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FAST TRACK

Identification of Proteins That Interact With BRCA1 by Far-Western Library Screening

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Abstract Protein–protein interactions control numerous biological processes. In the case of a protein with no known function, identification of interacting proteins may lend insight into its cellular function. Protein–protein interactions are often detected by yeast two-hybrid screening which is based on a transcriptional read-out. One limitation of this technique is that transcription factors, when used as bait, frequently impair the effectiveness of this screen because they give rise to high levels of false positives. The carboxyl terminus of the breast cancer tumor suppressor gene, *BRCA1*, contains two BRCT motifs, a motif found in several DNA repair and cell cycle checkpoint proteins. This region of BRCA1 also exhibits an intrinsic transcriptional transactivation activity when bound to DNA as a fusion protein, thereby limiting its use in yeast two-hybrid screen. In order to isolate proteins that interact with this domain of *BRCA1*, we utilized a Far-Western screen, a method based on direct protein binding. We used recombinant histidine-tagged BRCT as the primary protein probe. We isolated eight cDNAs that bind to the BRCT domain of BRCA1. Further analysis demonstrated that two of the clones encode for proteins that interact directly with the BRCT domain of BRCA1. J. Cell. Biochem. 83: 521–531, 2001.

Key words: BRCA1; BRCT domain; retinoblastoma-binding protein; RbAp46; breast cancer; proteomics; protein–protein interactions; ribosomal protein L31; DNA repair

Germline mutations in the BRCA1 tumor suppressor gene predispose carriers to breast and ovarian cancer and are responsible for approximately 50% of inherited breast cancer. The BRCA1 gene was isolated and mapped to chromosome 17q21 [Hall et al., 1990; Miki et al., 1994]. This gene encodes an 1863 amino acid protein with unknown function. Except for a RING finger motif in the amino terminus, two BRCT (BRCA1 carboxyl terminal) repeats in the carboxyl terminus and several nuclear localization signals, BRCA1 shares little similarity with other proteins. In addition, when BRCA1 homologs are compared between species, the majority of the protein is not well conserved outside of these motifs [Schrock et al., 1996; Szabo et al., 1996]. This lack of similarity to other proteins has rendered determining the physiological function of BRCA1 a difficult task.

To date, multiple functions have been attributed to the BRCA1 protein including DNA repair, recombination and transcriptional control. Initial insight into the function of BRCA1 emerged from studies demonstrating that BRCA1 colocalizes in the nucleus with rad51, a protein involved in homologous recombination and repair of DNA double strand breaks [Scully et al., 1997a,b]. BRCA1 has also been shown to be hyperphosphorylated in cultured cells in response to ionizing irradiation. Mouse embryonic fibroblasts derived from BRCA1 null mice are defective in transcription-coupled repair [Gowen et al., 1998]. An involvement of BRCA1 in transcription can be inferred from the finding that the carboxyl terminus of BRCA1 activates reporter gene transcription when bound to DNA as a fusion protein [Monteiro et al., 1996]. Additionally, BRCA1 activates transcription of the cyclin-dependent kinase inhibitor, p21, by p53 dependent and independent pathways [Zhang et al., 1998].

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To better understand the function of BRCA1 and the pathways in which it is involved, we sought to isolate proteins that interact with the BRCT domain in the carboxyl terminus of the protein. This domain is found in other proteins involved in DNA repair and maintenance of genome integrity such as p53BP, rad4, rad9, XRCC1, XRCC4 and others [Bork et al., 1997]. We used a Far-Western screening approach to identify proteins that bind BRCA1 BRCT. This approach has been employed successfully with several transcription factors resulting in the identification of Rb partner proteins RBAP-1 and RBP-3 [Defeo-Jones et al., 1991; Helin et al., 1992; Kaelin et al., 1992] and c-Myc interacting proteins Mad and Max [Blackwood and Eisenman, 1991; Ayer et al., 1993]. We modified this method to be more generally applicable by expressing BRCT with a histidine-tagged epitope. This allowed for single step protein purification of BRCT and detection with two different antibodies: an antibody directed to the histidine tag and an antibody directed to the BRCT domain of BRCA1. Here we demonstrate that BRCA1 BRCT exhibits different patterns of binding to interacting proteins in cells heterozygous for BRCA1 than in cells carrying two wildtype BRCA1 alleles. Additionally, we utilized the recombinant, epitope-tagged BRCT polypeptide to probe a cDNA expression library. Interactions between the recombinant BRCT and putative candidates were determined by direct binding of the proteins rather than by transcriptional readout. In contrast to the yeast two-hybrid system, the Far-Western approach is not complicated by the intrinsic transcriptional activation function present in the carboxyl terminus of BRCA1. This strategy led us to the isolation and analysis of eight different clones representing candidates for BRCA1 interacting protein.

MATERIALS AND METHODS

Cell Culture

All cells were maintained in 5% CO_2 at 37°C. Tissue culture products were purchased from Life Technologies. EBV-transformed lymphoblasts S753(4446C>G), 157 (3875del4), 185 (3875del4), and PB293 were originally established from both BRCA1 mutation carriers and non-mutation carriers [Castilla et al., 1994] and are available from Coriell repository. Lymphoblast cell lines from BRCA1 mutation carriers GM13715 (5382insC), GM14090 (185delAG), GM14095 (5256delG), and normal lymphoblasts GM15311, GM15351, and GM15431 were obtained from Coriell repository. All lymphoblast cell lines were maintained in RPMI 1640 supplemented with 20% FBS. MCF-7, MDA-486, and BT474 breast cancer cells carrying normal BRCA1 gene were obtained from the Lombardi Cancer Center and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. MCF10 immortalized breast epithelial cell line was obtained from the Lombardi Cancer Center and maintained in DMEM/F12 supplemented with 20 ng/ml EGF, 10 μg/ml Insulin, 0.5 μg/ml hydrocortisone, and 100 ng/ml Cholera toxin. Rabbit polyclonal antibodies against the histidine tag (sc-803) and against the carboxyl terminus of BRCA1 (sc646) were purchased from Santa-Cruz Biotechnologies.

Plasmids

Restriction and modification enzymes were purchased from Life Technologies and Boehringer Mannheim. Plasmids were constructed by standard protocols. pET-BRCT has been previously described [Yarden and Brody, 1999]. Briefly, the plasmid was constructed by digestion of full length BRCA1 cDNA with *Sac*I-*Xho*I from the pcBRCA1-385 plasmid followed by subcloning into *Sac*I-*Xho*I-digested pET28a (Novagen). The plasmid is propagated in *E. Coli* DH5α. For expression of histidine-tagged BRCT, the plasmid was transformed into BL21 DE3 pLysS strain (Novagen). GST-BRCT was previously described [Yarden and Brody, 1999]. To rescue inserts of putative clones, λpTripleX vectors (Clontech) were excised to generate pTriplEX plasmids (Clontech) according to the manufacturer's instruction's. Inserts were digested with *Eco*RI-*Xba*I and further subcloned into pcDNA3.1/his A, B, and C (Invitrogen) expression vectors in the three different reading frames.

Recombinant Protein Expression and Purification

BRCT polypeptide was produced as a histidine-tagged protein from the pET-BRCT construct. Culture of BL21 DE3 pLysS transformed with pET-BRCT was grown to absorbency of 0.6 at 600 nm and expression induced with 1 mM IPTG for 2–4 h. Cells were collected by centrifugation, resuspended in 1/10 of the original

volume in lysis buffer [150 mM NaCl; 1 mM EDTA; 50 mM Tris–HCl pH 7.5; 0.5% Np-40 plus a cocktail of protease inhibitors (Boehringer Mannheim)], lysed by sonication and then centrifuged for 20 min at 8000*g* to clear the lysate. The lysate, was loaded on a pre-equilibrated Ni-NTA nickel column (Qiagen) incubated rotating overnight at 4°C and purified by affinity chromatography. His-BRCT polypeptide was eluted with 0.25 M imidazole and dialyzed against PBS. Protein concentrations were determined by BCA protein analysis (Pierce). The yield and purity of eluted protein was determined by coomassie brilliant blue staining and Western blot analysis.

Synthesis of radiolabeled proteins from the different pcDNA vectors containing the putative clones was performed by the TNT coupled reticulocyte lysate system (Promega) in the presence of [^{35}S] methionine (Amersham) according to manufacturer's instructions.

Nuclear and Cellular Extract Preparation

Nuclear extracts from the different cell lines were prepared from subconfluent cultures as described by Dignam et al. [1983]. Briefly, cells were harvested and washed twice with ice-cold PBS and once with buffer A (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA supplemented with cocktail of protease inhibitors (BM) and phosphatase inhibitors ($NaVO_3$, NaPPi, NaF). Cell pellets were incubated with buffer A on ice for 15 min and then Dounce homogenized. Samples were centrifuged and pellets resuspended in buffer C (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol supplemented with protease and phosphatase inhibitors) for 15 min on ice. The samples were sonicated and centrifuged in a microcentrifuge at 16,000*g* for 15 min. Total protein lysates were prepared as described [Yarden and Brody, 1999]. Protein concentrations were determined by BCA protein assay (Pierce) and aliquots of extracts were stored at −80°C.

Far-Western Analysis

Denaturing conditions. his-BRCT was in vitro translated in the presence of [^{35}S]-methionine (Amersham). Twenty reactions were combined and used as a probe. The human placenta cDNA λTripleEX library was plated and protein expression was induced with IPTG according to manufacturer's instructions. Plaque proteins were transferred to nitrocellulose membranes at 37°C for 4–6 h. Membranes were denatured in HBB buffer (20 mM HEPES, pH 7.8, 5% glycerol, 0.1 M KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 0.02% polyvinylpyrrolidone) and 6 M guanidine–HCl for 30 min at room temperature and renatured in HBB buffer with 0.5 M guanidine–HCl twice for 2 h each at room temperature. Membranes were blocked with 5% nonfat dry milk in HBB buffer at 4°C overnight, incubated with [^{35}S]-labeled his-BRCT at 4°C overnight and then filters were washed extensively in HBB and exposed to autoradigraphy.

Nondenaturing conditions. Recombinant his-BRCT polypeptide purified from bacterial extracts was used as a probe to screen a human placenta λpTriplEX cDNA expression library (Clontech). The expression library was induced by IPTG according to manufacturer's instructions and proteins were transferred to nitrocellulose membranes at 37°C for 4–6 h. The filters were directly blocked with 5% nonfat milk in TBST buffer (10 mM Tris–cl pH 7.5, 150 mM NaCl, 0.05% Tween 20) at 4°C for overnight incubation. The membranes were then incubated with 3 μg/ml his-BRCT in TBST with 5% nonfat dry milk at 4°C overnight, washed five times in TBST at room temperature and then incubated with an HRP-conjugated antibody against the histidine tag (Santa-Cruz) for 1 h at room temperature. Membranes were washed extensively and positive clones were detected by chemical luminescence (Pierce).

Far-Western Blot Analysis

Cell extracts were resolved on 7.5% SDS–PAGE and then transferred to nitrocellulose membrane, blocked with 5% nonfat milk in TBST and incubated with purified 3 μg/ml his-BRCT or his-LacZ or His-CBFβMH11 overnight at 4°C. Membranes were extensively washed in TBST and detection carried out as described for the library screening.

DNA Sequencing and Sequence Data Analysis

Inserts of putative clones were rescued as plasmids and sequenced by automated sequencing using the M13 forward and reverse primers. Additional sequencing was carried out with sequence specific primers synthesized on the Expedite–nucleic acid synthesis system

(Millipore). To search for similarities to known sequences, we used the advanced version of the basic alignment program (BLAST) to search various sequence databases. EST assembly was done with Sequencher (Gene Codes). Sequences in the database overlapping original clones were placed into contigs. These contigs were extended bi-directionally via BLAST search until maximal length of sequence was obtained.

Northern Blot Analysis

Multiple tissue Northern blots were purchased from Clontech and Invitrogen. Probes used in Northern blots were generated by labeling of either cDNAs or purified PCR products of inserts with [^{32}P]dCTP (Amersham) using the Rediprime labeling kit (Amersham).

GST-Pull Down Experiments

GST-BRCT fusion protein was expressed in *E. coli* DH5α (Gibco/BRL) and immobilized on glutathione-sepharose (Pharmacia) beads as described [Yarden and Brody, 1999]. The immobilized GST-BRCT was incubated with [^{35}S]-labeled proteins synthesized by an in vitro reticulocyte coupled transcription/translation system according to manufacturer's instructions (Promega). The pull-down reaction was carried out in binding buffer (50 mM Tris–HCl pH7.5/ 150 mM NaCl/ 1 mM EDTA/ 0.3 mM DTT/ 0.5% Nonident P-40 and cocktail of protease inhibitors) for 4 h at 4°C with constant rotation. Beads were washed 3–5 times in binding buffer. Bound proteins were separated on 10–20% Tricine gels (Invitrogen) and visualized by autoradiography.

RESULTS

Optimization of Far-Western Technique

A recombinant fusion protein was constructed to express the BRCT domain of BRCA1 fused to an amino-terminal histidine tag. This protein, designated his-BRCT, was purified and used as a probe to identify BRCA1 interacting proteins by the Far-Western technique (Fig. 1). To optimize the conditions of the Far-Western hybridization, we initially used nuclear extracts from normal breast and breast cancer cells, as well as, normal and patient-derived transformed lymphoblasts. Extracts were electrophoresed on 7.5% SDS–PAGE gels, transferred to nitrocellulose filters and probed with his-

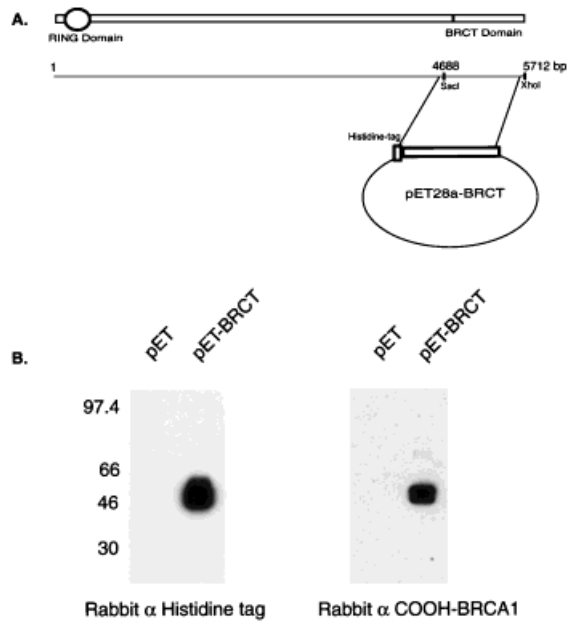

Fig. 1. His-BRCT protein expression. **A**: Schematic presentation of BRCA1 protein and cloning strategy of the BRCT domain of BRCA1 into the pET28a expression vector. **B**: Expression of his-BRCT was detected by Western blot analysis of 5 μg of bacterial extracts from BL21 DE3 pLysS transformed with pET-BRCT or pET vector only. One μg/ml of sc-803 antibody directed against the histidine tag and 1 μg/ml sc646 antibody directed against the carboxyl terminus of BRCA1 were used to detect the recombinant His-BRCT protein expression.

BRCT. We examined two different approaches. In the first, filters were directly probed with his-BRCT in a Tris–HCl based buffer, TBST. In the second, proteins bound to the filter were denatured with 6 M guanidine-HCL and renatured prior to binding with his-BRCT in HEPES-based buffer. We found that denaturation and renaturation did not increase sensitivity. This method was not used in subsequent experiments. Following the incubation with the protein probe in either buffer system, filters were probed with an affinity-purified rabbit polyclonal antibody, sc803, directed against the histidine-tagged fusion protein. To eliminate clones binding to the epitope tag instead of the BRCT polypeptide, we probed duplicate filters with different fusion proteins containing the same epitope tag such as His-lacZ or His-CBFβMH11 [Adya et al., 1998]. Results shown in Figure 2 demonstrate that proteins from nuclear extracts are able to interact specifically with his-BRCT compared to his-lacZ protein. Proteins bound to his-BRCT were from normal breast epithelial nuclear cell extracts HBL100 and MCF10A and from breast cancer cell lines

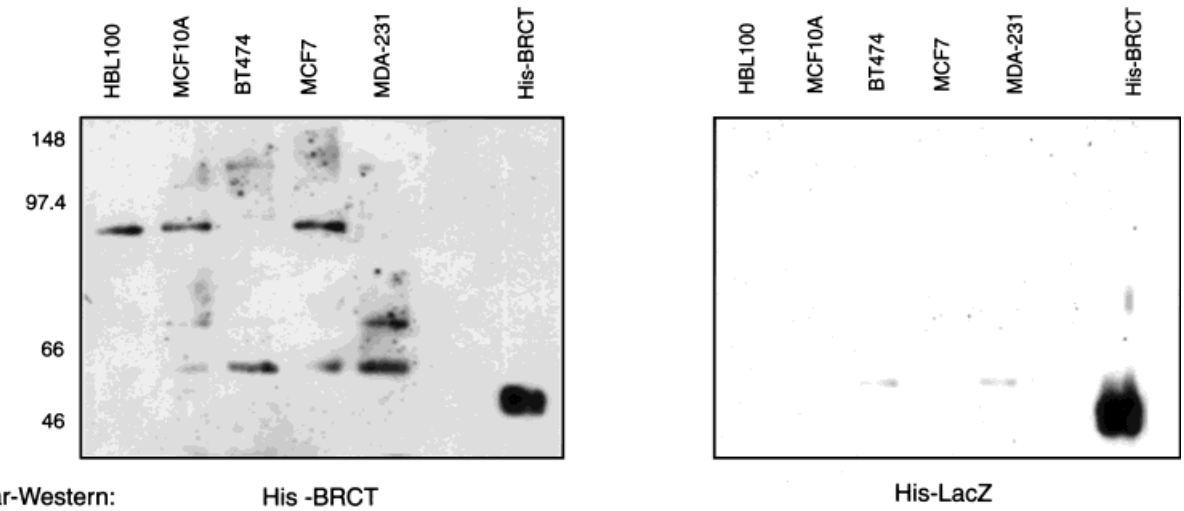

Fig. 2. Far-Western blot analysis of BRCT binding proteins in normal breast and breast cancer cell lines. Nuclear extracts (100 μg) from the different cell lines were resolved on 7.5% SDS–PAGE and transferred to nitrocellulose membrane, and incubated with 3 μg/ml his-BRCT recombinant protein or 3 μg/ml his-lacZ recombinant protein. Membranes were incubated with 1μg/ml rabbit polyclonal antibody directed against the histidine tag, followed by a secondary anti-rabbit antibody.

nuclear extracts, MCF-7, BT474, and MDA-231, all of which express wild-type BRCA1. In a similar Far-Western analysis, we noticed a different pattern of protein binding to his-BRCT, between normal lymphoblast extracts from cell line PB293 and breast cancer cell line extracts, MCF-7 or MDA-468. Interestingly, in the same experiment, the his-BRCT binding pattern observed in extracts from lymphoblasts of BRCA1 mutation carrier patients differed from the control (Fig. 3a). Cell lines established from patients samples #157 and #185 carry the germline mutation 3875del4 which causes a frameshift at codon 1252 and an early stop at codon 1262 [Castilla et al., 1994]. Cell line S753 was established from a 4446C>G germline mutation carrier. This mutation changes codon 1143 from arginine to a stop codon (R1143X). To confirm the differential binding to BRCT between normal lymphoblast extracts and hetrozygotes lymphoblast extracts with a mutated BRCA1 allele, we repeated the Far-Western analysis with total cellular proteins isolated from six lymphoblast cell lines. Three normal lymphoblast lines expressing two wild-type alleles of BRCA1 were compared to three additional hetrozygotes lymphoblast cell lines containing only one wildtype BRCA1 allele. Results shown in Figure 3b demonstrate the existence of 3–4 bands (~90–150 kd) binding to BRCA1 in normal lymphoblasts that are not expressed or able to bind BRCT in cells carrying BRCA1 mutations. The difference in binding patterns present in panels of Figure 3a and b may reflect the use of nuclear versus whole cell extracts, respectively. Taken together, the Far-Western analyses revealed that there are multiple proteins capable of interacting with BRCA1 and the pattern of binding is different in cells carrying BRCA1 mutations.

Library Screening

The recombinant protein his-BRCT was used as a probe to screen an expression library by Far-Western analysis according to conditions described above. We analyzed approximately 500,000 plaque forming units (pfu) from a human placenta λpTriplex expression library and identified 25 primary positive clones. After a secondary screen, 11 clones remained positive. Of those 11 clones, 8 remained positive and specific to BRCT after tertiary and additional screens. Purified plaque proteins were transferred to duplicate filters and hybridized to his-CBFβMH11 as a negative control. Representative results of the first and tertiary screen are shown in Figure 4. The cDNA insert of each clone was rescued from the λvector into the pTriplEX plasmid vector for DNA sequencing analysis. Each of the eight clones was found to contain a different sequence. The characteristics of eight of these candidates are shown in Table I. The expressed cDNAs can be grouped into three different classes based on computer searches of various DNA databases: (1) cDNA corresponding to sequences of known genes

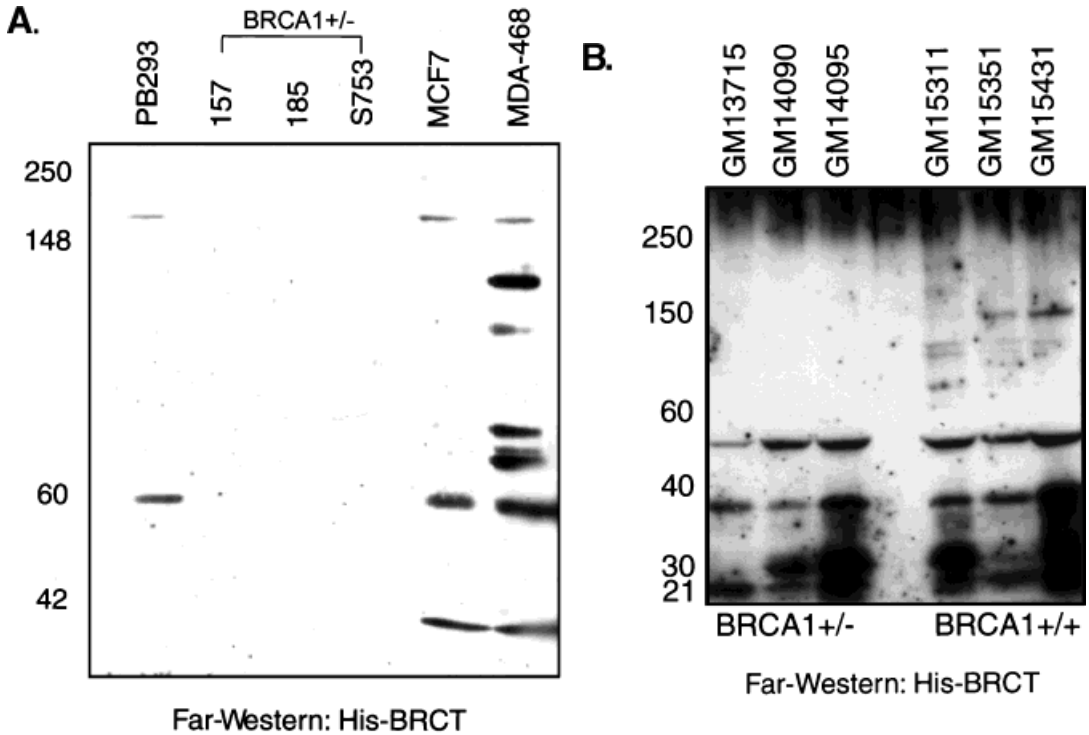

Fig. 3. Far-Western blot analysis of BRCT binding proteins in normal lymphoblast, BRCA1 mutation carrier lymphoblasts and breast cancer cell lines. **A**: Nuclear extracts (100 μg) from the different cell lines were resolved on 7.5% SDS–PAGE, transferred to nitrocellulose membrane, and incubated with 3 μg/ml his-BRCT recombinant protein. Membranes were incubated with 1μg/ml rabbit polyclonal antibody directed against the histidine tag, following by a secondary anti-rabbit antibody. **B**: Whole cell lysates (100 μg) from cells carrying two wild-type alleles of BRCA1 (GM15311, GM15351, and GM15431) or cells carrying one mutated allele of BRCA1 (GM13715, GM14090, and GM14095) were resolved on 4–20% SDS–PAGE and transferred to nitrocellulose membrane and then subjected to a Far-Western analysis as described above.

with described function; (2) identified genes and ESTs without known function; and (3) a novel sequence (i.e., not represented in any data base).

Inserts of the following five clones RY8, RY9, RY19, RY22, and RY24 correspond to sequences of previously described genes. Clone RY22 contains an insert of approximately 1 kb that is 100% identical to the RbAp46 protein from amino acids 223–425 (accession no. U35143). The interaction between RbAp46 and BRCT has been extensively characterized and described elsewhere [Yarden and Brody, 1999]. RY19 encodes for the full length ribosomal protein L31 (GenBank accession no. X69181) a protein known to be overexpressed in colon cancer and

TABLE I. Summary of Information on the Eight Different BRCA1 Interacting Proteins Candidates

Clone	Insert size (kb)	Most similar to	Nucleotide identity (%)	Gene
RY6	0.8	AC004526	98	Genomic clone NF1
RY8	1.5	AB006625	98	hPEG3
RY9	1.6	V00710.1/	98rRNA	rRNA
		Z71621	99	Wnt-13
RY12	0.7	AC008546	100	Unknown
RY19	0.8	X69181	99	RPL31
RY21	0.7	AC005261.1	100	Multiple ests
RY22	1.0	U35143	100	RbAp46
RY24	0.7	Z22572	97	CDEI

N/A-not available.

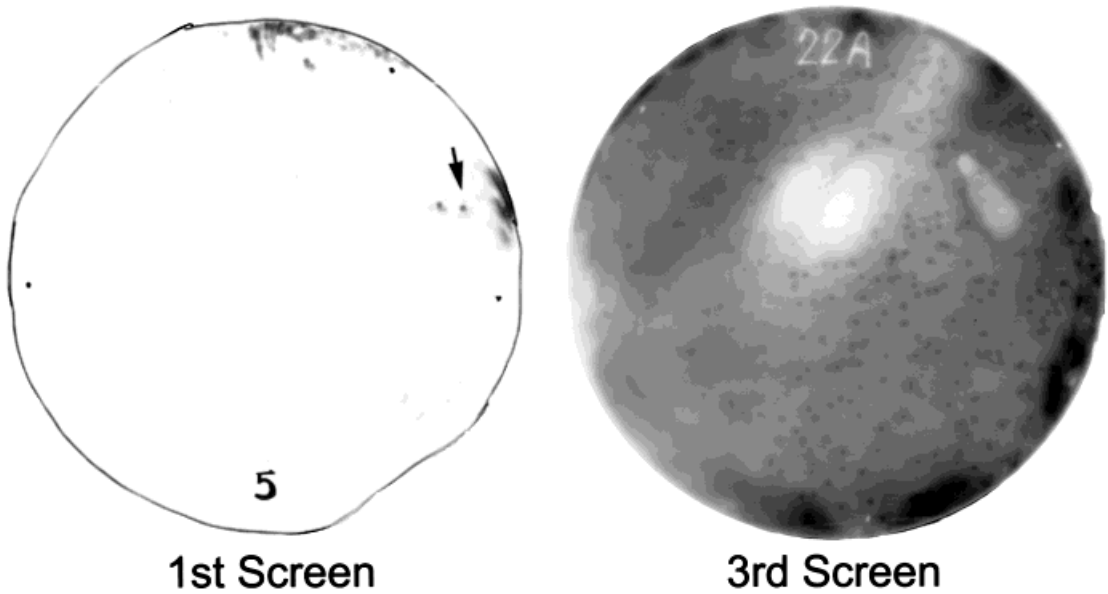

Fig. 4. Plaques corresponding to RbAp46 as detected at the primary and tertiary library screens by Far-Western analysis. Proteins expressed in λpTripleX plaques were transferred to nitrocellulose filters, incubated with 3 μg/ml his-BRCT or his-LacZ.

hematopoietic tumor cell lines [Chester et al., 1989; Shimbara et al., 1993]. RY8 corresponds to the imprinted gene *PEG3* (GenBank accession no. AB006625) [Kim et al., 1997]. Clone RY9 was a chimeric clone identical to mitochondrial rRNA 16S gene (GenBank accession no. V00710.1) and wingless-type MMTV-13 (GenBank accession no Z71621 [Katoh et al., 1996] on one end and corresponding to an unknown sequence on the other end. Based on a BLAST search with high throughput genome sequences (HTGS, the rough draft sequences of the human genome), this unknown sequence maps to chromosome 18. The clone RY24 is the gene encoding the centromere DNA element I (CDEI) binding protein (GenBank accession no. Z22572, [von der Kammer et al., 1994]). This protein was independently described as APLP2 (amyloid precursor like protein 2) based on its similarity with the amyloid precursor protein. Two clones, RY6, RY21 represent known sequences but of unknown function. The sequence of RY6 is embedded in a large genomic clone that also includes the NF1 gene (GenBank accession no. gbAC004526). RY21 corresponds to ESTs from several NCI-CGAP cDNA libraries including adrenal adenoma, colon carcinoma, glioma, as well as tonsillar cells, fetal brain, and liver and placenta. One clone, RY12, just recently matched a sequence of chromosome 5 BAC clone in the database using non-redundant searches, accession no. AC008546.

Confirmation of Expressed mRNA From Positive Clones

DNA probes were made from clones of known and unknown genes and ESTs to determine if these sequences are transcribed into mRNA. Multiple-tissue Northern blots were used to determine the size and tissue specificity of mRNA from the different clones (Fig. 5). RY22 corresponds to RbAp46 and encodes for 2.4 kb mRNA that is most highly expressed in heart and skeletal muscle tissues. High expression was also detected in kidney and placenta. Detectable, but lower expression was observed in liver, small intestine, and spleen. The RY8 clone corresponds to the imprinted gene PEG3 and encodes a mRNA of approximately 9.5 kb expressed almost exclusively in placenta. RY21 corresponds to numerous ESTs isolated from several different tissues represented in dbEST. Northern blot analysis with RY21 as a probe reveals two transcripts of 4.4 and 2.0 kb mRNA. The 2.0 kb transcript is expressed in all tissues but is highest in adipose tissue and esophagus. The 4.4 kb transcript is also expressed in all tissues but is most abundant in placenta. The insert of clone RY21 was 700 bp. We used the database searches to identify additional ESTs

identical to RY21. Some of these ESTs included additional 5′ and 3′sequence when compared to RY21. Interactive database searches were carried out to build a contig of clones extending RY21. Using the EST Assembly Machine program at Tigemnet (http://www.hercules.tige-m.it/cgi-bin/uniestass.pl), we were able to extend the contig containing RY21 to 3.0 kb of contiguous sequence. This sequence is exactly co-linear with a segment of chromosome 19 (BAC Clone CIT-HSO-444n2 accession no. AC005261.1) The sequence of the clone RY12 matched one recent entry in GenBank accession no. AC008546. When we used the insert of clone RY12 to probe a multiple tissue Northern blot, several transcripts were detected. (Data not shown).

Confirmation of Interacting Proteins

To test that the cDNAs isolated by the library screen can be translated into proteins, each insert was cloned and expressed. Because the plaques of pTriplex derived phages contain proteins translated from three reading frames, isolated inserts were subcloned in three frames. Using in vitro transcription/translation reactions, we were able to express in vitro proteins from clones RY8, RY19, RY21, RY22, and RY24 but not RY6.

To confirm that these clones bind to the BRCT domain of BRCA1, in vitro GST pull down experiments were performed incubating GST-BRCT immobilized on glutathione-sepharose beads with [^{35}S]-labeled in vitro translated proteins. Of the in vitro translated proteins tested, we found that BRCT bound specifically only to RbAp46 (RY22) and ribosomal protein L31 (RY 19) (Fig. 6). GST-BRCT did not bind to the other clones or to the negative control, in vitro labeled luciferase protein.

DISCUSSION

In this paper we demonstrated that Far-Western analysis, a method based on direct binding between BRCT and target proteins, can be used to isolate proteins that interact with BRCA1. We found that proteins present in normal breast and breast cancer cell lines bind to the BRCT domain of BRCA1. Interestingly, proteins from lymphoblast cells derived from BRCA1 mutation carriers (i.e., heterozygous for BRCA1 mutations) exhibit a differential protein binding pattern when compared to control cells

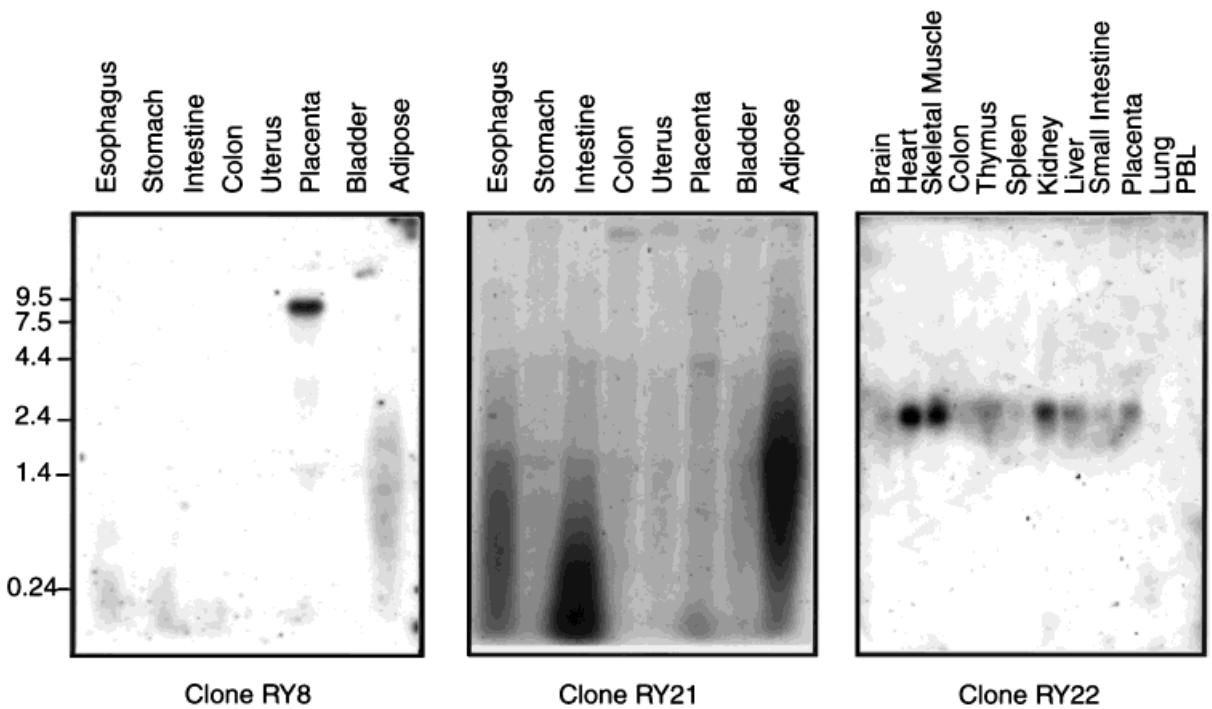

Fig. 5. Expression profiling of putative BRCT-interacting clones. Multiple tissue Northern blots were probed with the different clones isolated by Far-Western analysis. The clones are indicated under each panel. RY8 corresponds to PEG3 (left), clone RY21 (middle), and clone RY22 corresponding to RbAp46 (right).

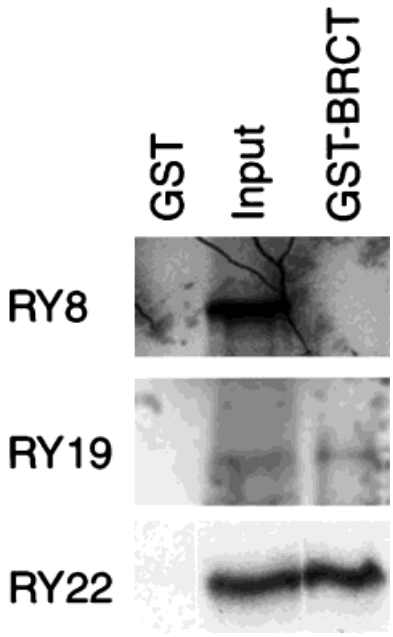

Fig. 6. BRCT binds to proteins RbAp46 and L31 in vitro. GST-BRCT immobilized on glutathione beads was incubated in a pull down experiment with an in vitro transcribed and translated putative clones isolated from the library screen. GST-BRCT complexes were then resolved on 10–20% tricine gels. BRCT-bound proteins were analyzed by autoradiography.

(i.e., wildtype for both alleles). This suggests that the presence of a single mutant allele can affect proteins in the cellular extracts available to bind the BRCT probe used in these experiments. One explanation is that the reduced expression levels of BRCA1 in heterozygous cells is sufficient to down regulate directly or indirectly the expression of its binding partners. It is intriguing to postulate that BRCA1, via its interactions with transcriptional co-repressors such as RbAp46 or CtIP, can modulate the expression of its additional binding partners. Another explanation is that the protein expressed from the BRCA1 mutant allele not only does not bind to its partners, but also can act as a dominant negative and down regulate expression of wildtype BRCA1 binding partners. Recently, Thangaraju et al. [2000] demonstrated that the truncated 5382insC BRCA1 protein acts as a dominant negative protein and abolishes BRCA1-dependent stress-induced apoptosis.

To identify candidate interacting proteins, we screened a human placenta cDNA expression library. We identified eight clones representing putative binding partners of the BRCA1 BRCT domain. Only one clone, RY19, encoding the ribosomal protein L31, included a full length cDNA. Although the function of this protein is not clear, it has been reported to be over-expressed in some types of colon cancers [Chester et al., 1989] and hematopoietic tumor cells [Shimbara et al., 1993]. Direct binding by Far-Western analysis and in vitro binding assays demonstrated that this protein can interact with the BRCT domain of BRCA1, possibly linking BRCA1 to the proteosome.

Other clones corresponded to partial cDNAs: RbAp46 (RY22) is expressed in multiple tissues with particularly high levels in heart, skeletal muscle, and kidney. To our knowledge, this is the first description of the expression profile of RbAp46 in human tissues. The expression pattern observed in human does not correspond completely to that reported in mouse [Qian and Lee, 1995]. RbAp46 mRNA is abundant in both human and mouse kidney tissue. However, expression of RbAp46 is high in human heart and muscle tissues, while the expression in the corresponding mouse tissues is relatively low. The interaction between BRCA1 and RbAp46 was described in detail elsewhere [Yarden and Brody, 1999]. RY21 is expressed in multiple tissues, however, we have not been able to extend the sequence based on database searches beyond 3.0 kb. The sequence of this clone is identical and co-linear to the genomic sequence (GenBank accession no. AC005261.1). No introns or large open reading frames are found in the extended sequence. At least three explanations are possible: (1) this sequence corresponds to the 3′ untranslated region (UTR) of a yet unidentified gene; (2) this gene codes for a structural mRNA of unknown function; and (3) this sequence represents a large exon and we have not yet identified the splicing junctions. Large exons are found in both the BRCA1 and BRCA2 genes. In BRCA1, exon 11 is about 3.0 kb [Miki et al., 1994] and in BRCA2, exon 11 is about 5.0 kb [Tavtigian et al., 1996]. In the future, we may be able to extend the RY21 contig of ESTs further which will enable us to clarify this issue.

Two of the clones correspond to the 3′UTR sequences of known genes. RY8 is a 1.6 kb sequence identical to part of the 3′UTR of the imprinted gene hPEG3. This gene is almost exclusively expressed in placenta. RY24 is a 0.8 kb sequence identical to part of the 3′UTR of the centromere DNA element I (CDEI) binding protein gene. If RY21 also proves to be a 3′UTR,

one may hypothesize that BRCA1 can bind RNA molecules. This is particularly intriguing as recently another BRCA1-interacting protein, BARD1, which interacts with the amino terminus zinc finger was identified in a complex with the polyadenylation factor, CstF-50 (cleavage stimulation factor)—[Kleiman and Manley, 1999]. This interaction inhibited polyadenylation in vitro and was assumed to prevent inappropriate RNA processing during transcription, perhaps at sites of DNA repair. Taken together these interactions may suggest that BRCA1 is part of a complex involved in processing of mRNA precursors. However, we cannot exclude that these clones represent false positives that appear to show specific binding under the conditions used. This is likely to be the case with PEG3 3′UTR (clone RY8) which is highly expressed in placenta. Its abundance in this tissue could potentially drive the interaction between the BRCT polypeptide and its mRNA.

In summary, Far-Western screening can complement other commonly used methods such as traditional and modified yeast two-hybrid screening. During the course of this work, we reported on the interaction between BRCA1 and RbAps (RbAp46 and RbAp48) while three other groups reported on the interaction between the BRCA1 BRCT and a transcriptional co-repressor called CtIP [Wong et al., 1998; Yu et al., 1998; Li et al., 1999]. Our screen did not detect the interaction between BRCA1 and CtIP. One possible explanation is that this particular protein is not expressed in the placenta. Indeed, a search of the EST and SAGE databases failed to identify any cDNA entries of CtIP from a placenta ESTs. Nevertheless, screening an expression library by Far-Western analysis has proven a useful method to isolate interacting proteins, as we reported on the interaction between BRCA1 and RbAp46 [Yarden and Brody, 1999].

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