RAD50 protein of *S.cerevisiae* exhibits ATP-dependent DNA binding

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ABSTRACT

RAD50 function of *Saccharomyces cerevisiae* is required during vegetative growth for recombinational repair of DNA double strand breaks, and during meiosis for initiation of meiotic recombination and formation of synaptonemal complex. RAD50 encodes a 153 kDa polypeptide which includes an amino-terminal ATP binding domain essential for function and two long heptad repeat regions. We show below that RAD50 protein purified from yeast exhibits ATP-dependent binding to double stranded DNA. Physical properties of the purified protein are also described. Models for RAD50 function *in vivo* are discussed.

INTRODUCTION

The *RAD50* gene of *S.cerevisiae* is required during vegetative growth for recombinational repair of double strand breaks (DSBs) (1,2,3) and for efficient mating type switching, a directed recombination event initiated by a programmed site-specific double strand break (A.McKee and N.K. unpublished observations).

During meiosis, *RAD50* is required for initiation of meiotic recombination (4–6), a process which also involves double strand breaks (6–8). In addition, *RAD50* function is required for formation of synaptonemal complex (SC) between homologous chromosomes during meiotic prophase (5, B.Byers, cited in 9, 10). The role of *RAD50* function in the latter process may be indirect, since SC formation may require a prior recombinational interaction between homologues (5, 11–13); however, a direct role for *RAD50* function in SC formation is not excluded.

The polypeptide predicted for RAD50 protein is 153 kDa (1312 amino acids). The protein contains an amino-terminal ATP binding domain (14). Mutations that alter critical conserved amino acids of the ATP binding site confer a null phenotype, and a specific class of non-null mutations which are primarily defective in meiosis (*rad50S*) alter non-conserved residues within the ATP binding domain (5). The remainder of the protein includes two long segments of heptad repeat sequence diagnostic of regions capable of forming alpha-helical coiled coils, one of which is of the same length as and exhibits significant amino acid similarity to the S-2 domain of the rabbit myosin heavy chain (14).

We report here the initial biochemical characterization of RAD50 protein purified from yeast which demonstrates the presence of ATP-dependent DNA binding activity. An updated discussion of possible *in vivo* roles for RAD50 protein is presented in the Discussion.

MATERIALS AND METHODS

Overproduction and purification of Rad50 protein

A plasmid containing the *RAD50* coding sequence fused at the ATG to the *PHO5* promoter (pNKY2076) (15) was introduced into the protease-defective *S.cerevisiae* strain BJ2667 (*MATa leu2 ura3-52 prb1-1122*; kindly provided by Elizabeth Jones, Carnegie-Mellon University). A 2 liter culture of such cells was grown to stationary phase in low-phosphate medium at 30°C (16). Cells were harvested and lysed essentially as described by Worland and Wang (17). Debris was eliminated by successive centrifugations at 15K rpm for 20 minutes and 5 minutes. The resulting supernatant (= Fraction 0) typically contained 450 mg of protein. Fraction 0 was adjusted to a total protein concentration of 5 mg/ml with buffer I [50 mM Tris-HCl, pH7.7; 1 mM EDTA, pH 8; 1 mM EGTA, pH 8; 10% glycerol (v/v); 1 mM phenylmethylsulfonyl fluoride; 1 mM β -mercaptoethanol] and brought to a KCl concentration of 0.1 M. Then, polyethyleneimine (Bethesda Research Laboratories) was added from a 10% (v/v) stock solution at pH 7.7 over a 15 minute period to a final concentration of 0.2%. After 15 minutes, the mixture was centrifuged at 8K rpm for 10 minutes. The resulting pellet was washed twice in buffer I containing 0.5M KCl and supernatants from both washes were combined (= Fraction 1). Fractionation with ammonium sulfate (35%–60%) yielded a pellet that was dissolved in 4ml of gT/200 (10% glycerol, 50mM Tris pH7.7, 2.3mM β -mercaptoethanol, 200 mM NaCl) to give Fraction 2, which was then fractionated on a Sephacryl S400-HR(Pharmacia) column equilibrated and run in gT/200. Fractions enriched for RAD50 protein were identified on SDS polyacrylamide gels (18) and pooled (= Fraction 3). Fraction 3 was diluted with 10% glycerol, 50 mM Tris, pH 7.7 to a conductivity equal to that of gT/100 (10% glycerol, 50 mM Tris, pH 7.7, 2.3 mM β -mercaptoethanol, 100 mM NaCl) and then loaded onto a 1 ml phosphocellulose (Whatman) column

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Figure 1. Purification of RAD50 protein. RAD50 protein was purified through a series of steps, described in detail in Materials and Methods. Fraction 0–5 are essentially: (0) crude extract, (1) KCl-washed PEI pellet, (2) 35–60% ammonium sulfate pellet, and peak fractions from (3) S400-HR, (4) phosphocellulose and (5) heparin agarose columns. (A). 0.3 μ g of protein from each fraction were analyzed on a 7% polyacrylamide-SDS gel stained with Coomassie-blue. Visual inspection suggests that RAD50 protein constitutes about 95% of total protein in Fraction 5; additional minor bands are visible by silver staining. M denotes markers of indicated molecular size in kDa. (B). Same as (A) for an independent preparation, 0.6 μ g of protein per lane. (C). Immunoblot analysis of a gel identical to that in (A) with polyclonal anti-RAD50 serum.

preequilibrated in gT/100. The column, run at 6 ml/hr, was washed with 5 column volumes of gT/100 and then with consecutive applications of 3ml each of gT/120, gT/150, gT/180 and gT/200. The majority of RAD50 protein eluted between 130–180 mM NaCl (= Fraction 4). Fraction 4 was diluted and fractionated on a 1ml column of heparin agarose type II (Sigma) equilibrated in gT/100; RAD50 protein eluted in two peaks, a major peak at 200 mM NaCl and a minor peak at 300 mM NaCl; protein eluting at 200mM NaCl is Fraction 5, which typically contains 80 μ g of protein. The amount of RAD50 protein present in Fraction 5 represents approximately 20% of the total RAD50 protein present in the crude lysate (Fraction 0). Protein concentrations were determined by Bio-Rad protein assay using bovine serum albumin as a standard.

DNA binding reactions and assays

DNA binding reactions were carried out at room temperature. Reaction conditions are specified in the figure legends. If Mg^{++} was omitted, no binding was observed. For nitrocellulose filter binding assays, each reaction was stopped by the addition of a 33-fold excess of ice cold wash buffer (33mM Tris pH 7.5, 13 mM $MgCl_2$, 5mM DTT) and filtered over a KOH-treated nitrocellulose filter (Schleicher and Schuell, BA85, 0.45 μ m) with a 1ml wash. Filters were dried and counted by Cherenkov radiation. ^{32}P -end-labeled λ H3 DNA was biotinylated using photoactivable biotin (Clontech; 19, 20).

Protein gels and immunoblotting

Proteins were separated on 7% polyacrylamide 1% SDS gels (18). Immunoblotting was performed according to Towbin et al. (21), using a 1:1000 dilution of anti-RAD50 serum 894 (22). RAD50 band intensities were quantified using the scanning densitometry program ImageQuant.

Analysis of physical properties of RAD50 protein at high ionic strength

Stokes radius (a) of RAD50 protein was determined from its position of elution from an S400-HR gel filtration column as compared with proteins of known molecular weight and Stokes radius (thyroglobulin, apoferritin, β -amylase, alcohol dehydrogenase, bovine serum albumin and carbonic anhydrase) and an excluded dye, dextran blue. Two separate determinations yielded Stokes radii of 148Å and 156Å. Gel filtration conditions

were 50mM Tris-HCl pH7.7, 200mM NaCl, 10% glycerol, 2.3mM β -mercaptoethanol. A sedimentation coefficient (s) was determined from the mobility of RAD50 protein in a 5–20% sucrose gradient (200mM NaCl, 50mM Tris pH7.5) relative to the mobilities of proteins of known s value (catalase, fumarase, lactate dehydrogenase, malate dehydrogenase). The sedimentation coefficient, $4.3 \pm 0.5 \times 10^{-13}$ sec, did not change if samples were pre-treated with DNaseI prior to analysis. Molecular weight (M) and frictional ratio (f/f_o) were calculated according to the following formulas (23): $M = 6\pi\eta Ns/(1 - \nu\rho)$; $(f/f_o) = a/(3\nu M/4\pi N)^{1/3}$. In determination of M , $\eta = 0.01002$ g $cm^{-1}sec^{-1}$, $\rho = 0.9982$ g/ cm^3 for H_2O (24), $\nu = 0.737$ cm^3/g for an unhydrated spherical protein with the amino acid composition of RAD50 protein (14, 25), $a = 152 \pm 4$ Å and $s = 4.3 \pm 0.5 \times 10^{-13}$ sec. Calculated $M = 2.82 \pm 0.3 \times 10^5$ g/mol, which is ~ 1.8 times the monomer molecular weight of 153,000. For calculation of frictional ratio, RAD50 was assumed to be a dimer and M was therefore taken to be 306,000; this calculation yielded $f/f_o = 3.4 \pm 0.1$. This frictional ratio was used to calculate the corresponding axial ratio for a prolate ellipsoid of 60 (26).

RESULTS

Purification of RAD50 protein from yeast

RAD50 protein was purified from yeast cells carrying the RAD50 coding sequence fused to the PHO5 promoter and grown in low phosphate medium to induce expression (Materials and Methods). Upon induction, RAD50 protein constitutes approximately 1% of total cellular protein (Figure 1A, lane 1), approximately 500 times higher than the wild type level. In the most highly purified preparation (Fraction 5) RAD50 constitutes about 95% of total protein; no significant proteolysis of RAD50 protein occurred during the purification procedure (Figure 1).

RAD50 protein exhibits ATP-dependent double stranded DNA binding activity

(1) Purified RAD50 protein will bind radiolabeled DNA to a nitrocellulose filter in the presence but not the absence of ATP (Figure 2A); the rate at which DNA is bound to a nitrocellulose filter at early times in the reaction increases approximately in proportion to the amount of RAD50 protein present (Figure 2B). Upon 100-fold dilution of RAD50 protein–DNA complexes,

Figure 2. Nitrocellulose filter binding assay of purified RAD50 protein. (A). 120 ng of Fraction 5 RAD50 protein was incubated with 75ng of 32 P-end-labeled HindIII-digested λ DNA (specific activity 1.8×10^3 cpm/ng) in a total volume of 15 μ l. Final concentrations of RAD50 protein (monomer) and DNA(bp) were 5×10^{-8} M and 8×10^{-6} M respectively; ATP was 1.3 mM (closed boxes) or absent (open triangles). Binding was assayed (Materials and Methods) after incubation for the indicated periods of time. Reactions contained 43 mM Tris-HCl pH 7.5, 13 mM MgCl₂, 18 mM DTT, 0.09 mg/ml BSA, 40 mM NaCl, 2% glycerol, and 0.46 mM β -mercaptoethanol. (B) Varying amounts of Fraction 5 RAD50 protein were incubated with 32 P-end-labeled HindIII-digested λ DNA (140ng; 15×10^{-6} M; 970 cpm/ng) in a 15 μ l reaction. Amounts of RAD50 protein were 20, 40, 80 and 160 ng [$0.9-7 \times 10^{-8}$ M (monomer)]; open boxes, closed triangles, open circles and closed boxes respectively. Assays were as in (A). Reactions contained 5 mM ATP, 43 mM Tris-HCl pH 7.5, 4 mM MgCl₂, 1 mM DTT, 0.09 mg/ml BSA, 53 mM NaCl, 2.7% glycerol, 1 mM EDTA, and 0.6 mM β -mercaptoethanol. (C) 80 ng of Fraction 5 RAD50 protein [3.5×10^{-8} M (monomer)] was incubated with 32 P-end-labeled HindIII-digested λ DNA (140ng; 15×10^{-6} M; 970 cpm/ng) in a 15ml reaction for the indicated periods of time (closed boxes). After incubation for 80 minutes, two separate 15 ml reactions were diluted with 1.5 ml reaction buffer containing 5mM ATP (open circle) or without ATP (closed circle) and were incubated for another 30 minutes at room temperature. Assays were as in (A). Reactions contained 5 mM ATP, 33 mM Tris-HCl pH 7.5, 8 mM MgCl₂, 1 mM DTT, 0.09 mg/ml BSA, 27 mM NaCl, 1.3% glycerol, 1 mM EDTA, and 0.6 mM β -mercaptoethanol.

most DNA remains bound by RAD50 protein (Figure 2C). (2) In reactions with biotinylated DNA as substrate, streptavidin-agarose precipitation of the DNA results in ATP-dependent co-precipitation of RAD50 protein (Figure 3). Since the fraction of RAD50 protein precipitated by streptavidin in the presence of ATP is the same as the fraction of DNA molecules precipitated, it appears that most or all of the RAD50 protein is bound to DNA.

	sup			pellets			
	1	2	3	4	5	6	7
DNA	B	N	B	B	N	B	-
ATP	-	+	+	-	+	+	-
Rad50p							
% Rad50p	62	110	44	12	9	39	=100
% cpm	55	100	56	45	0.4	44	-

Figure 3. Streptavidin precipitation of biotinylated DNA co-precipitates RAD50 protein. RAD50 protein (Fraction 5) was incubated with 32 P-labeled HindIII-digested λ DNA that had either been biotinylated (B) or not (N). 15 μ l reaction mixtures contained 80ng RAD50 protein [3.5×10^{-8} M (monomer)], 32ng DNA [3.3×10^{-6} M (bp)], and 50 mM Tris-HCl pH 7.5, 27 mM NaCl, 8 mM MgCl₂, 1.3% glycerol, 1 mM DTT, 0.3 mM β -mercaptoethanol, 0.09 mg/ml BSA, and if indicated, 5 mM ATP. After 60 minutes incubation at room temperature, samples were incubated with streptavidin agarose to specifically precipitate biotinylated DNA, and precipitates and supernatants were separated and assayed (Materials and Methods). The relative levels of RAD50 protein in the supernatant and pellet from each reaction are shown by an immunoblot of an SDS polyacrylamide gel and estimated by densitometry. As a standard, Lane 7 contains 100% of the total RAD50 protein present in the three reaction mixtures; values shown are expressed as percentages of the amount in Lane 7, ± 4 . The percentage of substrate DNA cpm precipitated by streptavidin in the same samples is also shown.

The presence of RAD50 protein in the reaction mixture does not change the fraction of biotinylated DNA precipitated (data not shown). Also, biotinylation of the substrate DNA does not affect the ability of RAD50 protein to bind to it, as measured by immunoprecipitation assays (15). (3) Highly specific anti-RAD50 antiserum will precipitate DNA in the presence of purified RAD50 protein and ATP; no precipitation is observed in the absence of ATP or with preimmune serum (15).

Nucleotide dependence of binding to double stranded DNA

RAD50-dependent binding to double stranded DNA is not observed in the absence of a nucleotide cofactor, is observed in the presence of rATP, dATP, ddATP or ATP γ S, and is not observed in the presence of ADP or other nucleoside triphosphates (Figure 4). The affinity of RAD50 protein for ATP is apparently relatively low: an ATP concentration of 2–5 mM was required for optimal filter binding activity (data not shown). High level ATPase activity is not detected under standard reaction conditions (≤ 1 molecule of ATP hydrolyzed per molecule of RAD50 protein per minute assuming that all of the protein is active).

Substrate dependence

RAD50-dependent DNA binding activity has not yet been characterized in detail. At a gross level, under conditions similar to those used for the binding reactions reported here, binding of RAD50 protein is not sensitive to the sequence or presence of ends on a duplex lambda DNA substrate and is able to bind single stranded M13 DNA (W.R., K.Reilly and N.K., unpublished observations). However, affinities for different substrates have not been examined under conditions that might reveal subtle differences (for example, initial binding to ends followed by subsequent binding elsewhere on the substrate molecule).

Figure 4. Nucleotide dependence of DNA binding by RAD50 protein. RAD50 protein [Fraction 4; 350ng; 1.5×10^{-8} M (monomer)] and 32 P-end-labeled HindIII-digested λ DNA [160ng = 1.7×10^{-5} M (bp)]; specific activity 360 cpm/ng) were incubated for 30 minutes at room temperature in the presence of the indicated nucleotide at a final concentration of 1.3 mM. 1,2,3 represent GTP, dGTP and ddGTP; 4,5,6 represent CTP, dCTP and ddCTP; 7,8,9 represent UTP, dTTP and ddTTP. Except for compounds 1–9, data represent the average of duplicate samples. Reactions contained 50 mM Tris–HCl pH 7.5, 67 mM NaCl, 3.3% glycerol, 0.8 mM β -mercaptoethanol, 18 mM DTT, and 0.09 mg/ml BSA.

Physical properties of RAD50 protein

In the presence of 200mM NaCl, RAD50 protein is an homogeneous asymmetric dimer: Stokes radius $\sim 152\text{\AA}$; sedimentation coefficient $\sim 4.3\text{S}$; calculated molecular weight 282,000 daltons, or ~ 1.8 times the monomer molecular weight of 153,000; frictional coefficient (calculated for a dimeric species) of 3.4 and axial ratio of slightly more than 60 (Materials and Methods). At a lower salt concentration (20mM NaCl), the conformation of RAD50 protein changes to a less asymmetric form: the protein exists as a heterogeneous mixture of species with sedimentation coefficients ranging from 4S to 11S but exhibiting a much smaller average Stokes radius than observed under higher salt conditions. The properties of these lower ionic strength forms are consistent with their being either dimers or a mixture of dimers and higher order oligomers.

DISCUSSION

Biochemistry of RAD50 protein

RAD50 protein binds double stranded DNA in an ATP-dependent fashion. Under the conditions examined thus far, binding is not grossly sensitive to overall DNA sequence composition or to the presence or absence of ends. RAD50 protein appears to bind DNA with reasonable affinity: under standard conditions, most of the RAD50 protein is bound to DNA after 60 minutes at a protein concentration of about 35nM (Figure 3), and dilution of such protein/DNA complexes does not result in rapid release of bound protein (Figure 2C). Although assay-dependent, quantitative differences in RAD50 protein binding to DNA are apparent, the fundamental, qualitative conclusion that purified RAD50 protein binds double stranded DNA in an ATP-dependent manner is clear. These observations are consistent with the possibility that direct contact with DNA is an important feature of RAD50 function. Detailed consideration of the stoichiometry of DNA binding in these initial experiments reveals a number of irregularities which suggest that RAD50 protein may preferentially load (cooperatively) onto a subset of DNA molecules in the population rather than distributing itself at random (Figures 2 and 3; 15); alternatively, oligomerization of

RAD50 protein at relatively low salt concentrations may account for these irregularities.

Binding of RAD50 protein to DNA requires an adenosine triphosphate nucleotide. Binding may not require ATP hydrolysis, since ATP γ S substitutes effectively for ATP. These observations are consistent with our previous speculation that *rad50S* mutations, which map in the ATP binding domain of RAD50 protein but not in consensus residues, might affect release of bound RAD50 protein from DNA (14).

At high ionic strength, RAD50 protein exists as a single asymmetric homodimer. At lower ionic strengths, the physical properties of RAD50 protein change, with the quaternary structure becoming more heterogeneous and less asymmetric. This pattern of changes is intriguingly similar to that of phosphorylated smooth muscle myosin, which changes from an extended 6S monomeric form (two heavy chains and four light chains; Stokes radius 185 $\text{\AA}$) to a folded 10S monomeric species (Stokes radius 125 $\text{\AA}$) and then to a folded dimeric species (27, 28). Moreover, this myosin transition specifically involves a bend within the S-2 domain of the heavy chain. Perhaps Heptad II of RAD50 protein, which shows significant similarity to the S-2 domain (14), is involved in an analogous transition, with a bend occurring specifically at the hinge region of this domain. The structural and functional significance of the ionic strength transition for RAD50 protein remains to be determined. All of the DNA binding experiments reported above were carried out at intermediate concentrations of NaCl (27–67mM). In the myosin case, several observations are consistent with the possibility that the observed structural transitions are biologically significant (28). It may also be of interest to examine the effect of ATP on the physical properties of RAD50 protein.

Additional aspects of RAD50 function

RAD50 protein probably works in concert with one or more additional proteins *in vivo*. The products of the *MRE11* and *XRS2* genes are obvious candidates, since *rad50*, *mre11*, and *xrs2* mutants have virtually identical phenotypes (29, 30). Purified Mre11 protein also exhibits DNA binding activity (K.Jozuka and H.Ogawa, personal communication).

The possibility that RAD50 protein is capable of additional and/or more complex activities remains to be investigated. An important component of RAD50 function may be its ability to recruit to its site of binding other proteins involved in DSB recombination, e.g. other members of the *RAD50* series epistasis group. More speculatively, since the predicted length of extended coiled coil region for a pair of RAD50 proteins interacting along their lengths is about the distance between lateral elements of the synaptonemal complex, RAD50 protein might be localized to the SC (14). The sequence of the protein does not provide any indication of a complex enzymatic activity; it resembles neither a helicase (31) nor a member of the emerging RecA family (32–35).

Models for the role of RAD50 function *in vivo*

Two general features of *RAD50* function seem clear. First, *RAD50* function acts at an early stage of recombination, both for DSB-promoted events in mitotic cells (3) and during meiotic recombination (reviewed in 5). In both situations *RAD50* function acts prior to *RAD52* function as shown by mutant phenotypes and/or epistasis analysis. Second, *RAD50* function is absolutely required for meiotic recombination (reviewed in 5) but is less essential for mitotic DSB-promoted recombinational repair. This

latter point is shown by the fact that *rad50* mutants are only partially defective in HO-mediated recombination in non-*MAT* sequences (3), in HO switching at *MAT* (A.McKee and N.K. unpublished results), and in zygotic lethality induced by irradiation of a donor strain prior to conjugation with an unirradiated recipient strain (J.Nitiss, personal communication). Most strikingly, a *rad50* null mutant is fully proficient for integration of linear DNA fragments introduced by transformation (E.A. and N.K. unpublished results cited in 5). These genetic observations are most simply explained if RAD50 protein participates directly in DSB repair and meiotic chromosome metabolism (rather than acting solely as a transcription factor, for example) and we will assume that this is the case in the discussion that follows.

We have previously suggested that *RAD50* is defective in a genome-wide homology search that is required both for recombinational repair of DSBs in mitosis and during meiosis for recombination and chromosome pairing (5). The relatively modest defects of *rad50* mutants in mitotic recombinational repair made it seem unlikely that RAD50 protein is the primary 'homology search' enzyme itself. We proposed instead that RAD50 protein might facilitate a homology search by stripping off inhibitory 'chromatin' proteins or by changing the topological state of the DNA (5). This proposal was based on the fact that RAD50 acts early in recombination, on the notion (6) that meiotic recombination might normally be under negative control by virtue of 'chromatin structure', and the finding that *rad50* mutants exhibit no discernible defect in integration of (naked) linear transforming DNA but are significantly defective in DSB repair when breaks are induced endogenously. In addition, we suggested, RAD50 protein might help recruit other proteins to the site of a recombination event.

Upon further reflection, we find it especially intriguing that RAD50 protein must act *after* the occurrence of DSBs in mitotic cells but *before* the occurrence of DSBs during meiosis. This difference could easily account for the stronger defect of *rad50* mutants in meiosis as opposed to mitosis since RAD50 function is required for occurrence of a crucial initial event (the DSB) in the former case but not the latter. When we considered this paradox in light of the possibility that RAD50 protein might alter chromatin structure, we were led to the general proposal that there is something similar about the sites where DSBs are destined to occur in meiotic chromatin and the sites of endogenous DSBs in mitotic cells, and that RAD50 protein recognizes this crucially similar feature. In this case, we could refine our original model to suggest that RAD50 protein first recognizes the appropriate chromatin feature and then acts to convert the DNA at this site into a form that 'reveals the nucleic acid to the recombination machinery' (5) and is appropriate for later steps in the recombination pathway.

It is now possible to speculate specifically about the nature of such a feature in light of the recent provocative observation of a 1:1 correspondence between DNase hypersensitive sites in mitotic chromatin and sites at which DSBs occur in meiosis (T.-C.Wu and Michael Lichten, manuscript in preparation). Since DNase hypersensitive sites are usually presumed to be places where nucleosome octamers are absent (either due to absence or disassociation of nucleosomal proteins), an economical hypothesis would be that nucleosomes undergo an analogous dissociation or disappearance spontaneously in the immediate vicinity of a DSB, and that RAD50 protein binds to such regions in both situations.

What then would RAD50 do upon binding to an appropriate chromatin feature? We originally suggested that RAD50 might strip away inhibitory proteins. In light of the finding that DSBs occur at places in the DNA that are already sensitive, it is possible to imagine that RAD50 serves simply as a recruiting agent, nucleating interactions amongst other proteins without necessarily altering the structure of DNA any further. However, we still favor the notion that RAD50 directly affects DNA structure as we originally proposed, for several reasons:

(1) The strongest argument to this effect is the simple fact that RAD50 function (and other gene products) is (are) required for occurrence of meiosis-specific DSBs. Recent observations suggest that these DSBs are probably made by a non-specific nuclease which cleaves at preferred positions throughout a 100bp region (L.Xu and N.K. manuscript in preparation). RAD50 (et al.) must either further alter 'chromatin structure' at the site of the break to permit formation of these DSBs and/or alter expression of a DSB nuclease, presumably also by affecting chromatin structure.

(2) A direct effect of RAD50 on chromatin structure is further supported by the fact that meiotic recombination within the rDNA of yeast is *RAD50*-independent, but is instead dependent upon *SIR2* function, which is now known to be a modulator of chromatin structure (36, 37, 38).

The current observation that RAD50 binds DNA is certainly consistent with the notion that this protein affects chromatin or DNA structure. Furthermore, this observation makes it unlikely that the postulated effects of RAD50 on chromatin or DNA structure would be mediated solely by interactions with nucleosomes or other DNA-bound proteins; direct contact with the DNA should be involved. Also, our enthusiasm for the above model is increased by hints that binding of a single RAD50 molecule may nucleate loading on of additional molecules (15); such a process would be perfect for identification of a preferred DNA binding region by a single RAD50 molecule followed by enlargement of the susceptible region by binding of additional RAD50 molecules.

Once loaded onto DNA at an appropriate site, RAD50 protein might then play additional roles. Homology searching might occur in the context of the RAD50/DNA complex and/or additional proteins may be encouraged to bind to carry out subsequent steps.

Although the above discussion is very speculative, it provides a framework than can guide both *in vivo* and *in vitro* analysis in the future.

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