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Rad52 and Ku bind to different DNA structures produced early in double-strand break repair

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ABSTRACT

DNA double-strand breaks are repaired by one of two main pathways, non-homologous end joining or homologous recombination. A competition for binding to DNA ends by Ku and Rad52, proteins required for non-homologous end joining and homologous recombination, respectively, has been proposed to determine the choice of repair pathway. In order to test this idea directly, we compared Ku and human Rad52 binding to different DNA substrates. However, we found no evidence that these proteins would compete for binding to the same broken DNA ends. Ku bound preferentially to DNA with free ends. Under the same conditions, Rad52 did not bind preferentially to DNA ends. Using a series of defined substrates we showed that it is single-stranded DNA and not DNA ends that were preferentially bound by Rad52. In addition, Rad52 aggregated DNA, bringing different single-stranded DNAs in close proximity. This activity was independent of the presence of DNA ends and of the ability of the single-stranded sequences to form extensive base pairs. Based on these DNA binding characteristics it is unlikely that Rad52 and Ku compete as 'gatekeepers' of different DNA double-strand break repair pathways. Rather, they interact with different DNA substrates produced early in DNA double-strand break repair.

INTRODUCTION

The toxicity of DNA double-strand breaks in eukaryotic cells is reflected in the multiplicity of pathways to repair them. Double-strand break repair follows one of two general mechanistic routes, non-homologous end joining or homologous recombination (1). These two distinct mechanisms necessarily require distinct sets of proteins. Proteins specifically involved in homologous recombination were originally defined as products of genes belonging to the *RAD52* epistasis group in *Saccharomyces cerevisiae* (2). In human cells these include the homologous gene products: Rad51, Rad52, Rad54, XRCC2, XRCC3, Rad51B, Rad51C, Rad51D, as well as a complex including Rad50, Mre11 and Nbs1. Proteins specifically involved in non-homologous end joining include the Ku70/80 heterodimer (hereafter referred to as Ku), DNA-PK catalytic subunit (DNA-PKcs) and the XRCC4–ligaseIV complex (3). The complex of Rad50, Mre11 and Xrs2 (the yeast equivalent of Nbs1) also plays a role in non-homologous end joining in *S.cerevisiae* (3,4).

Any double-strand break repair reaction must necessarily begin with recognition of DNA ends. For non-homologous end joining this is likely to be accomplished by Ku. Ku is a structure-specific DNA binding protein. It requires a free end for binding but can then migrate along DNA (5,6). The mechanism of DNA end-binding and inward translocation became clear with the solution of the atomic level structure of Ku. A co-crystal of Ku bound to DNA revealed that the protein forms a ring with DNA passing through it (7). Biochemical analysis suggests that DNA end-binding by Ku initiates a cascade of molecular events that leads to joining of the broken DNA ends. In this scenario, DNA-PKcs joins an end-bound Ku (8,9) which then leads to synapsis of the DNA ends (10). After processing to produce ligatable ends, if necessary, the XRCC4–ligaseIV complex completes the repair of the break (11).

A DNA end that will eventually be repaired by homologous recombination must be specifically processed to expose a single-stranded 3′ overhang. It is not clear if this end processing is the first step of homologous recombination repair or if some homologous recombination-specific protein first binds an unprocessed DNA end. Homologous recombination proteins that have been described to bind DNA ends include the Rad50–Mre11 complex and Rad52. The Rad50–Mre11 complex can bind to linear and circular DNA but assembles large oligomers only on linear DNA (12). The formation of Rad50–Mre11 oligomers on DNA with different end structures is modulated by ATP binding. ATP binding increases the preference for Rad50–Mre11 oligomer formation on DNA with 3′ overhangs (13). Rad50–Mre11 oligomers can tether DNA molecules and this could function at an early stage in double-strand break repair, perhaps a stage common to both non-homologous end joining and homologous recombination, to keep ends in close proximity for further processing (12).

Rad52 can also bind DNA ends; however, the importance of Rad52 to double-strand break repair goes beyond initial DNA end binding. *In vitro*, Rad52 has been shown to bind to both single-stranded and double-stranded DNA (14). Rad52 interacts with both RPA and Rad51, proteins also required for

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homologous recombination. Due to these interactions, Rad52 is described as a mediator in the formation of Rad51-directed formation of joint molecules between the broken DNA end and the double-stranded template DNA (15–19). Rad52 aggregates DNA and promotes annealing of complementary single strands (20). The full-length human protein forms heptameric rings alone and when bound to DNA (14,21). The DNA binding domain of Rad52 crystallized as an undecameric ring and its atomic level structure was solved (22,23). Single-stranded DNA modeled onto the undecameric ring structure has the bases exposed and available for pairing (23). The precise register with which some single-stranded DNA substrates bind to Rad52 and Rad52 protection of DNA ends from nuclease digestion have been interpreted to indicate a specific mechanistic role for Rad52 in double-strand break repair that requires its binding to DNA ends (24,25). This has lead to the prominent proposal that Rad52 is the 'gatekeeper' of homologous recombination (25,26). In this model, the binding of Rad52 to DNA ends precludes non-homologous end joining and begins a cascade of events culminating in repair by homologous recombination.

A competition between Ku and Rad52 for DNA end binding has been suggested to provide a mechanistic switching point between repair by non-homologous end joining and homologous recombination (25,26). We wanted to test the interesting prediction that Ku and Rad52 compete for binding to DNA ends. The binding of Ku to DNA and the binding of Rad52 to DNA have been described using a variety of techniques but not directly compared with any of them. Notably, many Rad52–DNA binding studies are done *in vitro* in conditions with relatively low concentrations of monovalent cations and lacking magnesium ions (14,24,25,27). These factors can have a dramatic effect of DNA–protein interactions and these conditions are very different from those used in Ku–DNA binding studies. In order to understand the relative DNA binding properties of these two proteins, Ku and Rad52, we have compared their interactions with a variety of DNA substrates under the same conditions. Using scanning force microscopy (SFM; also called atomic force microscopy) to visualize DNA–protein complexes we could simultaneously determine the percentage of DNA bound by protein and the position at which the protein was bound. In this way we could define DNA features preferentially bound by Rad52 and Ku, showing that these proteins bind to different DNA structures and do not compete for binding to similar structures.

MATERIALS AND METHODS

DNA substrates

Plasmid pDERI1, used in this study, is a 1821 bp derivate of pUC19 (28). Substrates with blunt ends and short 5′ or 3′ overhangs were made by linearization of pDERI1 with ScaI, BsaI or PvuI digestion, respectively. Singly nicked plasmid was obtained by digesting supercoiled pDERI1 (17 µg/ml) with DNase I (1 µg/ml) in a 30 µl reaction mixture containing 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$ and 360 µg/ml ethidium bromide. The reaction was carried out at 30°C for 30 min and stopped by the addition of a 0.1 vol of stop solution containing 5% (w/v) SDS, 50 mM EDTA, 30 µg/ml proteinase K and incubation at 65°C for 30 min.

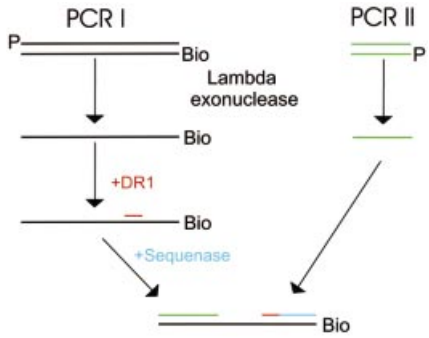

Figure 1. Schematic representation of the steps in synthesis of the DNA substrate with an internal single-stranded gap. The 810 bp PCR I DNA, shown in black at the left, formed the basis for building up the complete product. It was first digested with lambda exonuclease III to produce the bottom strand, to which oligo DR1, red, was hybridized and used as a primer for synthesis of double-stranded DNA, blue, toward one end. The 313 bp PCR II DNA, shown in green at the right, was identical in sequence to the first 313 bp of PCR I but has a 5′ phosphate on the opposite strand as PCR I. Digestion of PCR II with lambda exonuclease left a 313 nt single-stranded DNA complementary to the PCR I bottom strand. Upon annealing, this resulted in the final product with blunt ends and an internal 200 nt single-stranded gap.

DNA was purified by extraction with phenol and phenol/chloroform (1:1, v/v), precipitated with ethanol and dissolved in H_2O (glass-distilled; Sigma).

Relaxed covalently closed DNA was prepared by treatment of plasmid pDERI1 with calf-thymus topoisomerase I (Amersham). Reaction mixtures of 30 µl contained 1 µg of DNA, 35 mM Tris–HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% bovine serum albumin and 1.5 U topoisomerase I and were incubated at 37°C for 1 h. The reaction was stopped by the addition of 0.1 vol of stop solution containing 5% (w/v) SDS, 50 mM EDTA, 30 µg/ml proteinase K and incubated at 65°C for 30 min. After extraction with phenol and phenol/chloroform, DNA was purified over a GFX™ column (Amersham).

Substrates with a long 5′ overhang were produced as follows: plasmid pDERI1 was cut with XmnI and treated with a pre-determined saturating amount of *Escherichia coli* exonuclease III (10 U/µg DNA) for 20 min at 20°C in a 75 µl reaction mixture containing 66 mM Tris–HCl (pH 8.0) and 0.66 mM $MgCl_2$ (44 µg of DNA, 440 U exoIII). Exonuclease treatment produced 5′ single-stranded tails with an average length of 200 nt as determined by measuring the contour length of the remaining double-stranded DNA from SFM images. Subsequent digestion of DNA with long 5′ tails with AlwNI produced a substrate with a long 5′ tail at one end and a short restriction site overhang at the other end (6 nt 3′).

Linear DNA with a gap was made as follows (see schematic outline in Fig. 1): using the *URA*3 gene from *S.cerevisiae* as template DNA, an 810 bp PCR fragment was produced using primer U3 which was 5′ phosphorylated (GAAGGAAGAAC-GAAGGAAGGAGC) and primer Bio 5′ which was 5′ biotinylated (TTTCCCGGGGGGCCCGGGTTCTATACTG-TTGACCC). The PCR product was purified on a GFX™ column (Amersham). The DNA strand with a 5′-phosphate was digested by λ exonuclease (5 U/µg DNA). The reaction was carried out at 37°C for 1 h and stopped by heat

inactivation (95°C for 10 min). The resulting single-stranded DNA was dialyzed against TE buffer and hybridized with oligonucleotide primer DR1 (AGCGGTTTGAAGCAGG-CGGCGG anneals to position 526–548 of the PCR I bottom strand, see Fig. 1). Primer DR1 was extended for 30 min at 37°C in a reaction containing Sequenase™ DNA polymerase Version 2.0 (Amersham), 40 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$, 200 μM dNTPs and 50 mM NaCl. The partially double-stranded linear DNA produced with a long 3′ overhang was purified on a GFX™ column. A second PCR fragment of 313 bp, identical to the part of the 810 bp fragment described above, was produced using primer U3 (GAAGGAAGAAC-GAAGGAAGGAGC) and primer B313 which was 5′ phosphorylated (TTTTAGTAAACAAATTTTGGGACC). The PCR product was purified on a GFX™ column, before incubation with λ exonuclease (5 U/μg DNA) as described above, in order to digest the phosphorylated strand. The resulting single-stranded DNA was hybridized with partially double-stranded 810 nt long linear DNA with a long 3′ overhang produced as described above. The resulting linear DNA, with a 213 nt gap between 313 and 283 bp of double-stranded DNA with blunt ends, was resolved on a 2% agarose gel and purified from gel with a GFX™ column.

Proteins and DNA-binding reactions

Ku70/80 (Ku80, Ku70-his tag) was produced in baculovirus-infected Sf21 cells and purified as described (29). The human Rad52 protein was produced in *E.coli* FB810 carrying pET28a-hRad52 (30) and purified as described (16), with the following modification: a Ni^{2+}-NTA agarose column (Qiagen) was used for the first purification step, followed by a MonoQ column (Pharmacia). Protein purity was checked by SDS–PAGE and peak fractions with Rad52 were stored at –80°C.

Complexes of proteins and DNA were prepared in reaction mixtures (10 μl final volume) containing 24 μM DNA (concentration of nucleotides), 90 nM protein, 20 mM HEPES–KOH (pH 7.4), 30 mM KCl, 1 mM DTT and, if present, 10 mM $MgCl_2$. Reactions were carried out at 37°C for 15 min and then placed on ice. For SFM imaging, reaction mixtures were diluted 15–30 times in deposition buffer (5 mM HEPES–KOH pH 7.8, 5 mM $MgCl_2$) and deposited on freshly cleaved mica. After 30 s, the mica was rinsed with H_2O (glass distilled; Sigma) and dried with a stream of filtrated air. Images were obtained on a NanoScope IIIa or NanoScope IV (Digital Instruments; Santa Barbara, CA) operating in tapping mode in air with a type E scanner using silicon Nanotip cantilevers (Nanoprobes).

RESULTS

In order to compare Ku and Rad52 binding to different features of DNA we constructed a variety of defined substrates with the same sequence and length but different structure (shown schematically in Fig. 2). Linear DNA, 1.8 kb in length, with either blunt ends or short 3′ or 5′ single-stranded overhang ends was produced by restriction digestion of plasmid pDERI1. DNA with long 5′ single-stranded overhangs was produced from the same plasmid by digestion with a restriction enzyme followed by limited digestion with *E.coli* exonuclease III. Relaxed covalently closed circular and singly nicked circular forms of plasmid pDERI1 were also produced. A substrate with a defined central single-stranded gap, whose sequence is not the same as plasmid pDERI1, was also produced. Binding reactions with Ku and Rad52 were performed under the same conditions with respect to buffer components and molar amounts of protein and DNA. Importantly, the binding reactions analyzed by SFM did not contain a vast molar excess of protein as is common in binding reactions analyzed by biochemical methods. The DNA concentration was chosen to result in deposition of a sufficient density of molecules for convenient analysis. The concentration of protein was kept to a slight molar excess over DNA fragments in order to visualize complexes without prior purification away from free protein. All of the DNA–protein binding reactions were done with DNA concentrations of 24 μM with respect to nucleotides and protein concentrations of 90 nM Rad52 monomers or Ku70/80 heterodimers. This is equivalent to an ~14-fold molar excess of protein over DNA molecules for the 1.8 kb substrates and an ~6-fold molar excess of protein over DNA molecules for the 810 bp gapped substrate.

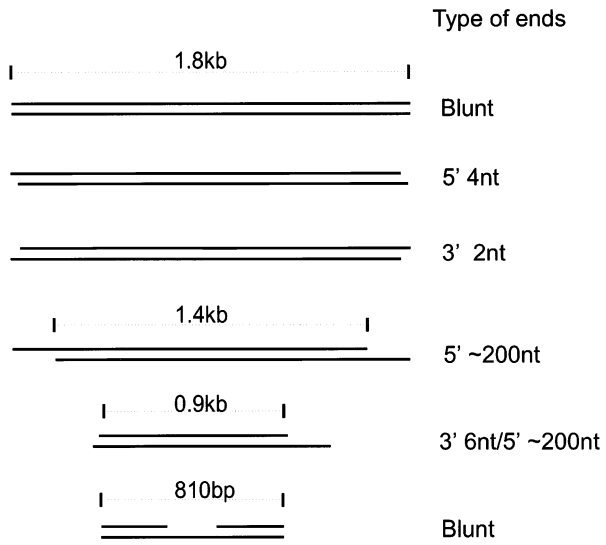

Figure 2. Diagram of the DNA substrates used in this study. The length as well as type of ends for each DNA is indicated. All but the substrate with the internal 213 nt gap were derived from 1.8 kb plasmid pDERI1. The substrate with the internal 213 nt gap was derived from the *S.cerevisiae URA*3 gene.

From the SFM images we determined the percentage of DNA molecules bound by protein for each of the different DNA substrates. The results for the 1.8 kb pDERI1-derived DNA substrates are summarized in Table 1. These DNA substrates had either no ends or interruptions, a single nick, blunt ends, short single-stranded overhang ends or long single-stranded ends. Rad52 binding reactions were done in two different conditions, one without magnesium ions for comparison with the most previous published studies and one with the same buffer, including magnesium ions, used for Ku–DNA binding (see Materials and Methods for details). As expected, Ku showed preferential binding to linear DNA with a similar percentage of linear DNA bound by Ku, 28–37% of DNA bound by protein, independent of the DNA end structure.

Table 1. Percentage of different DNA substrates bound by protein

DNA substrate	Ku	Rad52 – Mg^{2+}	Rad52 + Mg^{2+}
Linear blunt	28.5 (1180)	26.7 (409)	8.9 (190)
Linear 2 nt 3′	27.7 (1014)	48.5 (330)	19.8 (304)
Linear 4 nt 5′	34.0 (113)	24.4 (522)	17.4 (172)
Linear 200 nt 5′	36.6 (175)	25.7 (678)	18.5 (427)
Circle relaxed	8.8 (512)	29.6 (412)	12.5 (331)
Circle nicked	6.7 (430)	24.0 (58)	16.7 (102)

The percentage of DNA molecules bound by protein is listed for each reaction. The number in parenthesis is the total number of DNA molecules counted. Binding reactions with Rad52 were done in buffers without or with magnesium ions. The number of molecules counted was pooled from several independent experiments. No significant inter-experiment differences were noted.

Much less of the circular DNA, 7–9%, was bound by Ku. In contrast, Rad52 bound a similar percentage of all DNA substrates irrespective of the presence of an end. There was an ~2-fold preference for binding to DNA with a short 3′ single-stranded overhang (see Discussion). When Rad52 binding reactions were done in the same buffer used for Ku binding reactions, which most notably differed in the presence of magnesium ions, the percentage of protein-bound DNA dropped for all substrates. Again, under these conditions there was no evidence for Rad52 preferentially binding to DNA substrates with ends. The percentage of DNA bound by protein was even a bit higher for circular DNA than for some of the linear substrates. There were, however, distinct qualitative differences in the protein–DNA complexes formed by Rad52 on the different DNA substrates. Rad52 is presumed to function as a large DNA-bound oligomer. Notably, large DNA-bound Rad52 oligomers only formed on the DNA with a long single-stranded end (Fig. 3A). Rad52 bound to all other DNA substrates as small complexes, presumably monomeric in size (Fig. 3B).

For all binding reactions with linear DNA, the position of the DNA-bound proteins was also analyzed. The percentage of protein located at an end or at an internal position for the various substrates is summarized in Table 2. As is well known from other studies, Ku requires an end for DNA association but does not remain bound to ends. Here also we observed

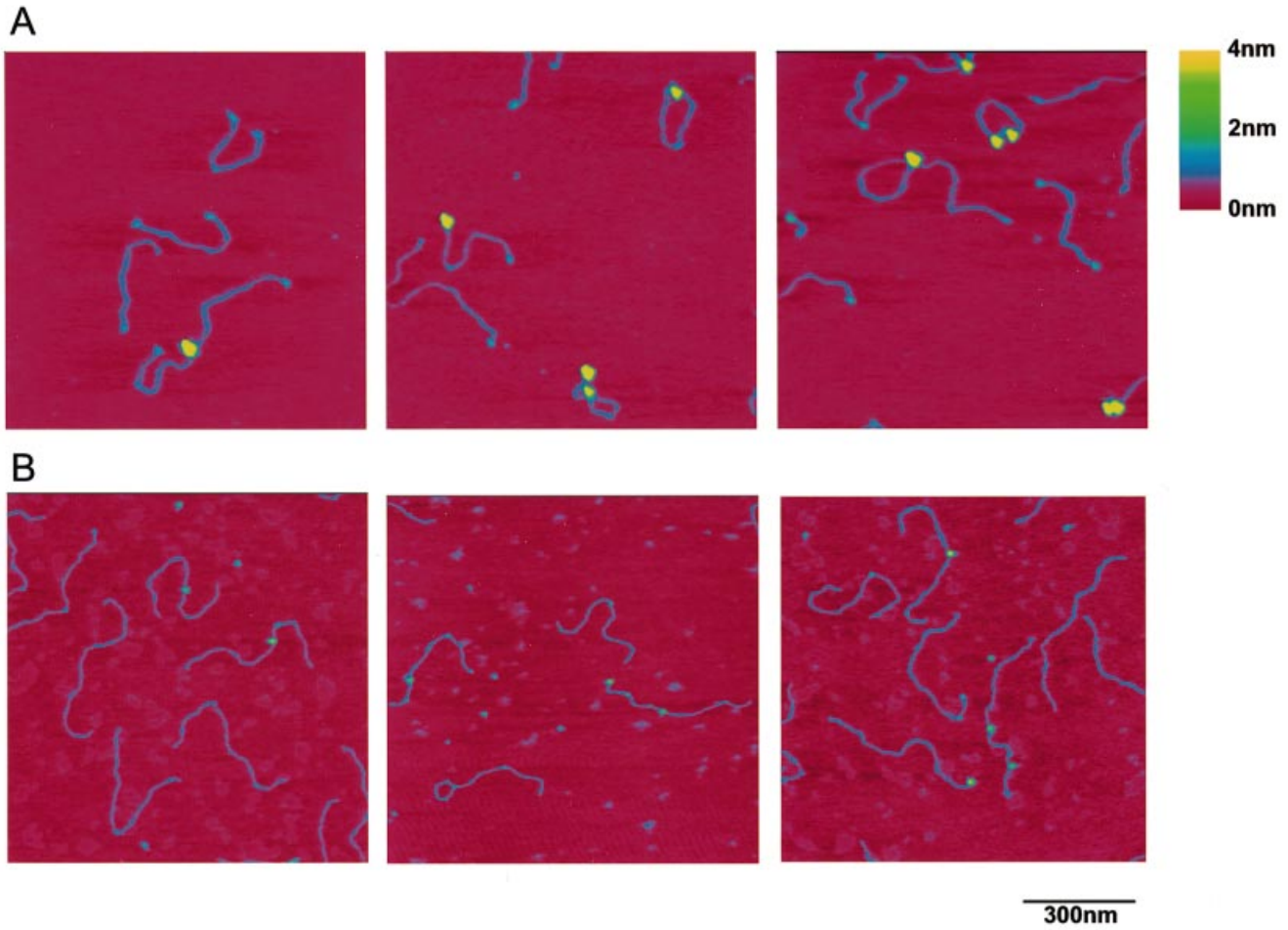

Figure 3. Human Rad52 forms large oligomers on long single-stranded DNA. (**A**) SFM images showing large Rad52 complexes formed in binding reactions including DNA with long single-stranded ends. The long single-stranded DNA ends not bound by protein appear as a small knob at the end of the substrate. (**B**) SFM images showing small Rad52 complexes formed in binding reactions including DNA with blunt ends (left), short 5′ overhang ends (middle) and short 3′ overhang ends (right). The scale bar indicates 300 nm in the *X* and *Y* dimensions and height is represented by color as shown by the bar at the right.

Table 2. Percentage of DNA–protein complexes with protein bound at an end

DNA substrate	Ku	Rad52 – Mg^{2+}	Rad52 + Mg^{2+}
Linear blunt	6.0 (336)	18.2 (109)	5.9 (17)
Linear 2 nt 3′	11.0 (281)	24.8 (160)	28.3 (60)
Linear 4 nt 5′	14.3 (38)	29.0 (127)	26.7 (30)
Linear 200 nt 5′	88.2 (64)	100.0 (174)	100.0 (79)

The percentage of DNA–protein complexes that have protein bound to an end is listed for each binding reaction. Data is from the same experiments as presented in Table 1. The total number of DNA–protein complexes for each data point is given in parenthesis.

only 6–14% of DNA-bound protein present at an end if it was blunt or a short single-stranded overhang. There was no difference in the size or structure of Ku located at DNA ends or at internal positions. The observation that most of the DNA-bound Ku remained at an end on the substrate with long single-stranded overhangs was at least in part due to the appearance of single-stranded DNA in these SFM experiments. Presumably due to irregular secondary structure and a very short persistence length, the single-stranded DNA appeared as a small knob at the end of the remaining double-stranded segment (e.g. see the protein-free DNA ends in Fig. 3A and protein-free DNA with a single-stranded gap in Fig. 5B). Thus, protein bound to any position along the single-stranded DNA appeared to be at an end of the double-stranded DNA segment. It is also possible that translocation of Ku along single-stranded DNA is inhibited by irregular DNA secondary structure.

DNA-bound Rad52 had no general preference for an end position on DNA with blunt or short single-stranded DNA ends, either in the presence or absence of magnesium ions (Table 2). In the presence of magnesium ions, ~20–30% of the protein–DNA complexes had Rad52 bound to a DNA end. However, if a long single-strand overhang was available, all of the Rad52 was found bound to the DNA ends. As mentioned above, the Rad52 structures on the long single-stranded DNA ends were also very different from those bound to double-stranded DNA. All of the Rad52 bound to long single-stranded DNA was in the form of large oligomers (Fig. 3A). Direct competition experiments were also done in which either Ku or Rad52 was combined with a mixture of DNA substrates; nicked circular, linear blunt-ended and linear with one long single-stranded end (Fig. 4). When presented with this choice of DNA substrates, the results were similar to those obtained with the DNA substrates used one at a time (Table 3). Ku was bound almost exclusively to the linear DNA substrates with more protein remaining at an end on the DNA with a long single-stranded overhang. Rad52 was found exclusively on the DNA with a long single-stranded end in large oligomeric complexes.

We wished to determine which feature of the DNA structure was most important for Rad52 binding, the molecular end or the single-stranded nature. For this purpose, we produced DNA substrates with different combinations of end structures and single-stranded regions. These substrates had either two blunt ends and an internal single-stranded gap or one end with a 6 nt single-stranded overhang and one end with a long, ~200 nt, single-stranded overhang (Fig. 5). Binding reactions with Ku or Rad52 were performed in the same buffer conditions, those including magnesium ions, and the location of the bound protein on the DNA determined. These results, presented as the percentage of DNA–protein complexes with protein bound at different locations, single-stranded or double-stranded regions at an end or internal position, are summarized in Table 4. Here also, Rad52 was bound exclusively to the single-stranded region, independent of the presence of a DNA end. As expected, most of the Ku had migrated to internal positions on the DNA. Again, an increased percentage of Ku was found at the single-stranded end, which may indicate that Ku is less able to translocate over long stretches of single-stranded DNA.

Rad52 has been described to have a single-stranded DNA annealing activity (20), it promotes the annealing of complementary DNAs and large Rad52 complexes have been visualized joining DNAs with long single-stranded regions (27). We also observed multiple DNA molecules joined within large Rad52 protein complexes; 42% of the protein-bound DNA with long single-stranded ends was in complexes including more than one DNA molecule (Fig. 5A). In some cases, the protein complexes joining two or more DNA molecules were obviously larger than those formed on a single DNA molecule (e.g. see Figs 3A and 5B). However, from these images we cannot determine if multiple DNAs were captured by a Rad52 oligomer or if Rad52 oligomers bound to different DNAs aggregated. In either case, single-stranded DNAs were brought into close proximity, which would presumably favor base pairing if that were possible. In our experiments, the different single-stranded DNA molecules to which Rad52 bound were identical and not complementary, demonstrating that extensive base-pairing interactions are not important for Rad52-induced DNA aggregation. Importantly, DNA ends were not required as Rad52-induced DNA aggregation was observed with a similar frequency with the internally gapped substrate, 30% of the protein-bound DNA was in complexes including more than one DNA molecule (Fig. 5B).

DISCUSSION

We have directly compared the ability of Ku and Rad52 to bind to DNA substrates with different end structures. Instead of a comparable binding preference for the same DNA structures, which would be required if these proteins compete in binding to broken DNA ends, we observe that Ku and Rad52 preferentially bound to different DNA structures. As expected, Ku bound to DNA with a free end. Rad52, however, had no apparent preference for binding to DNA ends above internal double-stranded positions on DNA. Rad52 did preferentially bind to single-stranded DNA and notably formed large oligomers on long single-stranded DNA independent of end or internal location on DNA fragments. We did not attempt mixing both proteins with a given DNA substrate. In SFM images, objects are distinguished by their size and shape. We would expect Rad52 monomers to appear smaller than a Ku heterodimer. However, Rad52 is known to form multimers, which would likely be larger than Ku. Based on size alone, it would be impossible to determine if a DNA-bound complex consisted of Rad52 multimers or a combination of Rad52 and Ku. However, because we did not observe

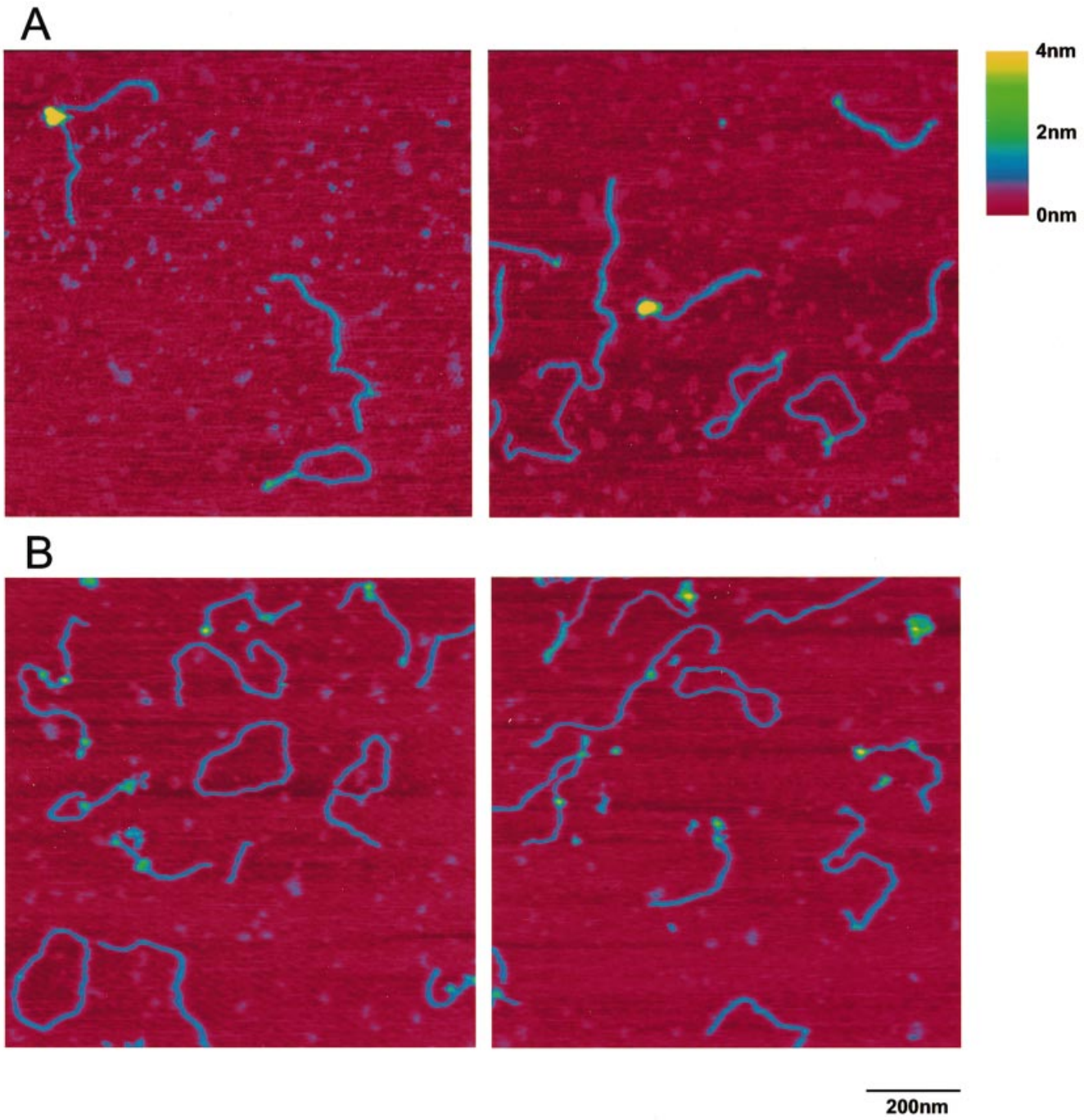

Figure 4. Competition for binding to a mixture of DNA substrates by human Rad52 or Ku. (**A**) SFM images from binding reactions including Rad52 and a mixture of 1.8 kb nicked circular DNA, 1.8 kb linear blunt-ended DNA and 0.9 kb DNA with a 200 nt single-stranded end. Rad52 bound exclusively to the long single-stranded DNA as large oligomers. (**B**) SFM images from binding reactions including Ku and the same mixture of DNA substrates as in (A). Ku bound almost exclusively to the linear DNA substrates independent of the end structure. The scale bar indicates 200 nm in the *X* and *Y* dimensions and height is represented by color as shown by the bar at the right.

any preference for Rad52 binding to DNA ends in the absence of Ku, any observable change in Rad52 binding to DNA ends would likely be positive, indicating cooperative activity rather than competition.

Given the binding characteristics we have observed it seems highly unlikely that there is a competition between Ku and Rad52 for binding to the same broken DNA. Thus, these two proteins probably do not function as a molecular switching point between the non-homologous end joining and the homologous recombination repair pathways. Ku associates with DNA via ends and Rad52 associates with single-stranded DNA. The choice of double-strand break repair via either non-homologous end joining or homologous recombination may in part be determined by the structure of the end at a DNA break.

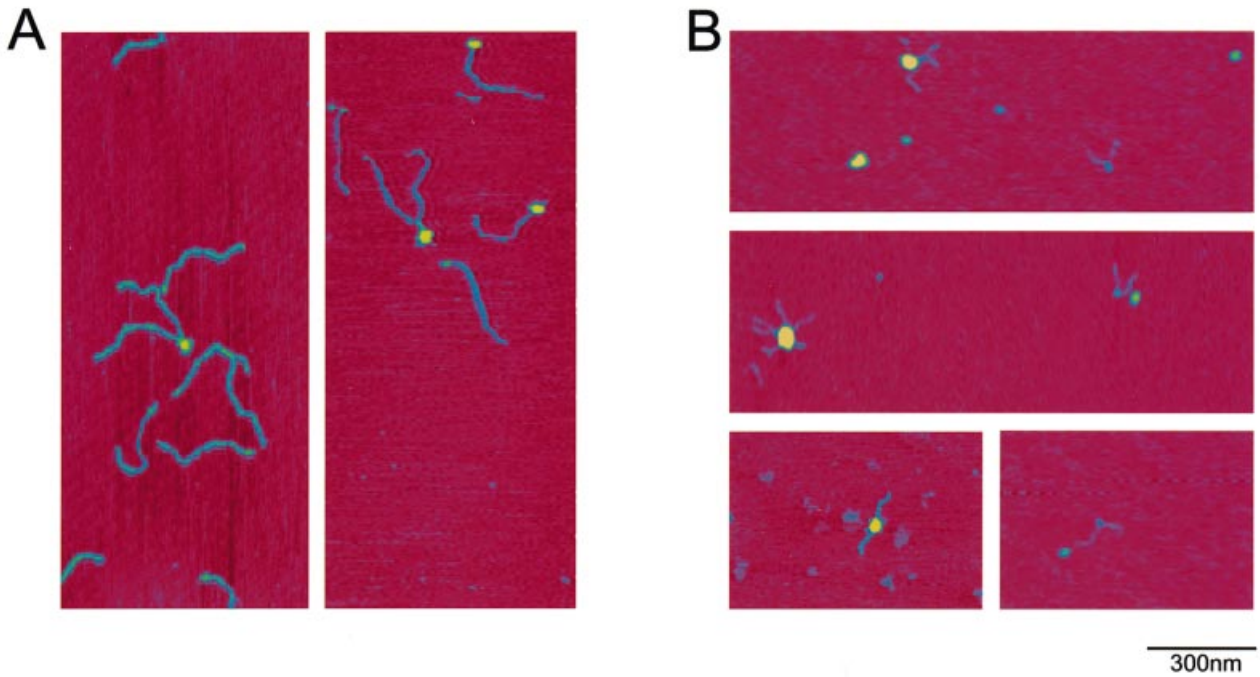

Figure 5. Human Rad52 complexes often aggregate single-stranded DNA. (**A**) SFM images from binding reactions including Rad52 and DNA with one long single-stranded end. Large Rad52 oligomers form on the single-stranded end of the DNA and often aggregate more than one DNA molecule. (**B**) SFM images from binding reactions including Rad52 and DNA with a central single-stranded gap. Large Rad52 oligomers form on the central single-stranded region of this DNA and often aggregate more than one DNA molecule. The scale bar indicates 300 nm in the *X* and *Y* dimensions, height ranges from 0 to 4 nm and is represented by color (red to yellow as in the scale bars of Figs 3 and 4).

Table 3. Competition between different DNA substrate for binding Rad52 or Ku

	Percentage of complexes formed on different DNA substrates	
DNA substrate	Rad52	Ku
Linear blunt	0 (0/94, 736)	61 (146/240, 395)
Linear 200 nt 5′	100 (94/94, 276)	39 (93/240, 259)
Circle nicked	0 (0/94, 146)	0.4 (1/240, 74)

The percentage of protein–DNA complexes on the different DNA substrates is listed. In parenthesis is the number of DNA–protein complexes on the given substrate over the total number of DNA–protein complexes, and the total number of DNA molecules counted for each substrate.

For instance, two DNA ends produced by breaking a chromosome can be effectively joined by non-homologous end joining. However, end joining would be impossible, and homologous recombination would be required, for repairing single DNA ends that occur when DNA damage is encountered during replication (31). Factors, such as the point in the cell cycle at which a DNA break occurs and the availability of a homologous partner for repair, are also likely to influence the choice of pathway used for repair of a double-strand break (32).

The preference for binding to long stretches of single-stranded DNA by Rad52 suggests that nucleolytic processing to form long single-stranded regions at a DNA break precedes Rad52 binding in the homologous recombination mechanism. The Rad52 monomers that bind to short single-stranded DNA ends may also be mechanistically important, though most models of Rad52 function involve large DNA-bound oligomers (19,24,26,33). It is interesting to note that the only DNA end that was preferentially bound by human Rad52 in our experiments was a short 3′ overhang, though this preference is diminished in binding reactions including magnesium ions. One striking feature of Rad52 binding to single-stranded DNA is the apparent specific phasing with respect to the end of some substrates (24). This would require a specific interaction at an end to initiate the phasing for which our results with short 3′ overhangs may provide some evidence. The preference for binding to a 3′ end is also interesting because double-strand break repair by homologous recombination requires joint molecule formation by a single-stranded DNA with a 3′ overhang. The preferential binding of Rad52 to 3′ ends provides an intriguing entry point for further analysis of the role of this protein in homologous recombination.

The *in vitro* activities described for Rad52 can be accommodated in at least two mechanistic pathways of homology-dependent DNA repair, homologous recombination and single-strand annealing. The Rad52-mediated aggregation of single-stranded DNA that we observed here, and previously described by others (25,27), is obviously consistent with a role in single-stand annealing. However, because the aggregation of single-stranded DNA did not require either complementary sequences or DNA ends, the relevance of *in vitro* annealing to *in vivo* repair mechanisms remains to be proven. The importance of Rad52 for homologous recombination is

Table 4. Location of protein bound to DNA with a terminal or central single-stranded region

Protein	Schematic of substrates	Single-stranded end	Blunt end	Single-stranded internal	Double-stranded internal
Ku		25	5	NA	70
Rad52		100	0	NA	0
Ku		NA	10.4	19.8 (+22.8)[a]	47 (+22.8)[a]
Rad52		NA	0	100	0

The percentage of DNA–protein complexes with protein located at the indicated position is listed for each binding reaction. All binding reactions were done in the presence of magnesium ions. The gapped DNA has a 213 nt single-stranded region between 283 and 313 bp of duplex DNA. Of this DNA substrate, 31.3% was bound by protein in a reaction containing Ku. Of the gapped substrate, 19.1% was bound by protein in a reaction containing Rad52. NA, not applicable.

[a]Of the DNA bound by Ku, 22.8% had protein at multiple sites, both single-stranded internal and double-stranded internal positions.

underscored by its interactions with several proteins involved in the process. *In vitro*, a careful balance between RPA, Rad51 and Rad52 is needed for optimal joint molecule formation (15,17,33,34). We expect that direct imaging experiments will help elucidate the way Rad52, RPA and Rad51 act together with DNA to eventually produce Rad51 filaments active in joint molecule formation.

We often observed single-stranded DNAs joined by Rad52 oligomers. In some cases the size of the protein complexes aggregating DNA appeared larger than those formed on a single DNA molecule [e.g. Fig. 5B and examples in Van Dyck *et al.* (27)] implying that aggregation occurred via interaction of multiple DNA-bound Rad52 complexes. However, in other cases the DNA was aggregated by Rad52 complexes that were no larger than those formed on single DNA molecules (e.g. Fig. 5A). This raises the possibility that additional DNA molecules could be captured by a DNA-bound Rad52 oligomer. The model of DNA bound to a Rad52 oligomer based on the atomic level structure places the DNA in a large grove encircling the complex (23). The proposed DNA binding groove is an iteration of identical DNA binding domains of Rad52 monomers and does not *a priori* require complete occupancy by a single DNA. The possibility that multiple DNA molecules could bind to a Rad52 oligomer suggests a function in keeping different DNA molecules in close proximity, to favor annealing of complementary sequences if present. In addition, this may allow for dynamic rearrangement of the single-stranded DNAs if they can exchange positions among the identical binding sites of the complex. Describing the dynamic interactions between Rad52 and single-stranded DNA will be essential to understanding the mechanistic role of this protein in DNA double-strand break repair.

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