BRCA1 interacts directly with the Fanconi anemia protein FANCA

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Fanconi anemia (FA) is a rare autosomal recessive disease characterized by skeletal defects, anemia, chromosomal instability and increased risk of leukemia. At the cellular level FA is characterized by increased sensitivity to agents forming interstrand crosslinks (ICL) in DNA. Six FA genes have been cloned and interactions among individual FANC proteins have been found. The FANCD2 protein co-localizes in nuclear foci with the BRCA1 protein following DNA damage and during S-phase, requiring the FANCA, C, E and G proteins to do so. This finding may reflect a direct role for the BRCA1 protein in double strand break (DSB) repair and interaction with the FANC proteins. Therefore interactions between BRCA1 and the FANC proteins were investigated. Among the known FANC proteins, we find evidence for direct interaction only between the FANCA protein and BRCA1. The evidence rests on three different tests: yeast two-hybrid analysis, coimmunoprecipitation from in vitro synthesis, and coimmunoprecipitation from cell extracts. The amino terminal portion of FANCA and the central part (aa 740–1083) of BRCA1 contain the sites of interaction. The interaction does not depend on DNA damage, thus FANCA and BRCA1 are constitutively interacting. The demonstrated interaction directly connects BRCA1 to the FA pathway of DNA repair.

INTRODUCTION

Fanconi anemia (FA) is a rare autosomal recessive disorder characterized by birth defects, developing pancytopenia and increased risk of malignancy. At the cellular level the disease is manifest by chromosomal instability and increased sensitivity to agents such as mitomycin C (MMC) and diepoxybutane (DEB) which form DNA interstrand crosslinks (ICL). Following exposure to such agents, abnormal chromosome structures, including quadriradials and breaks, are noted (reviewed in 1 and 2). From the eight known complementation groups (3), six genes (FANCA, FANCC, FANCD2, FANCE, FANCF and FANCG) have been isolated (4–10). They do not have significant homology to other entries in the databases and do not contain recognized functional motifs, although FANCF shares partial homology with a bacterial protein, ROM (9). The FANCG gene is the homologue of XRCC9, a gene mutated in Chinese hamster ovary cells defective in ICL repair (7).

The FANCA, C, F and G proteins form a nuclear complex (11–14). Additional evidence of association of FANC proteins is that FANCA, C, E, F and G interact by yeast two-hybrid analysis (15). The nuclear complex does not form in FANC-defective cell lines, except for FANCD1 or FANCD2 lines. The complex must be assembled for the FANCD2 protein to be modified by monoubiquitination, needed for a normal repair response (10,16). This suggests the FANCD2 gene, which is conserved outside of vertebrates (10), may function downstream from the complex. The modified FANCD2 protein colocalizes with the BRCA1 tumor suppressor protein during S-phase and following DNA damage and can be coimmunoprecipitated with BRCA1(16).

The BRCA1 tumor suppressor gene is a 220 kDa protein with several activities, including transcriptional activation and DNA repair (reviewed in 17 and 18). Cells deficient in BRCA1 show chromosome instability (19) and BRCA1 associates with numerous DNA repair proteins (20) in a super complex, the BASC model (21), which might play a role in DNA damage sensing and activation of repair proteins (18). BRCA1 also interacts with BRCA2, known to interact with RAD51 (reviewed in 18). If BRCA1 acts via homologous recombination (HR) to maintain chromosome integrity after double strand breaks (DSB), association with proteins involved in ICL or DSB repair might be expected. Therefore, interaction with the FANC proteins would not be surprising.

As a result of the colocalization of BRCA1 with FANCD2, we tested for direct protein–protein interactions between BRCA1

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and all of the FANC using the yeast two-hybrid system (22). The only direct interaction of BRCA1 and any FANC protein was found with FANCA. The interaction appears constitutive and is not damage-dependent. The amino terminal portion of FANCA and the central portion of BRCA1 (aa 740–1083) contains the sites of interaction. The results provide evidence that FANCA and BRCA1 directly interact and indicate that BRCA1 is an integral component of the FA pathway. In light of the reported coimmunoprecipitation of FANCD2 and BRCA1 the results presented here suggest the FANCD2 interaction with BRCA1 may occur via the FANC complex.

RESULTS

FANCA and BRCA1 interact in the yeast two-hybrid test

The colocalization of FANCD2 and BRCA1 in nuclear foci after DNA damage (15) prompted an inspection of the interactions of each of the FANC proteins with BRCA1 in the yeast two-hybrid system. All constructs had expression verified by immunoblot analysis (not shown). Only FANCA constructs gave interactions with BRCA1 constructs (Fig. 1), while no interaction was seen between BRCA1 and FANCC, FANCD2, FANCE, FANCF or FANCG constructs (data not shown). Sub-constructs showed that the N-terminus of FANCA and the central portion of BRCA1 were essential for the interaction. The FANCA/BRCA1 interaction was verified by constructs fused to either the binding domain or activation domain, except for amino acid residues 629–1313, 1049–1313 and 1308–1855 of BRCA1, which autoactivated when fused to the binding domain. The BRCA1 constructs, amino acids 629–1313 and 740–1083, were tested against all the other FANC constructs and did not show any interactions.

Since a stimulus for searching for protein interactions in the yeast two-hybrid system was the observed colocalization of FANCD2 with BRCA1, it was surprising not to find interaction for those two proteins. However, colocalization does not define a direct interaction, but might be dependent on additional proteins. The BRCA1–FANCA interaction indicates a specific reaction and suggests a central role for FANCA in associating the FANC complex with BRCA1.

The region of BRCA1 binding to FANCA contains several sites identified for breast cancer-related mutations. One rare polymorphism not related to breast cancer (Ser1040Asn) (23) was found in the BRCA1 construct recovered from a lymphoblast library. Two cancer-related mutations (Lys820Glu and Gly960Asp) were introduced by site-directed mutagenesis. None of these sequence alterations affected binding of the product to FANCA. Thus nothing indicates that the involved sites are disrupting interaction with FANCA as a loss of tumor suppressor function.

FANCA and BRCA1 coimmunoprecipitate in vitro

To confirm the interaction of BRCA1 and FANCA in the absence of DNA damage, an in vitro transcription/translation system was used. The specific T7 RNA polymerase promoter in the two-hybrid vectors produces protein fusions with epitope tags. BRCA1 amino acid residues 629–1313 coimmunoprecipitated with FANCA (Fig. 2A), confirming direct interaction in this system. Confirmation of the interaction and additional definition was achieved by coimmunoprecipitation of in vitro-translated BRCA1 (amino acids 740–1083) and the N-terminal region of FANCA (Fig. 2B), in agreement with the results of the two-hybrid system. Thus, the relevant domains of the two proteins do not appear to require additional factors or damaged DNA to promote interaction. The interacting regions of the proteins as determined by this test were the same as for the yeast two-hybrid system. In contrast, a C-terminal construct of BRCA1 did not coimmunoprecipitate with the N-terminal FANCA construct, again consistent with the two-hybrid results, demonstrating the specificity of the interaction for the central domain of BRCA1 (Fig. 2C). The negative result with the in vitro-translated C-terminal domain of BRCA1 rules out non-specific interaction of FANCA due to binding to the beads.

Coimmunoprecipitation of FANCA and BRCA1 in cell extracts

Given the direct interaction in yeast and in vitro between BRCA1 and FANCA, it seemed reasonable to search for interaction in human cell lysates. Using a FANCA-defective cell line (GM6914) that does not express detectable FANCA protein, as well as the same line complemented with a retrovirus carrying an intact FANCA gene (24), BRCA1 and FANCA coimmunoprecipitated (Fig. 3) using antibodies to BRCA1. In the absence of detectable FANCA protein, no precipitate was seen (Fig. 3A, lane 4). The interaction does not appear to be influenced by DNA damage in asynchronous cell cultures, suggesting that it is constitutive in fibroblasts (Fig. 3A, lanes 2 and 3). In the complemented cells producing FANCA there was no precipitated FANCA when antibodies to BRCA1 were not used (Fig. 3, lane 7), ruling out non-specific binding of FANCA to the beads. FANCA and BRCA1 coimmunoprecipitation was also observed in GM639 immortal normal human fibroblasts without DNA damage (Fig. 3B). Thus, the interaction seen in complemented FANCA-defective cells was not the result of overproduction of FANCA. Any of the three anti-BRCA1 antibodies used independently duplicated the results obtained with a mixture of all three (Fig. 3C). The coimmunoprecipitation reflected specific interaction, as shown by a control immunoprecipitation for NFkBp65 which did not coimmunoprecipitate with the BRCA1 protein in cellular extracts (Fig. 3D). Coimmunoprecipitation of FANCD2 and BRCA1 was not re-examined.

DISCUSSION

The findings presented define a direct interaction between FANCA and BRCA1. Interactions between FANC proteins and proteins other than additional FANC proteins are limited, but interaction between FANCC and FAZF, a zinc finger protein (25) and between FANCA and BRG1, a component of the SWI/SNF complex (26), have been noted. The interaction reported here links BRCA1 directly to the FA pathway, establishing interaction between two proteins with tumor suppressor function, both needed for normal repair of ICLs and DSBs.
What might the function of the interaction between BRCA1 and FANCA be? Based on the results reported here, it might be speculated that the BRCA1 protein acts in the detection of a DSB (or an intermediate in ICL repair) (Fig. 4) and possibly serves to bring DNA repair proteins to the site, since it interacts with numerous repair proteins (21). Among such proteins would be FANCA, constitutively interacting with BRCA1. FANCA could serve as a docking site or anchor point at the site of ICL damage for the FANC complex. FANCA, C, E and G are known to assemble in a nuclear complex required for the action of FANCD2. Alternatively, BRCA1 might localize FANCA to the site of DNA damage and then release it to initiate complex formation. The complex would allow ubiquitination of FANCD2, apparently a late function or terminal effector in the FA path, promoting ICL repair.

This model is compatible with the BASC model of genome surveillance in which BRCA1 is associated with numerous DNA repair proteins (18,21,27). The model makes several suggestions. First is the possible function of this complex as an ubiquitin ligase for FANCD2 (15). The BRCA1 protein contains a RING finger motif (17) and such domains are known to have ubiquitin ligase activity (28). Second, other proteins known to interact with BRCA1 include BRCA2, which associates with RAD51 (18), bringing the HR protein into association with the FANC complex. Thus interactions of RAD51 and the FANC complex may occur during ICL repair. Third, in addition to localization of repair proteins to the site of damage, this model does not rule out the BRCA1 and FANC complex from serving a structural role in stabilizing the intermediates required for ICL repair.

If indeed both BRCA1 and FANC function in DSB repair, then the question arises of whether they act in the same pathway—are epistatic—or different pathways, non-epistatic. Nothing rules against the BRCA1 protein’s acting in more than one pathway of DSB repair, perhaps by serving a general function important to more than one path. Similarly, the FA pathway seems limited to vertebrates and has not been shown to share components with other recognized ICL repair paths found in lower eukaryotes (29). Therefore open questions resulting from the observations described here include the functional result of the FANCA/BRCA1 interaction and whether this reflects a general or specific role of BRCA1 in ICL and DSB repair.

Figure 1. Two-hybrid analysis of BRCA1 and FANCA. Domains of BRCA1 (17) are indicated. Constructs producing interaction are filled; those not producing interaction are open. Numbers refer to amino acid position of the proteins. Fusions to the activation domain or binding domain are indicated by AD or BD, respectively. Colony staining for β-galactosidase activity is shown on the right.
MATERIALS AND METHODS

Bacterial and yeast strains

*Escherichia coli* strains XL1-Blue MRF’ and DH5α and DH10B (Stratagene and Life Technologies, USA) were used for cloning and propagation of plasmids. For yeast two-hybrid analysis, the *Saccharomyces cerevisiae* strains Y187 and AH109 (Clontech, USA) were used to transform the activation domain and binding domain constructs, respectively.

Plasmids and expression constructs

Yeast two-hybrid vectors pGBKT7 [GAL4 (1–147) DNA-BD, TRP1, kan, c-Myc epitope tag] and pGADT7 [GAL4 (768–881) DNA-AD, LEU2, amp, HA epitope tag] (Clontech) were used to generate two-hybrid constructs. BRCA1 constructs were amplified by polymerase chain reaction (PCR) from a full-length cDNA clone (5’ HA epitope-tagged BRCA1 in pcDNA, a gift from R. Scully). A 2.37 kb PCR product covering BRCA1 aa 1–784 was amplified with primers 5’-CACACCCGGGTATGGATTTATCTGCTTTGC and 5’-CAGGATCCAACGAGATACCTTTCTGA; the PCR product was ligated to pGADT7 and pGBK7. Constructs spanning BRCA1 aa 629–1313 were generated using primers 5’-CAGAATTCAATGGTTGTTCCGAGATAATAGAAATGACACAG and 5’-CTGTGTCATTTCATATTATCTTCGGAACAACCATGAATTAGTC. Primers 5’-CATCTCAGTTCAGAGACAACGAAACTGGACTC and 5’-CAGAATTCAATACAAACACCCAGGAT and 5’-CACAGTCGACTATCAGGTAGGTGTCCAG. A BRCA1 fragment encoding aa 629–1313 was digested with Bgl II (internal site at nt 2244). An internal Scal-BamHI fragment encoding aa 1049–1313 was also used.

An additional construct covering BRCA1 aa 629–1313, amplified from a human lymphoblast cDNA library (a gift from M. Buchwald) contained a Ser1040Asn polymorphism (23). Using the Quick-Change (Stratagene) site directed mutagenesis, two other mutations associated with breast cancer were introduced into a BRCA1 subclone encoding aa 740–1083 in pBluescript (Stratagene). To generate a Lys820Glu mutation, the primers were: 5’-GACTAATTCATGGTTGTTCCGAGATAATAGAAATGACACAG and 5’-CTGTGTCATTTCATATTATCCTTTCTGAACCAACCATGAATTAGTC. Primers 5’-CATCTCAGTTCAGAGACAACGAAACTGGACTC and

Figure 2. *In vitro* coimmunoprecipitation of 35S-labeled proteins. Samples were precipitated with either HA or c-Myc antibody as indicated. (A) HA-fused full length FANCA and c-Myc-fused BRCA1 (aa 629–1313). (B) HA-fused FANCA (aa 1–589) and c-Myc-fused BRCA1 (aa 740–1083). (C) HA-fused FANCA (aa 1–589) and c-Myc-fused BRCA1 (aa 1308–1855).
GAGTCCAGTTTCGTTGTCTCTGAACTGAGATG were used to introduce the Gly960Asp mutation. A full-length FANCA cDNA clone was kindly provided by M. Buchwald. The coding region was subcloned into pBluescript (Stratagene) and shuttled into pGBKT7 and pGADT7 using flanking engineered EcoRI sites. FANCA subclones encoding aa 1–589, aa 589–1291, and aa 691–1455 were generated. Sequences and reading frames for all constructs were confirmed by DNA sequencing. Expression was verified by western blotting.

Cell culture

SV40 transformed fibroblast cell lines GM6914 (FANCA) and GM639 (normal) were grown in α-MEM supplemented with 10% FCS. The cell line GM6914 also was functionally complemented with a pMMP retroviral vector containing the 5′-GAGTCCAGTTTCGTTGTCTCTGAACTGAGATG were used to introduce the Gly960Asp mutation.

A full-length FANCA cDNA clone was kindly provided by M. Buchwald. The coding region was subcloned into pBluescript (Stratagene) and shuttled into pGBKT7 and pGADT7 using flanking engineered EcoRI sites. FANCA subclones encoding aa 1–589, aa 589–1291, and aa 691–1455 were generated. Sequences and reading frames for all constructs were confirmed by DNA sequencing. Expression was verified by western blotting.

Figure 3. Coimmunoprecipitation of BRCA1 and FANCA from human fibroblasts. (A) Coimmunoprecipitation of FANCA with BRCA1 from corrected and uncorrected FANCA mutant cell line GM6914. Cells were treated with 15 Gy γ-radiation as shown and harvested after 16 hours, immunoprecipitated with anti-BRCA1 and western analysis performed. Blots were probed with anti-FANCA. Lane 1—whole cell extract, nonirradiated, corrected; lane 2—immunoprecipitate, irradiated, corrected; lane 3—immunoprecipitate, nonirradiated, corrected; lane 4—immunoprecipitate, irradiated, non-corrected; lane 5—marker; lane 6—immunoprecipitate, nonirradiated, non-corrected; lane 7—immunoprecipitate, corrected, using beads without anti-BRCA1. (B) Coimmunoprecipitation of FANCA with BRCA1 from cell line GM639. Lanes 1 and 2 are without and with 15 Gy γ irradiation respectively. (C) Coimmunoprecipitation of FANCA with three independent monoclonal antibodies against BRCA1 from the FANCA negative cell line GM6914, corrected with a FANCA retrovirus. Lane 1—Ab-1; lane 2—Ab-4 (Oncogene); lane 3—D-9 (Santa Cruz). (D) Anti-NFκBp65 probe of BRCA1 immunoprecipitation from GM639. Lane 1—whole cell extract; lane 2—immunoprecipitate; lane 3—supernatant from immunoprecipitation.

Yeast two-hybrid interaction assay

Yeast two-hybrid assays followed protocols provided by Clontech. Two-hybrid constructs were transformed into the yeast strains using a lithium acetate/ssDNA/PEG procedure (30). Transformants were selected on synthetic dropout (SD) plates lacking leucine (Y187 transformants) or tryptophan (AH109 transformants). Mating occurred on YPD agar plates, and subsequent selection for diploid yeast was performed on SD agar plates lacking leucine and tryptophan. To test for interacting proteins, the diploid yeast
were plated on SD lacking leucine, tryptophan, adenine, and histidine (AHTL), supplemented with 1 mM 3-amino-1, 2, 4-triazole (3-AT). All constructs in the GAL4 binding domain were tested for autoactivation by mating with an empty GAL4 activation domain vector followed by plating on SD lacking AHTL with 3-AT. A β-galactosidase assay was used to verify interactions.

Colony lifts using an X-gal assay for β-galactosidase activity were also performed to test for interacting proteins. After mating on YPD, diploid yeast were selected by growth on SD lacking leucine and tryptophan. A charged nylon membrane was placed on the surface of the colonies, and then submerged into liquid nitrogen for 10 seconds. After thawing, the filter was placed onto Whatman 3M paper prewetted in a Z buffer/X-gal solution (as described in the Clontech manual) and incubated at 30°C for 1–8 hours.

**Coimmunoprecipitation of in vitro-translated proteins**

The T7 TnT coupled transcription-translation system (Promega) was used to produce 35S-labeled epitope-tagged proteins from pGBK7T (c-Myc epitope) and pGADT7 (HA epitope) expression constructs, following the manufacturer's protocol. Coimmunoprecipitation of in vitro-translated proteins was based on a published protocol (16). Equal amounts of in vitro-translated protein products were combined and incubated at 30°C for 60 min. To each sample an equal volume of buffer A (20 mM Tris, pH 7.8, 50 mM NaCl, 3.5 mM EDTA, and 1.0% NP-40), protease inhibitors, and either anti-HA or anti-c-Myc antibodies (Santa Cruz Biotechnologies and Sigma) was added. Samples were mixed gently overnight at 4°C by rotation. Dynabeads conjugated with goat anti-mouse IgG (Dynal) were then added, followed by a 2 hour incubation at 4°C. Using magnetic separation, the Dynabeads were washed three times with buffer A. The Dynabeads were then suspended in SDS sample buffer, boiled, and separated on 8% denaturing polyacrylamide gels. Gels were dried and proteins were detected by autoradiography.

**Coimmunoprecipitation from cultured fibroblasts**

Fibroblasts were harvested by trypsinization and centrifugation. Cell pellets were washed once with PBS, then resuspended in 1 ml cell lysis solution (50 mM Tris, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 2 µg/ml aprotinin, 200 µg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 25 mM sodium pyrophosphate). Lysates were sheared with a 23 gauge needle, and cell debris was removed by centrifugation. Dynabeads (Dynal) coated with anti-mouse IgG were washed with excess PBS with 0.1% BSA prior to use. Preclearing of the lysates was done by adding Dynabeads and gently mixing by rotation for 4 hours at 4°C. The Dynabeads were removed by magnetic separation, and the lysates were then mixed with dynabeads that had previously been incubated at 4°C for 24 hours in PBS with 0.1% BSA and an equal mixture of three primary anti-BRCA1 monoclonal antibodies [BRCA1(AB1), BRCA1(AB4), and BRCA1 (D9) (Oncogene and Santa Cruz Biotechnologies)], and washed three times with PBS with 0.1% BSA. After gently mixing for 1 hour at 4°C, the beads were magnetically separated, washed with lysis buffer, brought up in SDS sample buffer, boiled, and analyzed by 8% denaturing polyacrylamide gel electrophoresis. Proteins were detected by western analysis using chemiluminescence (NEN) with rabbit polyclonal antibody to FANCA (15).
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