PROTEIN-PROTEIN INTERACTIONS BETWEEN THE BREAST CANCER SUSCEPTIBILITY GENE PRODUCT BRCA2 AND REPPLICATION PROTEIN A

by

Daniela Ionescu

A thesis submitted in conformity with the requirements for the Degree of Master of Science Graduate Department of Molecular and Medical Genetics in the University of Toronto

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PROTEIN-PROTEIN INTERACTIONS BETWEEN THE BREAST CANCER SUSCEPTIBILITY GENE PRODUCT BRCA2 AND REPLICATION PROTEIN A

A thesis submitted by Daniela Ionescu for the Degree of Master of Science (1999)
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Mutations in the BRCA2 gene predispose women to familial, early onset breast cancer. BRCA2 has been implicated in DNA double-strand break repair through its interaction with hRad51, which is known to be involved in this DNA repair pathway. Consistent with this proposed role in repair, cells deficient in BRCA2 are highly sensitive to γ-irradiation. Another protein involved in double-strand break repair is Replication Protein A (RPA), whose role is to remove secondary structure from ssDNA, and in collaboration with hRad52 and hRad51, to initiate the strand exchange reaction of homologous recombination. RPA has previously been shown in our laboratory to interact directly with the acidic activation domains of the transactivators VP16, GAL4, and p53 and the "activation" domain of the repair proteins hXPG and Rad2. Since BRCA2 also has an acidic domain at its N-terminus which, when fused to a DNA binding domain such as LexA, is capable of activating transcription in vivo, I examined whether the acidic transcriptional activation domain of BRCA2 binds hRPA. Using purified recombinant hRPA and GST-BRCA2 fusion proteins, I showed that, by affinity chromatography or co-immunoprecipitation, hRPA interacts directly with the N-terminal acidic domain of BRCA2. Moreover, this interaction is not mediated by DNA and is specific for human and not yeast RPA. Importantly, the cancer-predisposing mutation Y42C significantly compromised the ability of GST-BRCA2 to interact with hRPA, suggesting that this interaction may be biologically important. Using co-immunoprecipitation experiments, I showed that endogenous, full length BRCA2 and hRPA proteins also form a complex in cell extracts. In summary, I have demonstrated an in vitro and in vivo interaction between hRPA and BRCA2 and that this hRPA-BRCA2 interaction is sensitive to a point mutation found in familial breast cancer.
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INTRODUCTION

I. Overview of DNA Repair:

Damage to cellular DNA can result in the loss and alteration of genetic information, cell cycle arrest or cell death. It is therefore essential for any organism to evolve mechanisms to correct lesions which might result in mutations in order to preserve the integrity of the genome. DNA damage can be introduced by many agents, including reactive oxygen species generated by normal metabolism, errors in base-pairing introduced during replication, ultraviolet light, ionizing radiation and noxious chemicals. These different forms of damage are normally repaired by three main mechanisms: direct reversal, excision, and double strand break repair.

Direct reversal:

This is the simplest pathway of correcting an error, in which a damaged DNA residue is directly reversed to the original chemical form. One of the most studied direct reversal pathways is photoreactivation. In this pathway, a specialized enzyme called photolyase carries out a light-dependent reaction to convert cyclobutane dimers to their monomeric forms (Sancar, 1994; Sancar, 1990). Although photoreactivation is a fairly ubiquitous pathway, it has not been found in humans (Ley, 1993; Li Y. et al, 1993).

Excision repair:

Repair by excision can be divided into three types: base excision repair, nucleotide excision repair, and DNA mismatch repair. These three pathways have different specificities for distinct types and sizes of lesions.

i. Base excision repair

Base excision repair removes small lesions that do not substantially distort the DNA helix (Lindahl, 1997; Krokan et al, 1997; Seeberg et al, 1995). This pathway is usually initiated by DNA glycosylases, which recognize and remove the target
base, leaving an abasic site. Phosphodiester bond cleavage follows at this abasic site and the gap generated is then filled by repair-linked DNA replication. Most DNA glycosylases, including uracil DNA glycosylases, which act on uracil in DNA and on certain forms of oxidized cytosine and uracil, are specific for a particular type or a few related forms of base damage. Cleavage at the resulting abasic site involves an AP endonuclease; in mammalian cells, the major endonuclease is HAP1 (Demple et al, 1991; Robson and Hickson, 1991). Following the cleavage, one of two pathways differing in the size of the repair patch are utilized for completing the repair process. Short-patch repair, the major base excision repair pathway, results in the incorporation of a single nucleotide residue during DNA repair synthesis. The complete pathway of this branch of base excision repair has been fully reconstituted in vitro using human cell extracts and later with purified recombinant human proteins (Dianov et al, 1992; Wiebauer and Jiricny, 1990; Singhal et al, 1995; Kubota et al, 1996). Long-patch repair, on the other hand, generates a repair patch of 2-7 nucleotides (Wang et al, 1992; Frosina et al, 1994). Both PCNA and Replication protein A (RPA) have been shown to stimulate the long-patch mode of base excision repair (DeMott et al, 1998; Biswas et al, 1997; Wu et al, 1996; Li X et al, 1995). The dependence of this pathway on PCNA is also consistent with its requirement for DNA polymerases δ and ε (Kelman, 1997; Blank et al, 1994; Matsumoto et al, 1994). This is in contrast to the short-patch mode in which a different polymerase, DNA polymerase β, is responsible for repair synthesis (Kubota, 1996; Singhal et al, 1995).

ii. Nucleotide excision repair

Nucleotide excision repair (NER) acts on bulky, helix-distorting lesions (Van Houten, 1990), such as dipyrimidine photoproducts formed from two adjacent pyrimidine residues, which are the major lesions generated by UV irradiation (Pfeifer and Tornaletti, 1997). The lesion is first recognized and subsequently removed by two endonucleolytic incisions flanking the damaged site, followed by excision of a stretch of about 30 nucleotides from the damaged strand (Moggs et al, 1996; Huang and Sancar, 1994). In humans, recognition of DNA damage is
performed by XPA and RPA (He et al, 1995; Li L et al, 1995; Burns et al, 1996), whereas XPG and the heterodimer XPF-ERCC1 provide the two endonuclease activities (Matsunaga et al, 1995; Mu et al, 1996). The incision and excision processes are assisted by other factors, including XPC and TFIIH (Schaeffer et al, 1993; Weeda et al, 1990). PCNA, RPA, DNA polymerases δ and/or ε, and ligases fill in the single-strand gap in an analogous fashion to DNA replication (Shivji et al, 1995; Jackson et al, 1994; Ayyagari et al, 1995; Coverley et al, 1992; Zeng et al, 1994). Although the NER reaction can be arbitrarily divided in vitro into discrete steps of recognition, incision and excision, and repair synthesis, these reactions might act in a highly concerted manner in vivo. In fact, Zhigang He (1997) from our laboratory has provided evidence that the human NER proteins may exist in a preassembled multiprotein complex even in the absence of damaged DNA. Others have also presented evidence for a preexisting repair complex in yeast (Svejstrup et al, 1995; Rodriguez et al, 1998). The significance of NER in humans is vividly exemplified by the fact that three hereditary diseases, xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy, are caused by mutations in NER genes (Bootsma et al, 1998; Lehmann, 1987; Nance and Barry, 1992; Cleaver and Kraemer, 1989). Patients with xeroderma pigmentosum exhibit hypersensitivity to UV light and a marked increase in incidence of skin cancer, whereas those with Cockayne's syndrome and trichothiodystrophy show severe neurological problems including mental retardation.

An interesting phenomenon in both nucleotide and base excision repair is that genes actively transcribed by RNA polymerase II are known to be repaired at a faster rate than non-transcribed genes (Leadon and Cooper, 1993; Cooper et al, 1997; Bohr et al, 1985). This process of preferential repair of active genes is termed transcription-coupled repair. Furthermore, this higher repair rate of active genes is attributed to an enhanced rate of repair of the template strand as compared to the non-transcribed strand (Mellon et al, 1987, Mellon and Hanawalt, 1989). Whereas lesions in the coding strand have no effect on the progression of the transcription complex, bulky lesions such as pyrimidine dimers in the template strand block
transcription by stalling RNA polymerase both in *E.coli* (Selby and Sancar, 1990) and eukaryotes, including humans (Donahue et al, 1994). Such a stalled RNA polymerase may enhance the repair rate of the transcription-blocking lesions, at least in *E.coli* (Selby and Sancar, 1990), by virtue of an interaction with a protein named TRCF/Mfd for "transcription repair coupling factor". Mfd has the ability to displace the stalled RNA polymerase and recruit the UvrABC excision nuclease to the damaged site (Selby and Sancar, 1991; Selby and Sancar, 1993; Selby and Sancar, 1994). However, no such biochemical activity has been established for any eukaryotic proteins. A putative TRCF in eukaryotes might be encoded by the CSA/ERCC8 and CSB/ERCC6 genes. Mutations in these two genes lead to Cockayne's syndromes A and B, respectively. Cells from patients suffering from Cockayne's syndromes are defective in transcription-coupled repair (Venema et al, 1990; Van Hoffen et al, 1993); however, it is not clear if this deficiency actually causes the disease. In fact, other studies suggest that the primary defect in Cockayne's syndromes is a deficiency in transcription (Dianov et al, 1999; Tantin et al, 1997; Balajee et al, 1997; Dianov et al, 1997). Studies in yeast by Johnson Wong in our laboratory (PhD. thesis, 1999) also suggest that a wide spectrum of mutations causing a deficiency in transcription by RNA polymerase II can result in enhanced UV sensitivity, a hallmark of Cockayne's syndrome cells.

iii. DNA mismatch repair

DNA mismatch repair acts on mismatched and unpaired bases resulting from replication errors. Therefore it is the next line of defense against incorporation of replication errors, after the immediate proofreading activity of the DNA polymerase itself (Modrich and Lahue, 1996; Kolodner, 1995). In human cells, the hMSH2-hMSH6 or hMSH2-hMSH3 heterodimers have the ability to recognize different types of the mismatched base pairs (Acharya, 1996; Drummond et al, 1995; Palombo et al, 1996). The next step in the pathway involves the binding of these complexes to a heterodimer of hMLH1 and hPMS2 (Li and Modrich, 1995). The signal directing repair to the newly replicated strand is not known for eukaryotes. This is in contrast
to prokaryotes in which MutH uses hemi-methylation of DNA as a signal to
distinguish the two strands (Au et al, 1992). Recently, a novel human protein MED1
has been identified and shown to interact with hMLH1. Intriguingly, MED1 appears
to have some features of a candidate functional homologue of the prokaryotic
MutH, since it can bind methyl-CpG and has endonuclease activity (Bellacosa, 1999).
A nick is made in one strand of the duplex at a distance of up to 1 to 2 kilobases from
the mismatch. The nicked strand is then degraded to a point beyond the mismatch
and the resulting gap is filled, and thereby the mismatched base is corrected
(Kolodner, 1996; Sancar and Rupp, 1983). Biochemical studies show that in
eukaryotic cells, DNA polymerase δ is most likely the enzyme that performs repair
replication (Longley et al, 1997). This is also consistent with the known requirement
for PCNA (Gu et al, 1998; Johnson et al, 1996; Umar et al, 1996) and RPA (Lin et al,
1998) in mismatch repair, since these two proteins are known to stimulate the
processivity of DNA polymerase δ (Kelman, 1997, Podust and Hubscher, 1993;
Tsurimoto and Stillman, 1989).

II. Double Strand Break Repair:

Double-stranded breaks (DSBs) in DNA can be the result of ionizing radiation,
oxidative damage, and the actions of endonucleases. DSBs can also arise from
normal cellular processes such as replication, or as intermediates in V(D)J
recombination during lymphocyte development (Jeggo et al, 1995). Failure to repair
a broken chromosome can lead to the loss of genetic information, and inappropriate
repair of DSBs can result in defects such as chromosomal translocations. Eukaryotic
cells possess two distinct mechanisms for repairing double-stranded breaks in DNA.
(reviewed by Kanaar and Hoeijmakers, 1997). One pathway, end-joining, is
nonhomologous, while the other involves genetic recombination with an intact
homologous sister duplex.
i. Non-homologous end-joining

Non-homologous end-joining has also been called illegitimate end-joining because this repair pathway does not require homologous DNA sequences. The repair reaction can either be a simple ligation which is accurate, or it may involve processing of the DNA ends followed by a ligation event. Rodent cell lines, such as that derived from *scid* mice which are defective in DSBR, have been instrumental in the identification of genes involved in the non-homologous end-joining pathway (Thompson and Jeggo, 1995; Jeggo et al, 1995). Four genes corresponding to the complementation groups *XRCC4*-7, have been identified in this way, (Taccioli et al, 1994; Smider et al, 1994; Kirchgessner et al, 1995; Blunt et al, 1995).

DNA-dependent protein kinase (DNA-PK) is required for normal double-strand break rejoining activity in mammalian cells. DNA-PK consists of a catalytic subunit (encoded by *XRCC7*) and an associated DNA-binding component, the Ku complex of Ku70 and Ku80 (encoded by *XRCC6* and *XRCC5*, respectively) (Lees-Miller, 1996). The Ku heterodimer binds to dsDNA ends without sequence specificity, although it recognizes gapped and hairpin DNA structures as well (Falzon et al, 1993). In addition, the Ku protein complex has been shown to stimulate DNA end-joining (Ramsden and Gellert, 1998). The catalytic subunit of DNA-PK, which functions as a serine-threonine protein kinase, is activated upon binding to DNA breaks (Yaneva et al, 1997; Hammarsten and Chu, 1998). Although DNA-PK is able to phosphorylate many protein substrates *in vitro*, its physiological targets have not been established (Hartley et al, 1995; Anderson, 1993). One likely candidate is RPA, and a recent study suggests that DNA replication is critical for activating DNA-PK, which can then lead to RPA phosphorylation (Shao et al, 1999). The same study also demonstrated the existence of a multiprotein complex containing DNA-PK and RPA.

*XRCC4* encodes a protein which complexes with and enhances the activity of DNA ligase IV (Grawunder et al, 1998; Grawunder et al, 1997; Critchlow et al, 1997), suggesting that this enzyme is responsible for catalyzing the ligation step. The DNA-PK complex and *XRCC4* are not only involved in end-joining during DNA repair, but also in V(D)J recombination (Wilson et al, 1997; Shar et al, 1997) during
which the antigen receptor sites of antibodies are rearranged (Taccioli et al, 1993). Consequently, in addition to being deficient in double strand break repair, mutant cells with defects in components of DNA-PK are also unable to perform correct V(D)J rejoining of maturing immunoglobulin genes (Jeggo et al, 1997). Although end-joining has mainly been studied in mammalian cells, in vitro systems of end-joining dependent on DNA-PK have not been developed until recently (Baumann and West, 1998; Ramsden et al, 1997). These recent breakthroughs should allow further biochemical characterization of other protein factors important in this pathway of repair. A separate line of research, involving the identification of homologues of Ku70, Ku80, and XRCC4 in S.cerevisiae, not only pointed to the evolutionary conservation of this pathway in eukaryotes, but also permitted powerful genetic analysis in this well-studied organism (Feldmann and Winnacker, 1993; Feldmann et al, 1997; Milne et al, 1996). Interestingly, no S.cerevisiae homologue of the catalytic subunit of DNA-PK has been identified, although yeast has two proteins similar to it, namely Tel11 and Mec1, which may perform related functions.

ii. Homologous recombination

In eukaryotes, DSBR by homologous recombination occurs by two pathways, single-strand annealing and strand invasion. I will only briefly describe single strand annealing and place more emphasis on the latter pathway involving strand invasion as this is the major focus of my research.

In single strand annealing, a DSB in or near one of two directly repeated sequences leads to a recombination intermediate in which complementary strands from the two repeated sequences are annealed. Homologous complementary DNA sequences flanking the break are aligned and the intervening sequences are deleted. As a result, the end product contains only one copy of the repeated sequences, with a deletion of sequences originally present between the two repeats (Haber, 1995). In S.cerevisiae this mechanism involves the following genes: RAD1, RAD10, RAD52,
does not provide repair with high-fidelity since DNA is deleted in the process and, consequently, information is lost (Lin F.L. et al, 1990). As a result, this pathway of single strand annealing may only be important for the repair of DSBs within directly repeated non-protein-coding DNA sequences, where the loss of DNA that lies between the break and the site of alignment is unlikely to have major consequences.

DSBR involving strand invasion uses the second intact copy of a chromosome as a template to repair the break (Stahl, 1996; Haber, 1995). Hence, this mechanism ensures that information lost at the break site is restored. In *S. cerevisiae*, genes involved in this pathway of homologous recombination include those that belong to the RAD52 epistasis group (namely *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, XRS2*, and *MRE11* (Game, 1993; Shinohara and Ogawa, 1995, reviewed by Petrini et al, 1997). Mutations in these genes result in sensitivity to ionizing radiation and other DSB-causing agents, and are also associated with defects in meiosis (Petes and Pukkila, 1995). Within the RAD52 epistasis group, the *rad51, rad52 and rad54* mutants exhibit more severe repair and recombination defects, suggesting that their gene products may be the central components of the pathway of DSBR (Muris et al, 1993; Saeki et al, 1980). Genetic studies revealed that *RAD50, XRS2 and MRE11* are required for the initial processing of DSBs: this involves the generation of 3' overhanging ssDNA tails approximately 600 bases long (Cao et al, 1990; Ivanov et al, 1992). In yeast, the products of the *RAD51, RAD52, RAD54, RAD55, and RAD57* genes are then involved in the search for the homologous intact duplex, DNA pairing and strand exchange. The broken ends of chromosomal DNA are degraded to create a gap, which is repaired using an unbroken homologous template. Mechanistically, the broken ends initiate recombination by invading into the homologous dsDNA. As a result, heteroduplex DNA, consisting of paired strands from both the unbroken and broken recombination substrates, is present at the boundaries of the gap. Two Holliday junctions are formed at the boundaries of the heteroduplex and are subsequently resolved (see Figure 1).
In both human and yeast cells, a multiprotein complex appears to participate in the recognition of DSBs. In yeast, Mre11p interacts with Rad50p and possesses an exonuclease activity that may be involved in the processing of DNA ends before the strand exchange step (Kanaar and Hoeijmakers, 1997; Petrini et al, 1997). A third yeast protein, Xrs2p, has also been found in the Mre11p-Rad50p complex (Ajimura et al, 1993; Chamankhah and Xiao, 1999). In humans, hMre11, hRad50 and a protein called NBS1 also form a similar complex (Carney et al, 1998). The NBS1 protein is encoded by a gene mutated in the chromosome instability disorder Nijmegen breakage syndrome (Varon et al, 1998, Matsuura et al, 1998) and appears to be a functional homologue of Xrs2p. Using in situ visualization, Nelms et al (1998) were
able to show that hMre11 and hRad50 migrated to sites of DSBs, consistent with their recognition roles in DSBR, whereas initially hRad51 did not appear in these foci, presumably because it is involved in later reactions of DSBR.

Several of the RAD50-57 gene products which participate in the second stage of homologous recombination of DSBR show features of particular interest. For instance, Rad51 has significant homology with the *E.coli* RecA protein and is similar to RecA both biochemically and structurally (Ogawa et al, 1993; Benson et al, 1994; Gupta et al, 1997). Early experiments showed that mutations in *recA* caused a severe defect in homologous recombination and sensitivity to DNA-damaging agents (Weisemann and Weinstock, 1988). Presently we know that RecA also plays a regulatory role in the induction of the SOS response to DNA damage by catalysing the autoproteolysis of the LexA repressor (Kim and Little, 1993; Kogoma et al, 1993; Ennis et al, 1995; Howard et al, 1993). In turn, LexA cleavage leads to the induction of more than 20 genes, most of which are involved in DNA repair (Lewis et al, 1994; Roland et al, 1992; Thliveris and Mount, 1992). In addition to its function as a regulatory factor, RecA catalyses *in vitro* reactions that are indicative of a direct role in the recombination process. For instance, in the presence of ATP, purified RecA polymerizes on DNA and forms helical filaments (Stasiak and Egelman, 1986; Ogawa et al, 1993). In recombination, such extended nucleoprotein filaments on single-stranded DNA interact with naked duplex DNA to allow subsequent homologous strand exchange (Kowalczykowsky et al, 1994; Camerini-Otero and Hsieh, 1995).

Eukaryotic homologues of RecA share a high degree of sequence conservation within a "homologous core" domain of approximately 200 amino acids (Shinohara, 1993). However, the homologous eukaryotic Rad51 proteins, which include a yRad51p and additional RAD51-like proteins in human cells, such as DCM1 which is expressed specifically in meiosis, also have an N-terminal extension that is found in RAD51 but not in RecA, and are missing the C-terminal domain that is present in RecA (Ogawa et al, 1993; Shinohara et al, 1993). Yeast *rad51* mutants fail to repair DSBs, exhibit chromosomal loss, and harbor defects in recombination (Ogawa et al, 1993). Furthermore, vertebrate cells deficient in RAD51 also have broken
chromosomes, consistent with a putative role for RAD51 in DSBR (Sonoda et al, 1998). Biochemical studies of both the yeast Rad51p and human RAD51 revealed that, like E.coli RecA, they also form nucleoprotein filaments on DNA (Sung, 1994; Gupta et al, 1997; Benson et al, 1994; Baumann et al, 1996; Ogawa et al, 1993), promote homologous pairing, and initiate strand exchange reactions (Sung, 1994; Bishop, 1994; Shinohara, 1992). However, there are also significant differences between the proteins. For example, RecA binds preferentially to ssDNA (McEntee et al, 1981; Howard-Flanders, 1984). In contrast, the yeast and human proteins exhibit similar affinities for both ssDNA and dsDNA (Benson et al, 1994; Sung, 1994; Baumann et al, 1996). Furthermore, the strand exchange reaction mediated by RecA requires ATP (Kowalczykowski and Eggleston, 1994; Bedale and Cox, 1996), whereas this does not seem to be the case for RAD51, since human RAD51 alone hydrolyses ATP at a rate that is 200 times lower than that of RecA (Sung and Robberson, 1995; Milne et al, 1995). It seems that, even though strand exchange proteins are structurally and functionally conserved, the details of their actions differ. Nonetheless, RAD51 activity is analogous to that of RecA. RAD51 coats the ssDNA to form a filament that scans the genome for a homologous dsDNA sequence; the ssDNA-containing filament and the intact dsDNA then form a joint molecule before strand exchange occurs (Kanaar and Hoeijmakers, 1998) (see also Figure 1).

The ssDNA-binding protein SSB of E.coli has two roles in RecA-mediated strand exchange (Heyer and Kolodner, 1989; Kowalczykowski et al, 1994). First, SSB disrupts any secondary structure within ssDNA and thus facilitates formation of a continuous RecA-ssDNA nucleoprotein filament (Egner et al, 1987; Lohman and Ferrari, 1994). Second, SSB facilitates strand exchange by binding to displaced ssDNA produced by DNA heteroduplex formation (Chow et al, 1988; Lavery and Kowalczykowski, 1992). In both steps, physical interactions between RecA and SSB are not required for this stimulation (Muniyappa et al, 1984; Kowalczykowski et al, 1987). The human ssDNA-binding protein hRPA appears to play a role similar to that of SSB in RAD51-mediated strand exchange reactions (Sung, 1997). Formation of heteroduplex DNA by RAD51 alone is inefficient under conditions where the ssDNA contains secondary structure, but is significantly stimulated in the presence
of hRPA (Baumann and West, 1997). However, hRPA has little stimulatory effect when little secondary structure exists in the presence of low concentrations of divalent cations (Baumann and West, 1997). Recently, a direct interaction has been identified between RAD51 and hRPA (Golub et al, 1998), which is unlike the case in *E.coli*, where SSB does not need to directly interact with RecA to stimulate strand exchange.

In addition to RAD51 and RPA, RAD52 also binds ssDNA (Benson et al, 1998; Mortensen et al, 1996). In yeast, mutations in the RAD52 gene cause severe defects in recombination and sensitivity to ionizing radiation. Interactions between yeast Rad51p and Rad52p, as well as between the human RAD51 and RAD52 proteins, have been demonstrated (Muris et al, 1994; Milne and Weaver, 1993; Shinohara et al, 1992; Shen et al, 1996). Biochemical studies show that yeast Rad52p and human RAD52 facilitate the annealing of complementary ssDNA (Mortensen et al, 1996; Reddy et al, 1997), an observation that supports a role for RAD52 in the single-strand annealing pathway of homologous recombination of DSBR. However, a series of recent reports showed that RAD52 also stimulates the RAD51-mediated strand-transfer reactions of homologous recombination (Sung, 1997; Benson et al, 1998; Shinohara and Ogawa, 1998; New et al, 1998). The fact that this stimulation was only seen when Rad52p was bound to ssDNA prior to the addition of Rad51p suggested that one function of Rad52p may be to target Rad51p to ssDNA (New et al, 1998; Shinohara and Ogawa, 1998; Benson et al, 1998). yRPA was shown to stimulate joint-molecule formation when added to preformed Rad51p-ssDNA filaments; however, the addition of yRPA before Rad51p inhibited joint-molecule formation. This observation can be explained by the fact that, although yRPA may remove secondary structure from the ssDNA to allow for efficient filament formation by Rad51p, yRPA also competes with Rad51p for ssDNA binding. Interestingly, inhibition is overcome when Rad52p is incubated together with Rad51p and yRPA (Sung, 1997; Shinohara and Ogawa, 1998; New et al, 1998). This suggests a role for Rad52p as an adaptor between Rad51p and RPA, a role for which it is well-suited since it binds both proteins (Sung, 1997; Wold, 1997; New et al, 1998; Shinohara and Ogawa, 1998) (see Figure 2).
Cloning of the RAD54 gene showed that it belongs to the SWI/SNF2 family of DNA-dependent ATPases, which includes known transcriptional activators and helicases that can alter chromatin structure (Eisen et al, 1995). Cells lacking RAD54 have reduced levels of homologous recombination and are highly sensitive to ionizing radiation and other DSB-inducing agents (Essers et al, 1997). Based on the functions of the other members of the SWI/SNF2 family, RAD54 has been suggested to play a role in chromatin remodelling during homologous recombination. However, yeast Rad54p was shown to stimulate Rad51p-mediated homologous pairing reactions but, at the same time, prevent extensive branch migration (Petukhova et al, 1998). Coupled with the observation that both yeast and human RAD54 proteins can interact directly with RAD51 (Clever et al, 1997; Jiang et al, 1996), RAD54 may have a more direct role in DSBR.

In addition to Rad51p, significant homology with RecA has also been found in Rad55p and Rad57p (Lovett, 1994; Kans and Mortimer, 1992). These two yeast proteins form a heterodimer that functions with RPA to stimulate Rad51p in strand exchange reactions (Sung, 1997). In this regard, it should be noted that Rad55p can also physically interact with Rad51p (Johnson and Symington, 1995; Hays et al, 1995). The Rad55p-Rad57p heterodimer overcomes the inhibitory effect of RPA, reminiscent of the effect of Rad52p (Sung, 1997). Genetic and biochemical studies revealed interactions between Rad51p and Rad55p, and between Rad55p and Rad57p (Johnson and Symington, 1995; Hays et al, 1995). However, overexpression of
RAD51 and RAD52 suppressed defects seen in rad55 and rad57 null mutants, indicating that Rad55p and Rad57p are dispensable when Rad51p or Rad52p is abundant. Interestingly, a complex in yeast containing Rad51p, Rad52p, Rad55p, Rad57p and RPA has been identified and dubbed the "recombinosome" (Hays et al, 1995) (Figure 2). Although RAD51 (Shinohara et al, 1993), RAD52 (Bendixen et al, 1994; Muris et al, 1994) and RAD54 (Zdzienicka, 1995) have been identified in mammalian cells, no human homologues of RAD55 and RAD57 have been reported.

In S. cerevisiae, DSBs are not generally repaired by non-homologous end joining, but rather by homologous recombination strand exchange reactions that involve the products of the RAD50-57 group of genes (Haber, 1995). As a result, non-homologous end-joining can only be detected in a rad52 null mutant where homologous recombination is abrogated (Moore and Haber, 1996; Mezard et al, 1994; Kramer et al, 1994). In fact, yeast mutants defective for Ku70 or Ku80 show wild-type levels of radiation sensitivity, again suggesting that non-homologous end joining plays only a minor role in repairing DSBs in yeast (Haber, 1995). This is not surprising considering that homologous recombination through strand exchange reactions preserves the integrity of the genetic information and any changes occurring in the more compact S. cerevisiae genome could potentially be deleterious. In contrast, the mammalian genome contains many introns and repetitive non-coding sequences, and small changes resulting from illegitimate end-joining are less likely to cause deleterious alterations. Initially, mammalian cells were thought to use non-homologous end-joining rather than homologous recombination as the predominant mechanism for DSBR (Jackson and Jeggo, 1995). However, recent analyses suggest that both pathways are used to similar extents (Liang et al, 1998; Sargent et al, 1997; Liang et al, 1996; Jasin, 1996). Considering the high degree of conservation of the two pathways of DSBR, one major difference between yeast and mammalian cells appears to be the relative use of the two pathways. Another difference is that, although RAD51 is essential for the survival of ES cells and for early embryonic development (Lim and Hasty, 1996; Tsuzuki et al, 1996), all of the rad52 epistasis group mutants from yeast are viable (Muris et al, 1996; Muris et al,
Given that vertebrate genomes are considerably larger than the yeast genome, DSBs should occur much more frequently in mammalian cells than in yeast. Hence, the difference in genome size may explain why the absence of Rad51 causes cell death in mammalian cells (Lim and Hasty, 1996; Tsuzuki et al, 1996) but not in yeast cells (Muris et al, 1996; Muris et al, 1993; Shinohara et al, 1992).

It is important to note that genetic recombination can be either beneficial or detrimental, depending on whether exchanges occur between homologous chromosomes during meiosis or between identical sister chromatids. Recombination between homologous pairs of chromosomes may lead to the deleterious effect of LOH (loss of heterozygosity) (Moynahan and Jasin, 1997). In turn, LOH contributes to tumorigenesis if defective tumor suppressor loci become homozygous (Lasko et al, 1991). Studies suggest that sister chromatids may be the preferred homology partner for DSBR in mammalian cells (Liang et al, 1996) because they are identical and have no diverged repetitive elements which may be a barrier to recombination between homologs (Elliott et al, 1998; Chambers et al, 1996). This suggests that while homologous recombination may take place during S phase when sister chromatids are available, non-homologous end joining may be the predominant repair pathway at other times in the cell cycle.

III. Breast Cancer Susceptibility Genes BRCA1 and BRCA2

A lot of interest in the pathway of DSBR was generated by reports that the breast cancer susceptibility gene products of BRCA1 and BRCA2 interact with human RAD51 (Scully et al, 1997a; Sharan et al, 1997). Both BRCA1 and BRCA2 were initially identified using linkage analysis in families with multiple breast cancer cases (Easton et al, 1993; Narod et al, 1995; Wooster et al, 1994). Both genes were suggested to be tumour suppressors due to the fact that the wild type allele undergoes LOH in tumour cells of mutation carriers (Smith et al, 1992; Neuhausen and Marshall, 1994). Between 5% and 10% of all breast cancer cases and about 10% of all ovarian cancers are familial and can be attributed to mutations in these highly penetrant, autosomal recessive susceptibility genes (Lopez-Otin et al, 1998; Claus et
Within the familial form of breast cancers, BRCA1 was found to be mutated in almost all families with both breast and ovarian cancer, and in approximately half of the families with only breast cancer (Easton et al, 1993). BRCA2 mutations are responsible for virtually all the rest of the familial cases of breast cancer only, and has also been associated with male breast cancers and pancreatic cancers (Arason et al, 1993; Sigurdsson et al, 1997; Thorlacius et al, 1995).

i. BRCA1

The BRCA1 gene encodes a nuclear phosphoprotein of 1863 amino acid residues (Chen Y. et al, 1996; Miki et al, 1994) (figure 3). The N-terminus contains a zinc finger domain, a motif found in many transcriptional regulators such as TFIIIA and nuclear hormone receptors, suggesting that BRCA1 may also be a DNA-binding protein (Saurin et al, 1996; Borden and Freemont, 1996). Located in the C-terminus of BRCA1 are two tandem regions termed BRCT, an acronym for "BRCA1 carboxyl terminus" (Koonin et al, 1996). Interestingly, this BRCT domain is found in over 40 proteins involved in cell cycle regulation, including the mammalian p53-binding protein 53BP1 and the yeast Rad9 protein, as well as in proteins involved in DNA repair, including NBS1 and ligase IV (Callebaut and Moron, 1997). Although the functions of the BRCT domains remain unclear, it has been suggested they may act as sites for protein-protein interactions between DNA repair proteins (Callebaut and Moron, 1997). Support for this hypothesis has come from studies demonstrating a direct interaction between DNA ligase III and the BRCT domain of XRCC1, two proteins involved in base excision repair (Taylor et al, 1998). Further evidence came from the recent crystal structure of the XRCC1 BRCT domain which was found to homodimerize, suggesting that the domain may function as a module for protein-protein interaction (Zhang X. et al, 1998).
Figure 3: Domain Structure of BRCA1

BRCA1 has been suggested to play a role in transcriptional regulation, since it possesses an acidic domain which can activate transcription both in vitro (Haile and Parvin, 1999) and in vivo (Chapman and Verma, 1996; Monteiro et al, 1996) when fused to the GAL4 DNA-binding domain. Significantly, germ-line mutations of BRCA1 in breast cancer patients impaired this ability to activate transcription, suggesting that the loss of transcription activation function by BRCA1 may predispose the carriers to cancer. The mechanism of transcriptional activation by the BRCA1 acidic domain may be related to its ability to remodel chromatin, possibly through interacting with histone deacetylase (Yarden and Brody, 1999; Hu et al, 1999) although the activity of histone deacetylation has been associated with transcriptional repression rather than activation. BRCA1 may also have an ability to contact components of the basal transcription machinery (Haïle and Parvin, 1999). Consistent with a role for BRCA1 in gene transcription, BRCA1 was found to associate with the RNA polymerase II holoenzyme, and a mutation in the C-terminal acidic domain of BRCA1 reduced the effectiveness of this association (Maldonado et al, 1996; Scully et al, 1997c). The role of BRCA1 as a transactivator has been substantiated further by several reports demonstrating a direct interaction and synergistic activation with the transactivator p53 (Chai et al, 1999; Somasundaram et al, 1997; Zhang H. et al, 1998). The second BRCT domain of BRCA1 was found to interact with p53 and stimulate transcription of the CDK-inhibitor p21 (Chai et al, 1999; Somasundaram et al, 1997). Moreover, the BRCT repeats of BRCA1 were found to bind to the co-repressor CtIP (Yu et al, 1998) and this interaction was abrogated in cells treated with DNA-damaging agents, a response that correlates
with BRCA1 phosphorylation and p21 induction (Li et al, 1999). Interestingly, BRCA1 was also found to bind the proto-oncogene c-Myc and repress its transcriptional and transforming activity (Wang et al, 1998).

In addition to a role in transcription, BRCA1 has also been implicated in double-strand break repair through its association with RAD51 (Scully et al, 1997a; Baumann et al, 1996). However, it is not clear that the interaction between RAD51 and BRCA1 is direct since the purified proteins fail to interact (Golub et al, 1997). Immunofluorescence staining studies showed that RAD51 and BRCA1 co-localize in nuclear dot-like structures, termed nuclear foci, during the S phase of the cell cycle (Scully et al, 1997a). In addition, upon DNA damage, BRCA1 becomes hyperphosphorylated and undergoes relocalization, along with RAD51, within the cell (Scully et al, 1997b). All these observations are consistent with a role for BRCA1 in DNA repair. More evidence suggesting a DNA repair role for BRCA1 came from knock-out mouse studies. Mouse embryonic stem cells deficient in BRCA1 were found to be hypersensitive to ionizing radiation (Leadon et al, 1998), which causes double strand breaks. Interestingly, these BRCA1-/- cells are also deficient in transcription-coupled repair of oxidative damage, induced by hydrogen peroxide (Leadon et al, 1998). The association of BRCA1 with the RNA polymerase II holoenzyme (Scully et al, 1997c; Maldonado et al, 1996) may provide an explanation for a role of BRCA1 in transcription-coupled repair.

At the cellular level BRCA1 seems to be involved in some aspects of cell cycle regulation and progression. BRCA1 undergoes cell-cycle dependent changes in its expression (Rajan et al, 1997; Gudas et al, 1995; Gudas et al, 1996; Vaughn et al, 1996) and phosphorylation state (Chen Y. et al, 1996; Ruffner and Verma, 1997). Furthermore, the developmental pattern of mouse Brca1 expression (Lane et al, 1995; Marquis et al, 1995) also suggests a possible function for BRCA1 in cellular proliferation. Moreover, BRCA1-/- embryos revealed an early proliferation block after approximately 7.5 days (Gowen et al, 1996; Hakem et al, 1996; Liu et al, 1996). A similar phenotype was observed for mice bearing homozygous, loss of function mutations in Rad51, which also died early in embryogenesis. Like BRCA1-/- embryos, cells of Rad51-/- embryos also exhibited a proliferation defect (Lim and
Hasty, 1996; Tsuzuki et al, 1996) and the embryonic lethality of both Rad51-/- and BRCA1-/- mutations is partially suppressed in p53-/- embryos (Ludwig et al, 1997). This proposed cellular function of BRCA1 in proliferation is not consistent with findings that BRCA1 causes growth retardation when stably transfected into breast and ovarian cell lines (Holt et al, 1996), and introduction of antisense nucleotides seems to enhance cell proliferation (Rao et al, 1996; Thompson et al, 1995). The defect in BRCA1-/- embryonic cell proliferation may be due to the accumulation of unrepaired double-strand breaks.

ii. BRCA2

The BRCA2 gene encodes a nuclear protein of 3418 amino acids that bears no strong similarity to known proteins (Wooster et al, 1995; Tavtigian et al, 1996). A significant feature of this protein is the presence of eight copies of a 30-80 amino acid BRC repeat (Bork et al, 1996) (see figure 4). Comparison of BRCA2 from several mammalian species revealed that many of these repeats appear to be conserved (Bignell et al, 1997), but the only known function of the BRC repeats is that they are one domain for interaction of BRCA2 with RAD51 (Chen P.L. et al, 1998). Evidence suggests that, although BRCA1 and BRCA2 are not significantly related in sequence, they may function in an analogous manner.

![Figure 4: Domain Structure of BRCA2](image)

Like BRCA1, BRCA2 also has an acidic domain which can activate transcription in an *in vivo* assay (Milner et al, 1997). However, unlike the situation
for BRCA1, the involvement of BRCA2 in transcriptional regulation is not substantiated by additional evidence. As discussed below, most of the evidence to date points to a DNA repair role for BRCA2.

The first indication that BRCA2 may be involved in double-strand break repair came from Sharan et al (1997) who reported that BRCA2, like BRCA1, can also interact with RAD51 (Marmorstein et al, 1998; Wong, 1997). This interaction appears to be mediated by two distinct domains of BRCA2: one in the BRC repeats and another in the C-terminal region of the protein (Chen et al, 1998; Sharan et al, 1997; Wong et al, 1997). Additional evidence for a role of BRCA2 in DSB repair came from studies showing that disruption of murine Brca2 resulted in embryos that were hypersensitive to ionizing radiation (Sharan and Bradley, 1997; Connor et al, 1997a; Patel et al, 1998), a phenotype shared by Rad51 null embryos (Lim and Hasty, 1996). Significantly, cells derived from mice bearing a truncated version of Brca2 were shown to have an impaired ability to repair DSBs following γ-irradiation, as measured by a Comet assay that directly identifies DSBs (Connor et al, 1997a). Furthermore, Patel et al (1998) found that cells derived from mice bearing a different truncated version of Brca2 had numerous spontaneous chromosomal abnormalities, a phenotype also shared by Rad51-null embryos and cells (Lim and Hasty, 1996; Sonoda et al, 1998). A further link to Rad51 was demonstrated by experiments with developing mouse embryos showing that the patterns of Brca1, Brca2 and Rad51 gene expression are almost identical (Lane et al, 1995; Marquis et al; Rajan et al, 1997; Sharan et al, 1997). In all, these studies suggest that BRCA2 and RAD51 are involved in the same pathway of double-strand break repair.

At the cellular level, BRCA2, like BRCA1, also seems to be involved in some aspects of cell cycle regulation and progression. The expression of BRCA2 is also dependent on the cell cycle, with elevated levels at the G1/S transition and G2 and low levels in G1 (Rajan et al, 1996; Rajan et al, 1997; Connor et al, 1997b). This observation led to the hypothesis that BRCA2 may be involved in cell cycle checkpoint activation, in addition to a possible direct role in DSBR. However, cells derived from mice with a truncated Brca2 appear to have an intact checkpoint
activation (Patel et al, 1998). This observation argues that BRCA2 has a more direct role in repair, although the possibility remains that the C-terminal portion of Brca2, which was deleted in these studies, does not include the domains involved in cell cycle arrest. Nonetheless, BRCA2 is required for cellular proliferation since cells derived from mice lacking functional Brca2 show a proliferative defect (Connor et al, 1997a; Patel et al, 1998). In addition, Brca2, like Brca1 and Rad51, was shown to play an essential role in early embryonic development by studies showing that homozygous Brca2 mutant mice cannot survive embryogenesis (Sharan et al, 1997; Ludwig et al, 1997; Suzuki et al, 1997).

In summary, these data suggest that loss of functional BRCA1 or BRCA2 is a mutagenic event predisposing cells to neoplastic transformation. Whether or not BRCA1 and BRCA2 play direct roles in homologous recombination and double-strand break repair remains to be determined. The development of an in vitro DSBR system capable of recapitulating the unique features of this repair pathway, including a dependence on RAD52 epistasis group genes, should help elucidate the precise functions performed by the BRCA proteins. In fact, a recent report demonstrating the coexistence of BRCA1, BRCA2 and RAD51 in the same complex (Chen et al, 1998), lends further credence to the idea that BRCA1, like BRCA2, may function in DSBR and therefore makes it more imperative that in vitro assays of these functions be developed.

III. Replication Protein A

As mentioned previously, the ssDNA-binding protein Replication protein A (RPA) plays an important role in homologous recombination during double-strand break repair, by stimulating RAD51 in the strand exchange reaction (Heyer et al, 1990; Smith and Rothstein, 1995). In addition, RPA is required in many other processes in which there is involvement of ssDNA as a reaction intermediate, namely DNA replication (Brush et al, 1996; Newlon, 1997; Ishimi et al, 1994) and several pathways of DNA repair (Coverley et al, 1991; Guzder et al, 1995; He et al, 1995; Lin Y.L. et al, 1998; Biswas et al, 1997). The following will briefly review the subunit composition, structure, and proposed functions of RPA.
i. RPA subunit composition

Human hRPA was originally identified as an essential factor for SV40 DNA replication in vitro and, subsequently, homologues of RPA have been identified in many eukaryotes, including S.cerevisiae, S.pombe and X.laevis (Umbricht et al, 1993; Brill and Stillman, 1991; Erdile et al, 1991; Adachi and Laemmli, 1992; Ishiai et al, 1996). All eukaryotic RPAs are composed of stable complexes of three polypeptides. In humans, the subunits of RPA are 70, 32 and 14kDa in size, and are designated RPA1, RPA2, and RPA3, respectively (Umbricht et al, 1993; Ozawa et al, 1993). There is a high degree of homology between different homologues of eukaryotic RPA. In particular, the genes encoding the largest and the middle subunits of RPA show the highest degree of conservation, whereas the genes for the small subunit show weaker homology (Ishiai et al, 1996; Philipova et al, 1996). Interestingly, weak homology was even reported between RPA1 and E.coli SSB (Philipova et al, 1996), which are now considered functional homologues.

In S.cerevisiae, the genes for all three subunits of yRPA are essential (Heyer et al, 1990; Brill and Stillman, 1991; Brill and Stillman, 1989). In humans, the genes for each of the subunits have been mapped to 17p13.3, 1p35, and 7p22, for RPA1, RPA2, and RPA3, respectively (Umbricht et al, 1993; Ozawa et al, 1993; Umbricht et al, 1994). Intriguingly, the 17p13.3 locus where RPA1 maps is thought to encode a tumour suppressor gene since this locus undergoes loss of heterozygosity in tumour cells (Stack et al, 1995). High frequency loss of heterozygosity at this locus has been reported not only in sporadic breast cancer (Mackay et al, 1988; Coles et al, 1990), but also in other malignancies, including ovarian cancer and osteosarcoma (Phillips et al, 1993; Thorlacius et al, 1991). However, it remains to be established if RPA1 is the gene undergoing LOH in these different tumours.

It is worth-noting that a homologue of RPA2, namely RPA4, has been identified in human cells (Keshav et al, 1995). It shares 47% amino acid identity with RPA2 and is expressed primarily in placenta and colon mucosa tissues (Keshav et al, 1995). Although RPA1, RPA2 and RPA3 are expressed ubiquitously, the tissue-specific expression of RPA4 suggests that this may be a mode of modulating RPA
function. Importantly, RPA4 has been shown to be capable of forming a trimeric complex with RPA1 and RPA3, raising the possibility that hRPA may exist as two different complexes, with either RPA2 or RPA4 as the middle subunit (Keshav et al, 1995). It will be of interest to determine if these two forms of hRPA possess distinct biochemical functions in various RPA-dependent processes.

The fact that recombinant hRPA could be expressed in soluble form in E.coli only if RPA1 is co-expressed with both RPA2 and RPA3 suggests that the formation of a complex contains RPA2 and RPA3 is necessary for proper assembly and/or folding of RPA1. As expected, direct protein-protein interactions have been found between the C-terminus of RPA1 and RPA2 (Gomes and Wold, 1995; Kim et al, 1996), and between RPA2 and RPA3 (Lin et al, 1996).

ii. Structure of RPA

Although RPA was found to bind ssDNA with high affinity (Wobbe et al, 1987; Faiman and Stillman, 1988; Wold and Kelly, 1988), it also binds dsDNA, but with four orders of magnitude lower affinity (Wold et al, 1989; Brill and Stillman, 1989). Together, the three subunits of yRPA appear to contain a total of four or five potential ssDNA-binding domains (Brill and Bastin-Shanower, 1998; Bochkareva et al, 1998). There seems to be some controversy, however, as to the total number and locations of ssDNA-binding domains within the trimeric RPA complex. The large subunit was shown to have three ssDNA-binding domains (Brill and Bastin-Shanower, 1998), of which the tandemly repeated central region of RPA1 is necessary for high affinity binding (Kenny et al, 1990; Lin et al, 1996; Wold et al, 1989; Erdile et al, 1991). Recently, the Zn finger domain located C-terminal to the central region was shown to be required for optimal ss-DNA binding (Walther et al, 1999). In fact, this domain can interact directly with DNA and it also contributes to dsDNA binding by inducing a localized unwinding of the duplex (Lao et al, 1999). The second subunit also has one binding site (Bochkareva et al, 1998; Lin et al, 1996). It has been suggested that there is also a DNA-binding site on RPA3 (Bochkarev et al, 1997). Hence, in theory there is a total of five potential DNA-binding sites. RPA from several species has been shown to interact with approximately 30 nucleotides
of ssDNA per trimer (Atrazhev et al, 1992; Kim et al, 1992; Mitsis et al, 1993; Kim et al, 1994). Although the most stable binding of hRPA seems to require a DNA molecule of at least 30 nucleotides, it is also capable of interacting with 10 nucleotides (Bochkarev et al, 1997; Blackwell and Borowiec, 1994).

The determination of the crystal structure of the central DNA-binding domain of RPA1 complexed with an oligonucleotide has provided significant insight into the interactions between RPA and ssDNA (Bochkarev et al, 1997). This co-crystal structure shows that the DNA-binding domain is composed of two copies of a single DNA-binding subdomain, both of which interact directly with ssDNA. Interestingly, these domains are composed primarily of beta sheets and show a strong structural similarity to other ssDNA-binding proteins. Two types of interactions are responsible for RPA-DNA interactions: aromatic residues stacking with individual bases, and hydrogen bonding between side chains of amino acids and both the phosphate backbone and individual bases. In addition, these interactions are mediated by several flexible protein loops. Potential changes in the orientation of these flexible loops may account for a previously reported conformational change induced by interaction between RPA and DNA (Blackwell et al, 1996; Gomes et al, 1996).

This conformational change, involving both RPA1 and RPA2, results in RPA becoming a better substrate for phosphorylation (Blackwell et al, 1996; Gomes et al, 1996). In cells, RPA2 has been shown to be phosphorylated either in a cell cycle dependent manner (Fang and Newport, 1993; Din et al, 1990; Dutta and Stillman, 1992; Fotedar and Roberts, 1992) or in response to DNA damage induced by ionizing radiation or UV irradiation (Liu et al, 1993; Carty et al, 1994). Several serine residues in the N-terminal of RPA2 are phosphoreceptor sites in vitro (Fotedar and Roberts, 1992; Henriksen and Wold, 1994; Henriksen et al, 1996) for either DNA-PK or the ataxia-telangiectasia protein ATM (Brush et al, 1994). Although it seems plausible that RPA2 phosphorylation may be a mode of regulating DNA replication (Zernik-Kobak et al, 1997), attempts to show such an effect have failed (Lee and Kim, 1995; Brush et al, 1994; Henriksen and Wold, 1994). The precise biological effect of phosphorylation of RPA2 has so far remained elusive.
iii. Functions of RPA in DNA replication and DNA repair

hRPA was originally identified and purified as an essential factor for SV40 DNA replication in human cell extracts, and was purified from human cell extracts (Wobbe et al, 1987; Faiman and Stillman, 1988; Wold and Kelly, 1988). RPA is now known to be required for the localized unwinding by SV40 large T antigen of the origin during initiation of DNA replication. As well, studies also demonstrated that hRPA interacts specifically with DNA polymerase α, implying a role in elongation of replication (Dornreiter et al, 1992; Nasheuer et al, 1992; Braun et al, 1997). Similarly, hRPA also interacts with the viral initiator from Epstein-Barr virus, EBNA1 (Zhang et al, 1998). Taken together, RPA is believed to play a critical role in the initiation of chromosomal DNA replication. Consistent with the above, immunolocalization of hRPA showed that it is localized at replication foci where chromosomal replication occurs during S phase (Yan and Newport, 1995; Brenot-Bosc et al, 1995).

As mentioned throughout the introduction of this thesis, RPA performs multiple roles in DNA repair in addition to its roles in DNA replication. RPA is involved in the recognition of damaged DNA, as first demonstrated by the finding that RPA can interact with cisplatin-modified DNA (Clugston et al, 1992). Subsequently, research in our laboratory and others showed that RPA could also bind DNA modified by AAAF or UV irradiation (He et al, 1995; Burns et al, 1996). hRPA interacts specifically with several proteins required for nucleotide excision repair including XPA, together with which it cooperatively binds to damaged DNA, XPG, and XPF/ERCC1, the endonucleases of NER, (He et al, 1995; Matsuda et al, 1995; Lee and Kim, 1995; Li L. et al, 1995; Matsunaga et al, 1996). In addition, RPA has been shown to bind with a defined polarity to the undamaged strand of ssDNA and appears to function to position and stimulate the activities of NER endonucleases (deLaat et al, 1998). Protein-protein interactions, such as that with RAD52, also appear to be an important means by which RPA executes its functions in double-strand break repair (Park et al, 1996; Firmenich et al, 1995).
Direct contact with RPA may also be essential for the ability of transactivators to stimulate DNA replication, when binding sites for these activators are near origins of replication (Cheng and Kelly, 1989; Bosher et al, 1990; Guo and DePamphilis, 1992; Bennett-Cook and Hassell, 1991; Marahrens and Stillman, 1992; Cheng et al, 1992). Several transactivators, including VP16, GAL4 and p53, were shown to bind directly to RPA (Dutta et al, 1993; He et al, 1993; Li and Botchan, 1993). Importantly, there is a strong correlation between the ability of mutant forms of VP16 to activate viral replication and their ability to bind RPA in vitro (He et al, 1993; Li and Botchan, 1993).

Of the transcription activators that bind RPA, the interaction with p53 is the most interesting. Binding of p53 requires the N-terminal of RPA1 and some contribution by the C-terminus (Lin et al, 1996; Kim et al, 1996; Gomes and Wold, 1996). This interaction appears to inhibit the ability of hRPA to bind to ssDNA, and was proposed to be one of the mechanisms by which p53 inhibits S phase progression (Dutta et al, 1993). This hypothesis is, however, contradicted by another study which demonstrated a reduction of the ability of RPA to bind to p53 upon DNA damage, apparently as a consequence of phosphorylation of RPA (Abramova et al, 1997). In this latter hypothesis, DNA damage would trigger a cellular response in which an RPA-p53 interaction is disrupted, freeing p53 to perform its function as a checkpoint protein. Clearly, more research is needed to elucidate the molecular significance of interaction between p53 and RPA and the role of phosphorylation of RPA2 in this interaction.
Rationale

BRCA2 has been implicated in DNA double-strand break repair through its interaction with hRad51, a protein known to be involved in this DNA repair pathway. Another protein required for double-strand break repair is Replication Protein A, whose role is to remove secondary structure in ssDNA and to collaborate with hRad52 and hRad51 to initiate the strand exchange reaction during homologous recombination. RPA has previously been shown in our laboratory to interact directly with the acidic activation domains of the transactivators VP16, GAL4, and p53, as well as with regions within NER repair endonucleases human XPG and yeast Rad2 which when fused to the DNA binding domain of GAL4, acquire the ability to activate the transcription of a GAL4-dependent reporter construct in yeast cells (Bardwell et al, 1994). Since BRCA2 also has an acidic domain at its N-terminus which, when fused to a DNA binding domain such as LexA, is capable of activating transcription in vivo (Milner et al, 1997), it is possible that the acidic transcriptional activation domain of BRCA2 actually functions to bind hRPA rather than serve any role in transcription. The experiments reported in my thesis substantiate this view.

![Figure 5: Protein-Protein Interactions Involved in DSBR](image)

Figure 5: Protein-Protein Interactions Involved in DSBR
MATERIALS AND METHODS

I. Preparation of GST Fusion Proteins

The acidic transcriptional activation domain of BRCA2 (Milner et al, 1997) was expressed as a glutathione-S-transferase fusion protein from a pGEX vector (Pharmacia), and was constructed by Michael Shales in our laboratory using RT/PCR reactions to amplify BRCA2 cDNA spanning amino acids 18-105.

I introduced the mutation Y42C into the acidic domain of GST-BRCA2 using a site-directed PCR mutagenesis approach (Stratagene). The mutagenic primers used to amplify the DNA were 5'GCT CCA CCC TGT AAT TCT GAA CCT GC3' and 5'GCA GGT TCA GAA TTA CAG GGT GGA GC3'. Following digestion of the template plasmid with DpnI, the PCR product was used to transform DH5α E.coli cells (Life Technologies). The DNA of a resulting clone was sequenced to ensure that no mutations other than the Y42C change had been introduced.

GST-BRCA2 fusion proteins were expressed in DH5α cells and purified using glutathione-Sepharose 4B according to the manufacturer's instructions (Pharmacia). The acidic transactivation domain of the herpes virus transactivator VP16 was also expressed in and purified as a GST fusion protein (He et al, 1993) from E.coli TOPP2 cells. The preparations of the GST fusion proteins were estimated to be over 90% pure by Coomassie blue staining.

II. Purification of human RPA

Recombinant human replication protein A (hRPA) was purified from an overexpression strain of E. coli cells as described previously (Henricksen et al, 1994) with the following modifications. Lysate from 18 L of induced culture was fractionated on Affi-Gel Blue (100 ml) and hydroxylapatite (30 ml) columns. After dialysis, peak fractions from the hydroxylapatite column were applied to a single-stranded DNA agarose (Pharmacia) column. After loading, the column was washed with 2 column volumes each of buffer containing 0.5 M and 0.75 M NaCl, and then eluted using a buffer containing 1.5 M NaCl. Peak fractions containing hRPA,
identified by SDS-PAGE and Coomasie Blue staining, were pooled and dialyzed overnight against a total of 4L of ACB buffer (10 mM HEPES, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.1 M NaCl (Formosa et al, 1991). The total yield of hRPA was approximately 1 mg, which is estimated by Coomassie blue staining to be more than 90% pure.

III. Purification of yeast RPA

Recombinant S.cerevisiae replication protein A (yRPA) was purified from an overexpression strain of E. coli cells as described previously for hRPA (Henricksen et al, 1994). In brief, extract from 4L of induced culture was chromatographed on Affi-Gel Blue (100 ml) and hydroxylapatite (30 ml) columns. Peak fractions were first concentrated using Centricon columns (Amicon). ACB buffer was added to dilute the solution to a final concentration of 0.1 M NaCl, and this solution was applied to a ss-DNA agarose column (Pharmacia), followed by procedures identical to that used for the elution of hRPA. A total of 0.5 mg of yRPA was obtained, which was more than 90% pure by visual estimation using Coomassie blue staining.

IV. Preparation of HeLa cell extract

Extracts were prepared from frozen HeLa cell pellets according to published protocols (Sopta et al, 1985).

V. Purification of antibodies against hRPA

Recombinant trimeric hRPA, purified as described above, was used as an affinity ligand to purify antibodies in serum obtained from rabbits immunized with purified hRPA (He et al, 1995), following a protocol described by Harlow and Lane (1988). hRPA was first covalently coupled to Affigel-10 (0.5 ml) (Bio-Rad) according to published procedures (Truant et al, 1993) at a concentration of 1 mg/ml. Five ml serum was then passed through a 0.5 ml column containing immobilized hRPA, followed by washing with 20 column volumes of 10 mM TRIS buffer (pH 7.5), and 20 column volumes of 500 mM NaCl, 10 mM TRIS (pH 8.8), to remove unbound antibodies. Bound antibodies were eluted with acidic (5 ml 100 mM glycine pH 2.5)
and then basic (100 mM triethylamine pH 11.5) buffers, followed by overnight dialysis of combined eluate fractions against ACB containing 0.1 M NaCl.

VI. Affinity Chromatography

The detailed procedures used in affinity chromatography experiments have been described previously (Formosa et al, 1991; Ingles et al, 1991). All affinity chromatography experiments were performed at 4°C.

a. To assess binding of recombinant hRPA to GST-fusion proteins

For affinity chromatography with recombinant hRPA, increasing concentrations (namely 0.5 mg/ml, 1 mg/ml, and 2 mg/ml) of GST, GST-VP16, GST-BRCA2 and GST-BRCA2-Y42C were covalently bound to Affigel-10 (Bio-Rad). Twenty five μl columns containing these ligands were prepared and equilibrated with ACB buffer containing 0.1 M NaCl. Each column was then loaded with 2 μg hRPA followed by washing with 250 μl of IP buffer (25 mM HEPES, pH 7.6, 100 mM KCl, 4 mM MgCl2, 1 mM DTT, 12.5% glycerol, 0.02% NP-40) (He et al, 1995) containing 75 mM NaCl, and bound proteins were eluted with 100 μl of ACB containing 1.0 M NaCl. One half of the eluates were resolved by SDS-PAGE and analyzed for the presence of hRPA by western blotting using the affinity purified anti-hRPA antibodies.

b. To assess interaction of GST-fusion proteins with hRPA bound to ss-DNA columns

Affinity chromatography was also performed using recombinant hRPA, E.coli single-stranded DNA binding protein SSB (Pharmacia), and S.cerevisiae recombinant γRPA proteins immobilized on ss-DNA agarose columns (Pharmacia). A ligand concentration of 2 mg/ml each of hRPA, γRPA and SSB were prepared on 20 μl of ss-DNA agarose columns equilibrated with IP buffer containing 75 mM NaCl (He et al, 1995). Four μg of the GST-fusion proteins were loaded on each column
followed by washing with 200 μl IP buffer containing 75 mM NaCl (He et al, 1995). Elution of bound proteins was carried out with 100 μl of ACB containing 1.0 M NaCl. One half of the eluates was resolved by SDS-PAGE and analyzed for the presence of the GST-fusion proteins by western blotting using the anti-GST monoclonal antibodies. The inputs for hRPA, yRPA and SSB were run on SDS-PAGE and stained with Coomassie Blue to ensure that the ssDNA-binding proteins were in fact present in equal amounts on each column. In addition, after adding either hRPA, yRPA or SSB, flow throughs from the columns were analyzed with Bio-Rad Protein assay to test whether the entire input amount bound to ssDNA.

c. To assess binding of hRPA from HeLa cell extract to immobilized GST-fusion proteins

Twenty five μl affinity columns containing 1 mg/ml GST, GST-VP16, GST-BRCA2 and GST-BRCA2-Y42C were prepared, and then equilibrated with IP buffer (He et al, 1995) containing 75 mM NaCl. Each column was loaded with 400 μl of HeLa cell extract, washed with 400 μl IP buffer containing 75 mM NaCl, and eluted with 100 μl of ACB containing 1.0 M NaCl. One half of the high salt eluate of each column was analyzed by western blotting, after SDS-PAGE, using the affinity purified anti-hRPA antibodies.

VII. Co-immunoprecipitation of BRCA2 and hRPA

a. Purified GST-fusion proteins and recombinant hRPA

Twenty μl of Sepharose-protein A (Sigma) was first incubated for 1 hr with anti-hRPA rabbit serum and 2 μg hRPA in 100 μl ACB buffer containing 100 mM NaCl, followed by incubation for 1 hr with 5 μg of GST, GST-BRCA2 or GST-BRCA2-Y42C in 100 μl IP buffer containing 75 mM NaCl. GST-fusion proteins bound to hRPA were then separated from unbound proteins by pelleting the Sepharose-
protein A beads by centrifugation (4°C, 3000 rpm). The beads were then batch-washed 3 times each with 500 μl IP buffer containing 75 mM NaCl, followed by an elution step with 100 μl ACB buffer containing 1M NaCl. One half of these eluates were fractionated on SDS-PAGE, followed by western blotting with monoclonal anti-GST antibodies (Santa Cruz).

b. Endogenous proteins in HeLa Cell extract

One ml volumes HeLa cell extract, prepared as described above, were incubated with the following antibodies: anti-BRCA2 polyclonal antibodies (Oncogene research), rabbit IgG (Cooper Biomedical), rabbit anti-XPA antiserum, and control pre-immune serum (He al, 1995). Seventy five μl protein A (Sigma) beads were then used to precipitate the antibodies. The beads were then batch washed 4 times each with 1ml of IP buffer containing 75 mM NaCl, and then bound proteins were eluted with 200 μl ACB buffer containing 1M NaCl. One half of each of the eluates was analyzed by SDS-PAGE and western blotting using the affinity purified anti-hRPA antibodies.

VIII. Western Blotting

After SDS-PAGE fractionation, proteins were electrotransferred onto nitrocellulose (Schleicher & Schuell) and probed with the indicated antibodies. Two methods of detection of bound proteins were used: alkaline phosphatase and horse radish peroxidase. For the former method, the filters were incubated with alkaline phosphatase-conjugated secondary antibodies (Bio-Rad), followed by development with BCIP and nitroblue tetrazolium (Sigma), according to the manufacturer's specifications. With the horse radish peroxidase detection method, the filters were incubated with peroxidase-conjugated secondary antibodies (Bio-Rad), followed by development with the chemiluminescent substrate and luminol enhancer solutions of the Amersham ECL detection kit.
RESULTS

Purification of proteins required to test hRPA-BRCA2 interaction

Given that BRCA2 and RPA both appear to play a role in DSBR, I decided to assess if there is a direct protein-protein interaction between these two proteins. In order to explore this hypothesis, recombinant forms of the two proteins were first purified. All three subunits of hRPA were coexpressed in and purified from E.coli cells (Henricksen et al, 1994). As expected, hRPA was purified as a trimeric complex and appeared as three polypeptides of 70, 34, 14 kDa in size (Figure 6). Because of the anticipated difficulty of expressing the full length BRCA2 protein due to its large size (3418 amino acids), and since it is the acidic transactivation domain of other activator proteins that interacts with RPA, only the acidic transactivation domain of BRCA2 (amino acids 18-105) (Milner et al, 1997) was expressed in E.coli as a GST-fusion protein. As a positive control for a known interaction with hRPA, the transactivation domain of the herpes virus protein VP16 was also expressed and purified as a GST-fusion derivative (He et al, 1993). Since there is a naturally existing cancer-predisposing mutation that maps to the acidic domain of BRCA2, it seemed possible that this Y42C mutation of BRCA2 might affect any observed binding of BRCA2 to hRPA. In view of this, the Y42C mutation was introduced by site-directed mutagenesis into the acidic domain of GST-BRCA2, and this mutant protein was also purified (Figure 6). Both wild-type and mutant GST fusion proteins purified as a 37 kDa polypeptide in addition to a smaller, approximately 33 kDa polypeptide (figure 6). The 37 kDa polypeptide migrated at the expected size of the full-length fusion protein, whereas the approximately 33 kDa polypeptide appears to be a C-terminal truncated version of GST-BRCA2, lacking about 35 amino acids of the C-terminal acidic domain, as it is recognized by a monoclonal anti-GST antibody (Figure 8, lanes 2 and 3).
Figure 6: Purified Proteins

GST and its derivatives, GST-BRCA2 (the acidic domain of BRCA2), GST-BRCA2-Y42C, and GST-VP16 (the transactivation domain) were purified using glutathione-Sepharose from *E.coli* cells transformed with the respective pGEX vectors. A vector leading to over-expression of the trimeric complex of hRPA was used to prepare recombinant hRPA in *E.coli* and cell extracts were chromatographed on Affi-gel Blue, hydroxylapatite, and finally on ssDNA. All proteins were dialysed with ACB buffer containing 100 mM NaCl, and then analyzed and quantitated using SDS-PAGE fractionation followed by Coomasie Blue staining.
Recombinant hRPA binds directly to the acidic domain of BRCA2

To assess whether the acidic domain of GST-BRCA2 could bind directly to hRPA, I used the affinity chromatography techniques previously used in our laboratory. Western blot experiments with purified polyclonal antibodies against all three subunits of human RPA were used to monitor hRPA polypeptides present in the high salt eluates from columns containing immobilized GST-fusion proteins. As the concentration of immobilized GST-VP16 on the columns was increased from 0.5 to 1.0 or 2.0 mg/ml, there was a corresponding increase in the recovery of RPA1 and RPA2 in the salt-eluted fractions (Figure 7, lanes 2-4). These results confirm the earlier observation of He et al (1993) and Li et al (1993). As the concentration of immobilized GST-BRCA2 was increased, there was also a corresponding increase in the recovery of hRPA in the high salt eluates from the columns (lanes 8-10). The hRPA did not bind the GST control columns (lanes 5-7). Significantly, the mutation Y42C severely compromised the ability of the acidic domain of BRCA2 to bind hRPA. Although there seems to be a larger percentage of the full length fusion proteins in the wild type GST-BRCA2 preparation as compared to that in GST-BRCA2-Y42C (Figure 6), a co-immunoprecipitation shown in Figure 8 indicates that both the full length and truncated fusion proteins bind to hRPA. Overall, results from Figure 7 suggested that the activation domain of BRCA2 might contact hRPA directly, and that the Y42C mutation may affect this interaction.

To confirm the interaction between GST-BRCA2 and hRPA, I also performed a co-immunoprecipitation experiment using recombinant GST-fusion proteins, hRPA, and anti-hRPA antibodies. Western blotting with a monoclonal anti-GST antibody of the high salt eluates from the antibody bound proteins showed that GST-BRCA2 was precipitated in the same complex as hRPA by anti-hRPA antibodies (Figure 8, lane 5). Interestingly, both the full length and the truncated versions of wild type GST-BRCA2 were able to bind hRPA, suggesting that the truncation of approximately of 35 amino acids did not affect the binding. It is interesting to note that truncating the C-terminal 45 amino acids from this acidic domain of BRCA2 only modestly hindered the ability of this domain to activate transcription (Milner
Figure 7: GST-BRCA2 binds recombinant hRPA directly

Purified recombinant hRPA was chromatographed over 25 ul Affi-gel columns containing immobilized GST-VP16, GST, GST-BRCA2, or GST-BRCA2-Y42C, each at increasing ligand concentration of 0.5, 1 and 2 mg/ml. The columns were washed with 10 column volumes of IP buffer containing 75 mM NaCl, followed by elution with ACB buffer containing 1 M NaCl. Lane 1 represents 5% of hRPA input, while lanes 2-13 are one half of the high salt eluates. SDS-PAGE analysis was followed by western blotting using affinity purified rabbit polyclonal anti-hRPA antibodies.
Figure 8: Recombinant RPA Binds GST-BRCA2 But Not mutant GST-BRCA2-Y42C

hRPA was first incubated with anti-hRPA rabbit serum and 20 µl Sepharose-protein A, followed by a second incubation with GST-fusion proteins. The protein A beads were washed 3 times with 500 µl IP buffer containing 75 mM NaCl, followed by elution with 100 µl ACB buffer containing 1 M NaCl. Lanes 1-3 represent 4% of GST-fusion protein inputs, while lanes 4-6 are one half of the high salt eluates. The proteins were analyzed by western blotting with anti-GST monoclonal antibodies.
et al, 1997), strengthening the correlation between this ability to bind hRPA and ability to activate transcription. The GST moiety alone was not able to bind hRPA (lane 4). As seen in the previous experiment, the introduction of the Y42C mutation into the acidic domain of BRCA2 disrupts this interaction of GST-BRCA2 with hRPA (lane 6). These experiments indicate that hRPA can bind directly to the activation domain of wild type but not mutant GST-BRCA2.

The BRCA2-RPA interaction is species-specific and is not mediated by DNA

As hRPA is a DNA binding protein, it may be possible that the protein-protein interaction detected in the experiments of Figures 7 and 8 may be indirectly mediated by both proteins binding to DNA independently. If this were true, GST-BRCA2 would have to be capable of binding to DNA in addition to hRPA. To assess this possibility, the GST-BRCA2 fusion protein was chromatographed on a ssDNA agarose column. As seen in Figure 9, western blotting of the high salt eluates from the ssDNA agarose columns using monoclonal anti-GST antibodies showed that GST-BRCA2 did not bind to ssDNA directly (lane 5), a result suggesting that the BRCA2-hRPA interaction is direct and not mediated by DNA.

Given that the acidic activation domains of other transactivators, such as GAL4 and VP16, are able to bind both yeast and human RPA, it was interesting to test if the BRCA2-hRPA interaction is also evolutionarily conserved. To do this, I also used this affinity chromatographic approach employing immobilized ssDNA. ssDNA-agarose was used to bind the E.coli ssDNA-binding protein (SSB), the S.cerevisiae RPA homologue (yRPA), and human RPA. In order to determine whether the DNA-binding proteins were present on the columns in equal concentrations, the inputs for hRPA, yRPA and SSB were run on SDS-PAGE and stained with Coomasie Blue, while Bio-Rad Protein Assay ensured that no protein was left in the flow-through from loading each of those columns. Various GST-fusion proteins were then loaded on these columns. As seen in Figure 9, western blotting of the high salt eluates from the ssDNA agarose columns using monoclonal anti-GST antibodies showed that GST-BRCA2 bound selectively to the column containing hRPA (lane 10), but not to the columns containing
Figure 9: BRCA2 Can Bind with RPA to SS-DNA

GST-Fusion proteins were passed over 20 µl ssDNA columns containing either no protein ligand (lane5), or ssDNA agarose columns that had first been loaded with 40 µg E.coli SSB (lane 6), 40 µg yRPA (lane 7), and 40 µg hRPA (lanes 8-11). The columns were washed with 10 column volumes of IP buffer containing 75 mM NaCl, followed by elution with with ACB buffer containing 1M NaCl. Lanes 1-4 represent 2.5% of GST-fusion proteins input, while lanes 5-11 are one half of the high salt eluates. The proteins were resolved by SDS-PAGE and analyzed by western blotting using monoclonal anti-GST antibodies.
either SSB or yRPA (lanes 6 and 7). Consistent with the experiment described in Figure 8, both the full length and partially truncated versions of GST-BRCA2 bound to hRPA. As expected, GST-VP16 was also able to bind hRPA (lane 9), whereas GST by itself did not (lane 8). This result suggests that the direct interaction between RPA and GST-BRCA2 is species-specific, in that only the human proteins interact, and that this BRCA2-hRPA interaction can take place while hRPA is bound to ssDNA. It is also interesting to note that the interaction between BRCA2 and hRPA was detected either in the presence (Figure 9, lane 10) or absence of DNA (Figure 8, lane 5). This is in contrast to a report that the p53-hRPA interaction is abrogated when RPA binds DNA (Dutta et al, 1993).

The acidic domain of BRCA2 binds hRPA present in HeLa cell extract

Since purified recombinant proteins were used in each of the binding experiments described in Figures 7, 8, and 9, it was important to test if hRPA present in HeLa cell extract could also bind GST-BRCA2. HeLa whole cell extract was first passed through affinity columns containing immobilized GST-fusion proteins as ligands. Western blot experiments with purified anti-hRPA antibodies were then used to monitor for the presence of RPA1 and RPA2 polypeptides in the salt-eluted fractions from each column. As expected (He et al, 1993), hRPA was present in the eluate from the GST-VP16 column (Figure 10, lane 4), but not from the GST column (lane 3). The acidic domain of BRCA2 was also able to selectively bind hRPA from cell extract (lane 5) and the Y42C mutation appeared to reduce the affinity of BRCA2 for hRPA, although to a lesser extent with this experimental protocol than was seen in the experiments with purified recombinant proteins. It is possible that in cell extracts these proteins may exist within larger multiprotein complexes, and it is possible that other proteins within this complex may affect the overall affinity of the hRPA-BRCA2 interaction.
Figure 10: RPA Present in HeLa Cell Extract Binds GST-BRCA2

HeLa cell extract was passed over 25 µl columns containing 1 mg/ml GST, GST-VP16, GST-BRCA2 or GST-BRCA2-Y42C. The columns were washed with 16 column volumes of IP buffer containing 75 mM NaCl, followed by elution with 100 µl ACB buffer containing 1 M NaCl. Lane 2 represents 5% of HeLa cell extract input and lane 1 contains 1 µg purified hRPA as marker. Lanes 3-6 represent one half of the high-salt eluates. hRPA was detected by western blot analysis using polyclonal anti-hRPA antibodies.
Endogenous BRCA2 and hRPA exist as a complex *in vivo*

Having detected an interaction between the acidic domain of BRCA2 and hRPA from cell extract, I also wished to examine if the full length endogenous BRCA2 protein and hRPA could be co-immunoprecipitated from cell extracts where both exist at physiological concentrations. As seen in Figure 11, when the cellular BRCA2 was immunoprecipitated with anti-BRCA2 antibodies, hRPA was also present in this immunoprecipitate (lane 3). This data suggests that the two proteins may exist in a complex within cells. No hRPA could be detected if the immunoprecipitation was performed using control rabbit IgG (lane 4). As a positive control, I also used this co-immunoprecipitation procedure to detect a previously described RPA-XPA interaction (He et al, 1995). As expected, hRPA was co-immunoprecipitated along with XPA by anti-XPA but not by the control pre-immune serum. This result suggests that the relatively abundant hRPA protein can be found in cell extracts in what are likely to be different multiprotein complexes, namely with BRCA2 and possibly other double-strand break repair proteins such as RAD51 and RAD52 (Chen et al, 1998; Scully et al, 1997; Sharan et al, 1997), and with XPA and other nucleotide excision repair proteins.
Figure 11: BRCA2 and RPA in HeLa Cell Extract Co-immunoprecipitate

HeLa cell extract was incubated with anti-BRCA2 polyclonal antibodies (lane 3), rabbit IgG control antibodies (lane 4), anti-XPA serum (lane 5), or pre-immune serum (lane 6). Antibodies and the associated proteins were co-immunoprecipitated using 75 µl protein A beads. The beads were washed 4 times with 1 ml IP buffer containing 75 mM NaCl, followed by elution with 200 µl ACB containing 1 M NaCl. One third of the high salt eluates were resolved on SDS-polyacrylamide gel followed by western blotting with purified anti-RPA antibodies.
My experiments indicate that hRPA interacts directly with the N-terminal acidic domain of BRCA2. Using purified recombinant hRPA and GST-BRCA2 fusion proteins, I first showed with three different experimental protocols employing either affinity chromatography or co-immunoprecipitation that these two proteins can interact directly, and that this interaction is species-specific (Figures 7, 8, and 9). Using co-immunoprecipitation experiments, I also showed that endogenous, full length BRCA2 and hRPA proteins also form a complex in cell extracts (Figure 11). This interaction between BRCA2 and hRPA appears by several criteria to be weaker than the interaction of the potent transactivator VP16 with hRPA. When I used more stringent conditions, namely 100 mM rather than 75 mM NaCl in my experiments, I was unable to detect the BRCA2-hRPA interaction, while VP16 still interacts well with hRPA in buffers containing 100 mM NaCl (data not shown; see also He et al 1993). In addition, with similar ligand concentrations, consistently more hRPA was found in eluates from the GST-VP16 columns, than those from GST-BRCA2 columns (Figures 7 and 10).

There are additional differences between the VP16-hRPA and BRCA2-hRPA interactions. Although both interaction domains have the ability to function as acidic transcriptional activation domains (Milner et al, 1997; Sadowski et al, 1988; Cousens et al, 1989), and both appear capable of binding hRPA, VP16 can also interact with yeast RPA (He et al, 1993), whereas the "activation" domain of BRCA2 specifically interacts only with hRPA and not with yeast RPA (Figure 9). The lack of interaction between BRCA2 and yRPA could be the consequence of divergence of the yeast and human RPA sequences. Given that the activation domain of VP16 does not share any significant amino acid similarity with the BRCA2 activation domain except for a high percentage of acidic amino acids, it is easily conceivable that the interactions of those proteins with RPA may be somewhat different.
One mutation, from a tyrosine to a cysteine at amino acid 42, in the acidic N-terminal domain of BRCA2 is associated with an elevated risk of breast cancer (Friend et al, 1995). Introduction of this Y42C mutation into the GST-BRCA2 polypeptide compromised the ability of recombinant GST-BRCA2 to bind to hRPA, suggesting that this BRCA2-hRPA interaction might be important for BRCA2 function in cells. Although the same mutation also inhibited the transcriptional activation potential of this same BRCA2 domain (Milner et al, 1997), there is currently no compelling evidence for involvement of BRCA2 in transcription. An acidic activation domain was also found in another DNA repair protein, XPG (Bardwell et al, 1994), which is an endonuclease involved in nucleotide excision repair and has no role in transcription. The "activation" domain of XPG mediates an important interaction between XPG and RPA (He et al, 1995; de Laat et al, 1998). In view of this, I suggest that BRCA2 does not normally function in transcription. Rather, coupled with other evidence pointing to a role of BRCA2 in DSBR, our evidence for a direct interaction with hRPA may further substantiate the notion that BRCA2 probably functions as a DNA repair protein.

My co-immunoprecipitation experiment with HeLa cell extract suggests that BRCA2 and hRPA physically associate in vivo (Figure 11). In addition, both BRCA1 and RAD51 proteins have also been found to interact with BRCA2 (Chen J. et al, 1998; Chen P.L. et al, 1998), suggesting that BRCA1, BRCA2 and RAD51 function, at least in part, in a multiprotein complex (Scully et al, 1997; Sharan et al, 1997). It is not surprising that BRCA2 can interact with multiple proteins simultaneously, since it is an extremely large protein and the domains interacting with different proteins may be distributed along its length (see Figure 4). BRCA1, BRCA2, RAD51 and hRPA may even exist as a large multiprotein complex in the absence of DNA damage, analogous to the nucleotide excision repairisome identified by He and Ingles (1996). That both BRCA1 and BRCA2 may function together in the pathway of homologous recombination to repair double-strand breaks in DNA is also consistent with the observation that both BRCA1 and BRCA2 undergo similar changes in nuclear localization upon DNA damage (Chen J. et al, 1998).
It should be noted, however, that a number of observations also link BRCA1 to transcription. For example, ectopic expression of BRCA1 leads to activation of transcription of the cell cycle regulator p21 (Somasundaram et al, 1997). BRCA1 has been shown to interact with p53 (Zhang H. et al, 1998), RNA helicase A (Anderson et al, 1998) and CtIP (Yu et al, 1998; Wong et al, 1998), proteins which are each thought to be involved in transcriptional events. Moreover, BRCA1 has been found as a component of different RNA polymerase II holoenzyme preparations (Maldonado et al, 1996; Scully et al, 1997c; Anderson et al, 1998). However, the holoenzyme purified by Maldonado et al also contained the DNA repair proteins RAD51 and RPA. The recent finding that BRCA1 interacts with components of both a histone deacetylase complex and a histone acetylase complex may reconcile the two seemingly conflicting roles of BRCA1 in transcription and repair (Yarden and Brody, 1999). Modulation of chromatin structure has long been known to be important in a number of cellular processes involving different DNA transactions, including transcription, repair, replication and recombination (Hassig and Schreiber, 1997; Smith and Stillman, 1991; Almouzni and Wolfle, 1993; Gaillard et al, 1996). Therefore, one of the functions of BRCA1 could be that it acts as a bridging molecule or adaptor to bring the chromatin remodelling activities to sites of DNA repair. During DNA repair, chromosomal remodelling may be necessary before and after removal of lesions, such that acetylation of histones could open the chromatin structure facilitating repair, while a subsequent deacetylation of histones may be required after the repair of any lesions.

To better define the multitude of functions proposed for both the BRCA proteins, an approach involving the use of assays dependent on these two proteins would be invaluable. However, a major obstacle in this approach is the large size of both BRCA1 and BRCA2, 220 and 400 kDa respectively, making them difficult to express and purify as full-length recombinant proteins. Recently, Kurimasa et al (1999) succeeded in transiently expressing, although not purifying, the even larger 470 kDa catalytic subunit of DNA-dependent protein kinase (DNA-PK) by constructing a contiguous 14 kb cDNA. This allowed better definition of the role of the catalytic subunit of DNA-PK in the non-homologous repair of double-stranded
breaks. Similar expression vectors would allow one to examine what effect cancer predisposing mutations in BRCA2 have on the ability of cell extracts to repair double strand breaks.

The availability of full-length BRCA2 protein in the form of recombinant protein would also assist in the biochemical characterization of its functions. Specifically, given that BRCA2 interacts with and localizes with RAD51, a pivotal protein in strand-exchange during homologous recombination (Sonoda et al, 1998; Sung, 1994; Gupta et al, 1997; Baumann et al, 1996), it will be of great interest to test if addition of BRCA2 would modulate the in vitro activity of the strand invasion step, a step that also depends on the presence of RPA. A modulating activity on the strand exchange reaction has also been identified for yeast and human RAD52 (Sung, 1997; Benson et al, 1998; Shinohara and Ogawa, 1998; New et al, 1998) and RAD54 (Petukhova et al, 1998) and for the yeast RAD55-RAD57 complex (Sung, 1997), for which no human homologues have yet been identified.

Our observation that BRCA2 interacts with hRPA has not yet provided proof of the involvement of this interaction in DSBR, since RPA is involved in many different pathways of DNA repair. To provide further support that the observed BRCA2-hRPA interaction is involved specifically in the homologous recombination repair pathway, the immunoprecipitation experiments described in Figure 11 could be used to detect in the same immunoprecipitated complexes the presence of proteins such as RAD51, RAD52 and others known to be involved in DSBR. These experiments might provide some evidence for the existence of a multiprotein DSBR recombinosome that includes both BRCA2 and hRPA.

Conceivably, dysfunction of this DSBR pathway may be required for the evolution of most hereditary breast cancers. Interestingly, it has been shown that deficient repair of radiation-induced DNA damage is a predisposing factor in familial and some sporadic breast cancer (Parshad et al, 1996; Jyothish et al, 1998). Insufficient repair of double-strand breaks likely accounts for the aberrant chromosomal structures observed in cells derived from Brca2 -/- mutant mice (Connor et al, 1997a; Patel et al, 1998). If an interaction between hRPA and BRCA2 in breast cells is compromised by the Y42C mutation, as I have shown to be the case
in my *in vitro* experiments, this may account for the predisposition to breast cancer. One can also envisage that mutations within hRPA could have the same consequence. Such mutations would likely be found in the gene encoding the largest subunit of RPA since RPA1 is known to be the subunit directly interacting with other known acidic transactivation domains (Dutta et al, 1993; He et al, 1993; Li and Botchan, 1993). Interestingly, the 17p13.3 locus where the RPA1 gene maps (Umbricht et al, 1994), undergoes loss of heterozygosity in various human malignancies, including breast and ovarian cancers (Stack et al, 1995; Mackay et al, 1988; Coles et al, 1990; Thorlacius et al, 1991). In collaboration with our laboratory, the Andrulis laboratory has identified an amino acid polymorphism present in the human RPA1 polypeptide. It will be of great interest to see if this polymorphism affects one of the many functions of RPA, including the binding of hRPA to BRCA2 or to other DNA repair proteins. This polymorphism could even exert an effect on DNA repair by simply affecting the cellular concentrations of RPA and therefore its distribution within the different multiprotein complexes that affect DNA replication, recombination and repair.
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