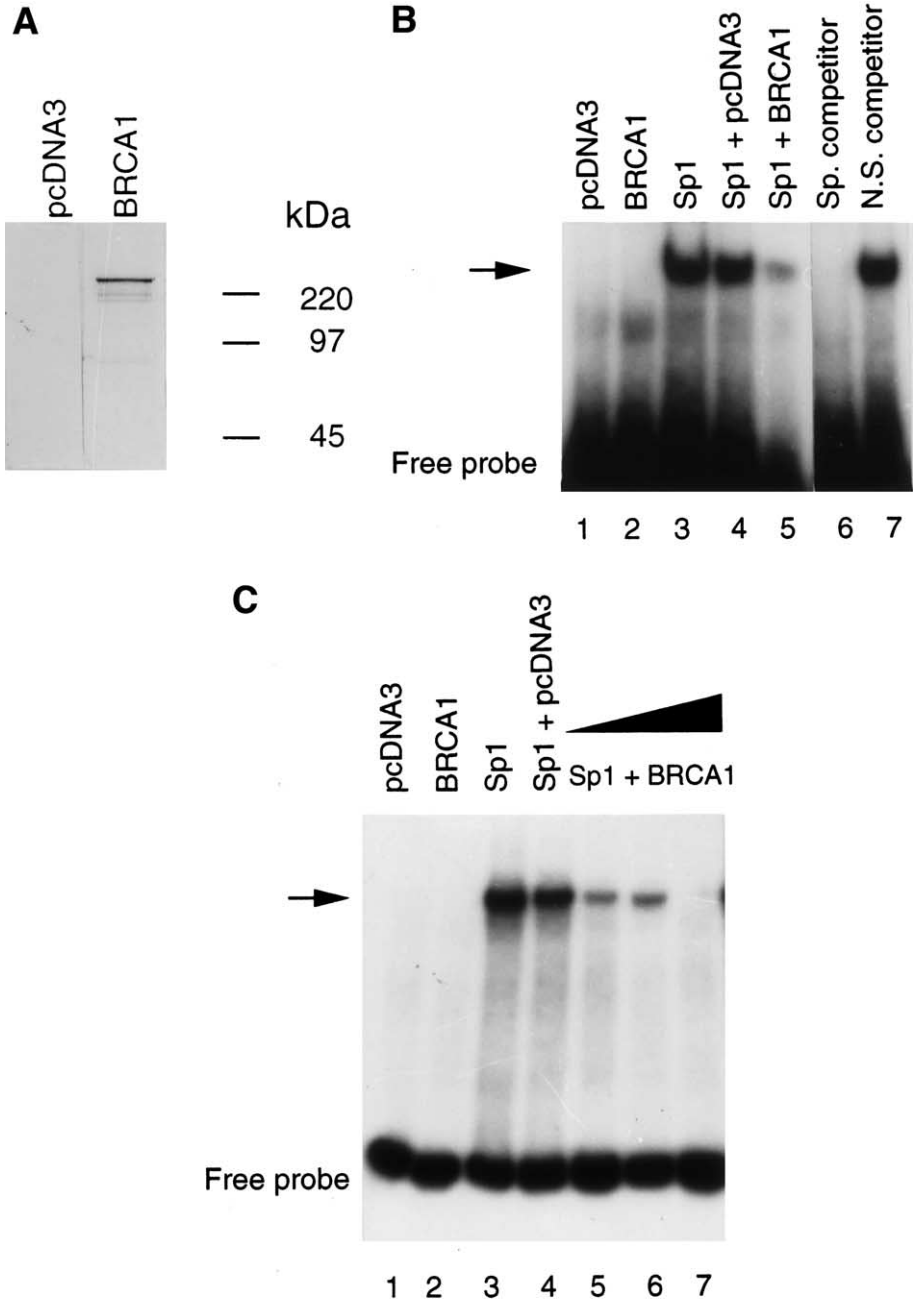

Fig. 3. EMSA analysis of BRCA1–Sp1 interactions at the IGF-IR promoter. A: In vitro transcription/translation reactions were performed with the TnT® System using 1 μg of pcDNA3 or BRCA1 vector. Translation reactions were performed in the presence of [^{35}S]methionine (or unlabeled methionine for EMSA) using rabbit reticulocyte lysates and electrophoresed through 8% SDS–PAGE. B: A double-stranded oligomer (nt −380/−357, probe A in Fig. 1A) was end-labeled with [γ-^{32}P]ATP, and used in binding reactions with 1 μl of in vitro translated BRCA1 (lane 2) or control pcDNA3 reaction product (lane 1), Sp1 (0.375 f.p.u.) (lane 3), Sp1 and pcDNA3 (lane 4), or Sp1 and BRCA1 (lane 5). Competition experiments were performed using excess of the unlabeled probe (Sp., specific competitor, lane 5) or a control oligomer lacking Sp1 site (N.S., non-specific competitor, lane 7). The arrow denotes the position of the DNA–Sp1 complex. C: A labeled promoter fragment (−40/+115, probe B in Fig. 1A) was employed in EMSA with BRCA1 (5 μl) (lane 2) or control pcDNA3 reaction product (lane 1), Sp1 (0.375 f.p.u.) (lane 3), Sp1 and pcDNA3 (lane 4), or Sp1 and 0.5, 1, or 2 μl of BRCA1 (lanes 5–7, respectively).

3. Results

Increased expression of the IGF-IR gene is a typical hallmark in breast tumorigenesis [5]. Levels of IGF-IR mRNA and protein are highly augmented in well and moderately differentiated carcinomas and decrease at advanced metastatic stages [18]. Because BRCA1 functions as a tumor suppressor capable of arresting growth of mammary cells, whereas mutant BRCA1 are unable to halt proliferation, we examined whether BRCA1 is able to control IGF-IR gene transcription. In previous studies we showed that BRCA1 suppressed the activity of co-transfected IGF-IR promoter constructs in Chinese hamster ovary, Saos-2, and MCF-7 cells [13]. Deletion analysis demonstrated that most of the suppressive effect of BRCA1 was ascribed to a fragment located between nt −476 and −188 in the IGF-IR 5′-flanking region, a region previously shown to include four specific Sp1 sites (at nt −399/−394, −378/−373, −374/−369, and −193/−188) (Fig. 1A) [7]. To address the potential functional interactions between BRCA1 and Sp1 in regulation of the IGF-IR gene, co-transfections were performed in Sp1-null *Drosophila* Schneider cells using BRCA1 and Sp1 expression vectors, along with the p(−476/+640)LUC reporter. In these cells, Sp1 induced a ∼175-fold stimulation of the IGF-IR promoter, while BRCA1 suppressed 45% of the Sp1-induced transactivation (Fig. 1B). These results replicate our previous data [13].

To examine the transcriptional effect of BRCA1 in different breast cancer-derived cell lines, we analyzed the T47D, MDA-MB-231, and HCC1937 cells. For this purpose, cells were co-transfected with a BRCA1 vector, along with the p(−476/+640)LUC reporter and a β-gal vector (pCMVβ). As shown in Fig. 2A, expression of BRCA1 suppressed promoter activity by ∼42–45%. On the other hand, co-transfection of MCF-7 cells with a vector encoding a truncated BRCA1 mutant (185delAG) had a reduced effect (Fig. 2B).

To assess whether the transcriptional activity of BRCA1 was associated with specific binding to the IGF-IR promoter, EMSA was performed using in vitro translated BRCA1 (Fig. 3A). The ∼220-kDa protein was employed in binding assays using an oligomer comprising nt −380/−357 of the IGF-IR promoter. This fragment is included in the region shown to mediate the BRCA1 response, and contains two overlapping Sp1 elements. As shown in Fig. 3B, lane 2, BRCA1 did not display any noticeable binding, whereas incubation of the DNA fragment with recombinant Sp1 generated one retarded band (lane 3). Band formation was abrogated when reactions were performed in the presence of a ∼40-fold molar excess of unlabeled probe (lane 6), but not when a control oligomer lacking Sp1 sites (nt −305/−325) was employed (lane 7). Addition of BRCA1 protein (lane 5), but not empty pcDNA3 translation product (lane 4), prevented the formation of the Sp1–IGF-IR promoter complex.

EMSA was also performed using a genomic DNA fragment (nt −40/+115) that includes the initiator element. Since the initiator acts in vivo in concert with a TATA box or upstream elements (including Sp1) to direct transcription initiation, we examined whether the effect of BRCA1 was associated with specific binding to this region. Incubation of the DNA fragment with BRCA1 did not generate any retarded band (Fig. 3C, lane 2). Despite the fact that this region, which contains ∼70% G-C nucleotides, lacks canonical Sp1 sites, addition of Sp1 protein generated a shifted band (lane 3). Addition of BRCA1 abrogated Sp1–DNA complex formation in a dose-dependent manner (lanes 5–7).

To investigate whether the ability of BRCA1 to prevent Sp1 binding to the IGF-IR promoter, and to repress Sp1-stimulated promoter activity, was associated with interaction with Sp1, co-immunoprecipitation experiments were performed. For this purpose, untransfected Saos-2 and MCF-7 cells, which express both proteins, were lysed and immunoprecipitated with anti-BRCA1 or anti-Sp1. Precipitates were electrophoresed through 8% SDS–PAGE, transferred to nitrocellulose membranes, and blotted with anti-BRCA1 (C-terminal) (Fig. 4A,B, upper panels) or anti-Sp1 (lower panels). Results obtained showed that, in both cell lines, immunoblotting with anti-BRCA1 identified the 220-kDa protein in anti-Sp1 immunoprecipitates. Likewise, immunoblotting with anti-Sp1 identified the 95- and 106-kDa bands in anti-BRCA1 precipitates.

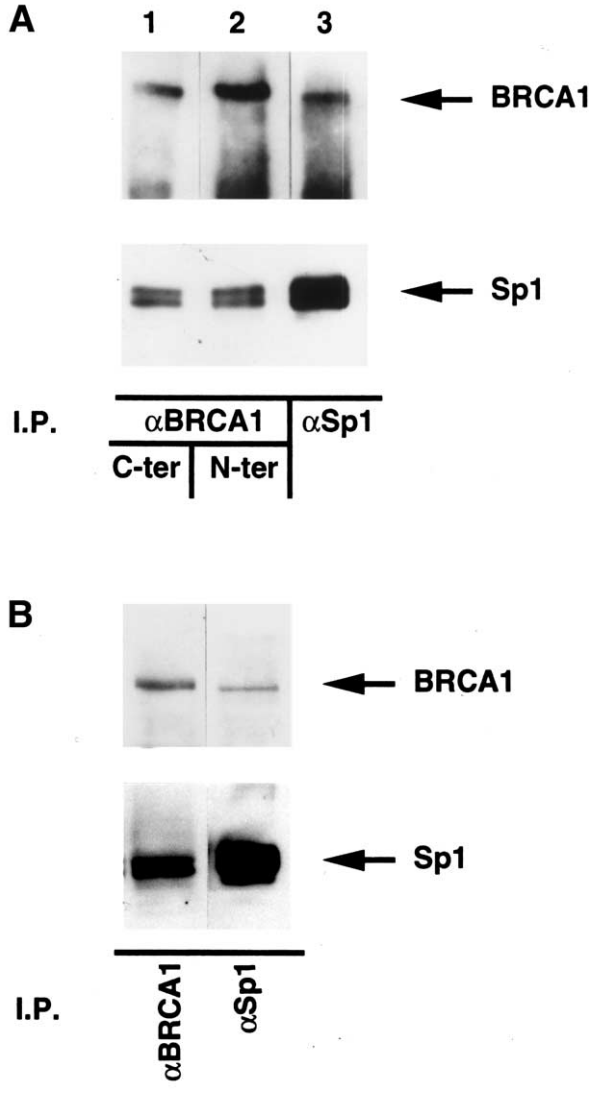

Fig. 4. In vivo association of BRCA1 and Sp1. Saos-2 (A) and MCF-7 (B) cells were lysed and immunoprecipitated with antibodies against the C- or N-terminal domains of BRCA1 (Saos-2), or anti-C-terminal BRCA1 (MCF-7), or with anti-Sp1. Precipitates were separated through 8% SDS–PAGE, blotted, and incubated with anti-BRCA1 (C-terminal) (upper panels) or anti-Sp1 (lower panels). The positions of the ∼220-kDa BRCA1 and 95- and 106-kDa Sp1 proteins are denoted by the arrows.

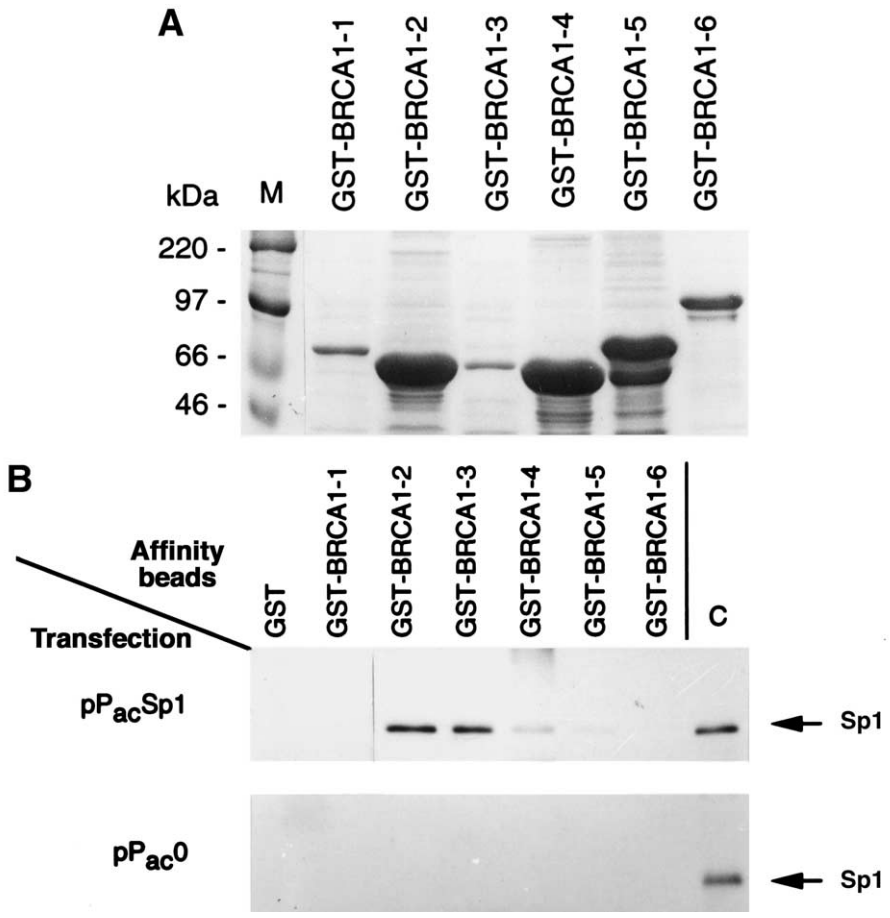

Fig. 5. Identification of the Sp1-binding region of BRCA1. A: Six GST–BRCA1 fusion proteins were generated in *E. coli*. The figure shows a Coomassie blue-stained gel. M, molecular weight markers. B: Equal amounts of GST–BRCA1 fusion proteins (and control GST) were bound to glutathione beads, after which they were incubated with extracts of Sp1-transfected Schneider cells (upper panel), or control cells (lower panel). Bound proteins were recovered, electrophoresed, and immunoblotted with anti-Sp1. C, control samples consisting of Sp1-transfected cells immunoprecipitated with anti-Sp1 and immunoblotted for Sp1.

No BRCA1 or Sp1 was detected in control samples precipitated with preimmune rabbit serum (not shown).

To identify the BRCA1 domain(s) involved in Sp1 interaction, six GST–BRCA1 fusion proteins spanning the entire sequence were generated in *E. coli* (Fig. 5A). GST–BRCA1 proteins were bound to glutathione Sepharose beads and incubated with lysates from Schneider cells that were transfected with an Sp1 vector ($pP_{ac}Sp1$), or control cells transfected with empty $pP_{ac}0$. Bound proteins were released, separated through SDS–PAGE, and immunoblotted with Sp1 and GST antibodies. Results obtained showed that partially overlapping GST–BRCA1 fragments 2 (aa 260–553) and 3 (aa 502–802) exhibited the highest affinity to Sp1 (Fig. 5B). Fragment 4 (aa 785–1064) bound Sp1 with reduced affinity. No binding was seen when lysates from cells transfected with empty $pP_{ac}0$ were employed (lower panel).

4. Discussion

The IGFs have been recognized as major regulators of mammary epithelial cell and breast cancer cell growth [19]. The IGF-IR, which mediates the mitogenic actions of the IGFs, has been identified as a central player in breast carcinogenesis [5,20]. Increased expression of the IGF-IR gene has been associated with radioresistance and tumor recurrence, as well as with development of resistance to chemotherapeutic agents [21,22]. Consistent with the tumor suppressor role of BRCA1, we showed that the IGF-IR promoter is repressed by BRCA1, and that the regulatory region responsible for this effect includes a cluster of Sp1 sites [13]. Furthermore, coexpression experiments demonstrated that BRCA1 repressed the Sp1-induced transactivation of the IGF-IR gene, suggesting that the mechanism of action of BRCA1 involves potential interaction(s) with Sp1. In the present study we investigated the physical association between BRCA1 and Sp1 in transcriptional regulation of the IGF-IR gene.

To explore whether the transcriptional activity of BRCA1 was associated with DNA binding to specific IGF-IR promoter regions, EMSA experiments were performed using full-length in vitro translated BRCA1. Results of EMSA performed under a variety of experimental conditions showed that BRCA1 did not display any substantial binding activity towards probes encompassing Sp1 sites and the initiator element. These results suggest that the effect of BRCA1 involves, most probably, protein–protein associations with additional DNA binding transcription factors. A recent paper has demonstrated that recombinant BRCA1 binds strongly to DNA, although without sequence specificity [23]. BRCA1 showed a preference for branched DNA structures and generated protein–DNA complexes that included multiple DNA molecules.

Furthermore, we cannot discard the possibility that lack of DNA-binding activity of the in vitro translated BRCA1 resulted from its lower specific activity in comparison to the *E. coli*-expressed BRCA1 used by Paull et al. [23].

In previous studies we showed that the IGF-IR promoter is highly responsive to Sp1 and that transcriptional control by Sp1 occurs primarily through a cluster of four GC boxes in the 5′-flanking region [7]. The results of the present study are consistent with a model in which physical association between BRCA1 and Sp1 prevents Sp1 binding to high-affinity sites in the IGF-IR promoter, with ensuing reduction in promoter activity. BRCA1 domains involved in Sp1 binding include the exon 11-encoded sequence. Interestingly, the same domain was recently shown to bind STAT1 following interferon-γ stimulation [24]. Mechanisms of action involving interactions with, and sequestering of, Sp1 were reported for other tumor suppressors, including Rb [25] and VHL [26]. Furthermore, estrogen receptor α suppressed IGF-IR promoter activity in aortic smooth muscle cells via a mechanism that involves inhibition of Sp1 binding to the promoter [27].

However, suppression of the IGF-IR promoter is not limited to BRCA1. We have previously demonstrated that tumor suppressor WT1 binds both upstream and downstream of the IGF-IR gene transcription start site and suppresses promoter activity in functional assays [14]. Likewise, p53 suppressed promoter activity via a mechanism that involves interaction with the TATA box-binding protein [8]. Taken together, these studies suggest that the IGF-IR gene constitutes a common downstream target to various families of tumor suppressors. Inhibitory control of the IGF-IR gene, with ensuing reduction in the levels of cell surface IGF binding sites, has been postulated to keep the receptor in its 'non-mitogenic' mode, thus preventing from the cell to engage in any type of mitogenic activity [28]. The moderate inhibitory activity of BRCA1 (~42–45% inhibition) compared to that of WT1 (~82–87%) [9] and p53 (~85–90%) [8] probably reflects its moderate efficacy as a growth suppressor in comparison to other tumor suppressors.

In conclusion, we have presented evidence that transcription of the IGF-IR gene in breast cancer-derived cell lines is under the inhibitory control of BRCA1. The mechanism of action of BRCA1 involves specific binding to Sp1, thus preventing from this zinc-finger protein from binding to, and transactivating, the IGF-IR promoter. Loss of BRCA1 function in familial and sporadic breast cancer may result in constitutive gene activation by Sp1, with ensuing increases in IGF-IR mRNA and binding, and enhanced activation of cell surface receptors by locally produced and/or circulating IGFs.

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