Identification of Residues Required for the Interaction of BARD1 with BRCA1*

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The breast and ovarian cancer predisposition gene product BRCA1, binds to BARD1 at its N terminus. In cells BRCA1 is found as a heterodimer with BARD1 and may represent the functionally active form of BRCA1. Using yeast two-hybrid and split-hybrid screens we have identified 16 independent missense mutations which prevent the ability of the BARD1 N terminus to heterodimerize with BRCA1. With reference to the recent structure of the BARD1-BRCA1 RING complex (Brzovic, P. S., Rajagopal, P., Hoyt, D. W., King, M-C., and Klevit, R. E. (2001) Nat. Struct. Biol. 8, 833–837) we note two classes of mutation; those that map to the hydrophobic core forming the BARD1:BRCA1 interface and are substitutions of leucine, and those that map to residues forming intramolecular contacts either in helical packing, or in the conserved zinc chelating cysteine residues of the RING itself. The directed mutation of charged residues predicted to play a role in the interaction could not alone prevent heterodimer formation suggesting that, while polar interactions may participate in the specificity of the interaction, they are not crucial. Together these data provide functional evidence for the requirement of a hydrophobic interface and illustrate that disruption of the tertiary structure by mutations away from the interface itself are able to prevent formation of the heterodimer.

The breast and ovarian cancer predisposition gene, BRCA1, is relevant to several biological processes, from DNA damage repair, centrosome duplication, and protein degradation, to chromatin remodeling and transcriptional activation (reviewed in Refs. 2 and 3). The N-terminal binding protein of BRCA1, BARD1, may participate in some of these activities since it associates with BRCA1 and RAD51 in nuclear dots during S-phase (4) and after DNA damage with BRCA1 and PCNA (5). Importantly BRCA1 protein bearing the disease-associated C61G mutation, which occurs in a zinc-ligating residue of the BRCA1:BARD1 heterodimer each molecule is composed of three structural elements: the RING itself and two α-helices which in the primary sequence flank the RING, but in the three-dimensional structure are antiparallel to each other and closely packed together (1). The helices interface in a hydrophobic core formed by two helices from each molecule, whereas the RING motifs themselves interact little. The structural data suggests that the interaction is augmented by polar interactions. Here we show that mutations in single leucine residues of the BRCA1 hydrophobic face to either proline or charged amino acids are able to inhibit the interaction with BRCA1, whereas changes in polar residues predicted to play a role were not correlated with inhibition of the interaction. Mutations in residues located away from the helical core, but in areas significant for BARD1 structure stabilization also inhibit heterodimer formation. This information provides a functional:structural framework for understanding the potential pathology of newly identified polymorphisms and/or mutations in BARD1 and BRCA1 N-terminal regions from patient material.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Human BRCA1 and BARD1 cDNAs were used as template in PCR. For ligation into pLexA (Qiogene, Harefield, United Kingdom) the forward primers were synthesized with additional 5′ linker sequences, (5′ to 3′) GAATTCCTCGGATCCGTCATT (containing a BamHI site) in-frame with codons 2–7 of BRCA1 or codons 27–32 of BARD1. The reverse primers consisted of the linker sequences (5′ to 3′) GGCCTCGAGCTCGACG (containing a SalI site) and the complement of codons 105–111 of BRCA1 or 134–140 of BARD1. Each PCR fragment was digested with BamHI and SalI and ligated into a similarly digested pLexA plasmid.

For ligation into pSHM.1-LacZ, referred to in this study as pVP16-LacZ (Qiogene, Harefield, UK), the forward primers were synthesized with 5′ linker sequences (5′-3′) CGCGTTGAGTTCCCTCTAGA (containing a BamHI site) in-frame with BARD1 codons 27–32, 49–55, or 87–93.

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† The abbreviations used are: Pol II, polymerase II; aa, amino acid(s).
The reverse primers contained linker sequences (5'-3') CCGGGG-GATCCCAAGATCT (containing a BglIII site) and the reverse comple-ment of BARD1 codons 134–140, 113–119, 81–87, or 43–49. Different combinations of primers were used to generate BARD1 N-terminal variants and each PCR fragment was digested with BamHI and BglIII and ligated into similar pLexA-BRCA1 digested plasmid. All con-structs were sequenced. One pVP16-BARD1 aa 27–119-LacZ construct was found to contain an A insertion before the BARD1 sequence. This was utilized as a frameshift LacZ and BARD1 negative control.

**Yeast Assays, Library Screen, β-Galactosidase Assay, and Plasmid Rescue**—The yeast two-hybrid strain, L40 (a kind gift of Richard Good-man, Volum Institute, Portland, OR), and yeast split-hybrid strain, Y1596 (Inc., Harefield, UK), were used in two-hybrid and split-hybrid assays as described by Shih et al. (15). β-Galactosidase colony filter assays were performed as described previously (16). Plasmid rescue was performed as described in Ref. 17. pVP16-BARD1-LacZ colonies containing bacteria were discriminated from pLexA-BRCA1 plasmid transformants by selection on X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopynoside).

In situ PCR transcripts were prepared from yeast in urea/ SDS cracking buffer (18), resolved by 6% SDS-PAGE, and blotted as described (19). A 1:2000 concentration of monoclonal αVP16 (Santa Cruz, sc-7545) or αLexA (sc-7544) antibody was used followed by incubation with an horseradish peroxidase-conjugated anti-mouse antibody and ECL reaction (Amersham Biosciences, Inc.) detected by short exposure to x-ray film.

**PCR Mutagenesis and Generation of BARD1 Random Mutant Libraries**—Mutagenic PCR explored the 1 in 500 bp error rate of Taq polymerase (Promega). The VP16-BARD1 aa 27–119-LacZ construct was used as template and reactions contained standard PCR buffer and dNTP concentrations. The primers used annealed to elements in the VP16–BARD1 construct with a random fashion across BARD1 residues 27–119. The split-hybrid system was observed, either on solid plates or liquid culture (Table I). We conclude that the earlier transfection initiation of BARD1 would be unlikely to alter its ability to bind BRCA1.

**Functional Screen for Single Amino Acid Changes Which Disrupt the BARD1-BRCA1 Interaction**—The results above and those of others (20) suggest that the flanking helices and the RING of BARD1 are required for the interaction with BRCA1. To establish which residues were required we performed a yeast split-hybrid screen (15) on a library mutated in a random fashion across BARD1 residues 27–119. The split-hybrid strain contains two integrated reporters. In the first, the coding region of the Tet-repressor TetR gene is fused to the nuclear localization signal of SV40 and its expression is controlled by binding sites for lexA in its promoter. In the second, the coding region of yeast HIS2 is regulated by the Tet operator. Since the expression of TetR depends on the formation of a complex between VP16-BARD1-LacZ and LexA-BRCA1, the system gives growth on media lacking histidine when complex formation is prevented. Indeed the yeast split-hybrid strain, Y1596, failed to grow on selective media lacking histidine when transformed with pLexA-BRCA1 aa 2–111 and pVP16-BARD1 aa 27–119-LacZ, whereas pLexA-BRCA1 aa 2–111 and the truncated BARD1 aa 48–119 construct allowed growth (data not shown).

### RESULTS

**The RING Domain and Flanking Helices of BARD1 Are Required for the Interaction with BRCA1**—Amino acids 26–119 of BARD1 and 1–109 of BRCA1 make up the smallest stable BRCA1-BARD1 N-terminal heterodimers in vitro (20). To establish, functionally, in eukaryotic cells, which regions of the BARD1 N terminus were required for interaction with BRCA1 we tested subdivisions of BARD1 (shown in Table I) for the ability to bind the N terminus of BRCA1, in a yeast two-hybrid assay. Importantly BARD1 polypeptides were flanked by the VP16 transactivation domain and 3’ by the LacZ gene. The LexA DNA-binding protein was fused to BRCA1 residues 2–111 or used alone. Table I illustrates that BARD1 amino acids 27–140 or 27–119 allowed growth on selective medium lacking histidine, indicating an interaction. Conversely frameshift BARD1 27–119, pLexA alone, or any other BARD1 fragment failed to allow growth in the absence of histidine indicating that residues on either side of the RING domain are required for the interaction. All constructs allowed growth on media with histidine but selective for the inclusion of the pLexA and VP16-LacZ plasmids (data not shown). Immunoblot analysis confirmed there was approximately equal expression of fusion proteins from each construct (data not shown).

| BARD1 amino acids within VP16-BARD1-LacZ (boxes represent conserved cysteine residues of the RING domain) | Growth on -histidine |
|---|---|
| BRCA1 LexA only |
| 27–140 | ++ |
| 27–119 | ++ |
| 49–87 | - |
| 27–87 | - |
| 49–119 | - |
| 27–49 | - |
| 87–119 | - |
| 87–140 | - |
| shift (27–119) | - |
| (2–119) | ++ |

**Summary of yeast two-hybrid experiment showing regions of BARD1 required for heterodimerization with BRCA1.**
The BARD1 library was generated within the pVP16-BARD1 vector. The library was screened for the interaction with BRCA1 using a two-hybrid system. The screen revealed high levels of tolerated amino acid substitutions, with the most nonconserved substitutions being proline substitutions at codon 102. Sequencing clones selected by the split-hybrid system revealed that no substitution at codon 102 was found in the absence of another mutation, suggesting that an Asp at this position is unlikely to be part of the BARD1:BRCA1 interface. Since growth in the two-hybrid system requires that the proteins be correctly folded to retain their binding function, it is interesting to note that potentially disruptive amino acid substitutions such as Gly and Pro were found. Although correct folding can only be inferred from this data, we can conclude that the inclusion of potentially disruptive amino acids such as Pro into this helix do not, simply by their very presence, affect BARD1 folding enough to prevent the interaction with BRCA1. We theorize that the behavior of the substitution is also greatly influenced by the role of the amino acid replaced.

**Types of Mutation Revealed in the Screen**—Generation of the BARD1 N terminus library exploited the 1 in 500 bp error rate of Taq polymerase. However, errors generated by Taq polymerase are frequently transitions (21) and the degeneracy of the genetic code means that amino acid substitutions by this method are limited. Nevertheless nonconserved substitutions that could be brought about by transitions include Ala → Thr, Trp → His, Trp → Gin, Ala → Gly, Ile → Thr, His → Tyr, Met → Thr, Ala → Gly, Ala → Lys, and Leu → Pro. The split-hybrid screen described above of BARD1 randomly mutated constructs with BRCA1 selected 6/8 transitions CTG → GTG to CC(T/G)T, (Leu → Pro), one transversion TGT → TGG (Cys to Trp), and one compound change ATT to TAT (Ile to Tyr), presumably generated by two separate transversions. Therefore of the many transitional substitutions possible the screen selected Leu to Pro changes, while some substitutions arose by transversion, and one by more complex changes. These observations suggest that the range of mutations available to the screening process was not significantly restricted.

**D102P Cannot Inhibit Formation of the BARD1:BRCA1 Heterodimer**—Since the split-hybrid system is an assay for loss of function and the substitution to Pro introduces the most structurally disruptive amino acid, it was important to establish whether changes to proline in other residues within the helices might prevent the interaction. Based on the NMR structure (1), Asp-102 is a surface residue on the C-terminal helix, proximal to the RING structure. It is acidic and protrudes into the solvent. It is not predicted to have either intra- or intermolecular contacts. We screened a library in which codon 102 had been replaced with degenerate nucleotides so that all amino acids should be represented at that position. Sequencing clones selected by the split-hybrid system revealed that no substitution at Asp-102 was found in the absence of another mutation, suggesting that an Asp at this position is not required for the interaction with BRCA1. Furthermore, sequencing clones that were selected in the two-hybrid screen which selects for the formation of the heterodimer, revealed many substitutions at codon 102 (see Fig. 1) confirming that this residue is unlikely to be part of the BARD1:BRCA1 interface. Since growth in the two-hybrid system requires that the proteins be sufficiently correctly folded to retain their binding function it is interesting to note that potentially disruptive amino acid substitutions such as Gly and Pro were found. Although correct folding can only be inferred from this data we can conclude that the inclusion of potentially disruptive amino acids such as Pro into this helix do not, simply by their very presence, affect BARD1 folding enough to prevent the interaction with BRCA1. We theorize that the behavior of the substitution is also greatly influenced by the role of the amino acid replaced.

**Substitution of Leucine Residues at the BARD1:BRCA1 Interface by Hydrophobic Amino Acids Is Tolerated by the Interaction**—Several of the mutations found in the split-hybrid screen (Leu-44, Leu-101, and Leu-111) map to the hydrophobic core formed by the four helices of BARD1 and BRCA1 (Fig. 2). To establish whether these residues functionally form part of a hydrophobic interaction face, degenerate libraries at codon 44 and 111 were independently examined in the split-hybrid system (to select for substitutions inhibitory to heterodimer formation) and the two-hybrid system (to select for tolerated substitutions). The results are shown in Fig. 1. Both polar (Gln) and charged amino acids (Arg, Asp) at codon 44 result in inhibition of heterodimer formation, whereas hydrophobic substitutions (Try, Ala, Ile, and Phe) are tolerated. The requirements at codon 111 are less strict as hydrophobic (Ile, Ala, and Val) and polar residues (Thr and Gln) are tolerated but the basic residues Arg and Lys are not. These observations confirm the view that substitution to amino acids other than Pro can inhibit the interaction. They show that a hydrophobic core is required for the interaction between BARD1 and BRCA1 since hydrophobic substitutions are tolerated, whereas hydrophilic, particularly charged substitutions are not. Importantly, despite the extent...
of the hydrophobic core, a single charged residue within it is able to prevent the formation of the heterodimer.

No Evidence for the Requirement for Electrostatic Interactions between BARD1 and BRCA1—From the NMR structure it has been inferred that electrostatic interactions between four charged residues of BARD1 and BRCA1 may augment the hydrophobic interactions and form part of the binding face (1). We had screened a degenerate codon library for one of these, Arg-43, and created a degenerate codon 43 library which was then tested in the yeast-split hybrid system with pLexA-BRCA1 aa 2–111. No substitution of Arg-43 was found in the absence of another, already characterized, heterodimer inhibiting mutation. We can conclude therefore that Arg-43 has no significant role in the interaction since heterodimer inhibition required a second mutation elsewhere in BARD1, although we cannot discount a slight contribution to electrostatic interactions from this residue.

Directed substitution of a residue, Arg-43, potentially forming part of an electrostatic interaction with BRCA1, could not inhibit heterodimer formation alone, suggesting that either electrostatic interactions are not part of the BARD1-BRCA1 interaction or at least that removal of one of the several charged residues required for the interaction between BARD1 and BRCA1. The initial screen was random and blind, selecting only for single amino acid changes that prevent the interaction of BARD1 with BRCA1. Of the 15 mutations identified in that screen 14 occurred in the two areas corresponding to the N- and C-terminal helices (Fig. 1), demonstrating their importance in the interaction. In the 40 or so residues that make up the RING domain only one mutation was found, and this in a zinc-ligand, suggesting that any direct interactions of the RING with BRCA1 are slight, and that intramolecular contacts with the helices are flexible. No mutations occurred in charged or polar residues. No mutations, apart from those at the hydrophobic residues that are required for zinc binding at site 2 in the RING (see Fig. 2). Whereas Arg, although charged carries a large side chain which may allow it to fill the hydrophobic pocket yet leave the charge in contact with the solvent. While more data is required to elucidate the precise mechanism of the inhibition it is clear that mutations in residues predicted to make contacts between secondary structural elements of the BARD1 N terminus can inhibit the interaction with BRCA1.

The C71W mutation is within one of the four cysteine residues required for zinc binding at site 2 in the RING (see Fig. 2). While one might predict that a loss of a zinc-binding residue would have serious consequences for the structure of the RING domain in the absence of further mutations in the remaining three chelating site II residues this remains conjecture. It is possible that the substitution to Trp may be largely responsible for the loss of structural integrity which leads to an inhibition of heterodimer formation.

DISCUSSION

These data arise from eukaryotic assays and identify residues that are required for the interaction between BARD1 and BRCA1. The initial screen was random and blind, selecting only for single amino acid changes that prevent the interaction of BARD1 with BRCA1. Of the 15 mutations identified in that screen 14 occurred in the two areas corresponding to the N- and C-terminal helices (Fig. 1), demonstrating their importance in the interaction. In the 40 or so residues that make up the RING domain only one mutation was found, and this in a zinc-ligand, suggesting that any direct interactions of the RING with BRCA1 are slight, and that intramolecular contacts with the helices are flexible. No mutations occurred in charged or polar residues. No mutations, apart from those at the hydrophobic face of the four helical bundle occurred on surface residues. We cannot conclude that such mutations might not have presented in an exhaustive screen, however, the fact that several of the mutations described in the helical regions occurred twice and three times in the screen suggests that they represent the most common population.

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charged interactions is insufficient to prevent binding. The nature of the initial screen means that weak, co-operatively acting residues could not be identified.

Of the helical substitutions identified here we find that mutation of leucines to charged residues in the hydrophobic core of the helical interactions do indeed prevent heterodimerization, whereas substitutions to other hydrophobic residues are tolerated. These results support the view that BRCA1 and BARD1 interact via hydrophobic interactions between the helices. In the absence of evidence for a large role for electrostatic interactions it appears that the BRCA1-BARD1 interaction at the N terminus relies largely on hydrophobic interactions.

Interestingly the screen also identified several residues predicted to form intramolecular contacts. The majority of these residues are involved in the packing between helices (see Fig. 2), emphasizing the importance of the connection between the helices for binding to BRCA1. These are also chiefly hydrophobic interactions as they are substitutions from leucine or isoleucine. Furthermore, replacement with charged residues, with one exception, inhibits the interaction with BRCA1.

The relevance of the C71W mutation is not clear. The data concerning the effect of mutations of zinc-chelating residues on the structure of the RING domain and proteins bound to the N terminus is not consistent. Workers using mammalian two-hybrid (6), rabbit lysate recombinant proteins (13), or bacterial

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