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Purification and characterization of the human Rad51 protein, an analogue of *E.coli* RecA

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In bacteria, genetic recombination is catalysed by RecA protein, the product of the *recA* gene. A human gene that shares homology with *Escherichia coli recA* (and its yeast homologue *RAD51*) has been cloned from a testis cDNA library, and its 37 kDa product (hRad51) purified to homogeneity. The human Rad51 protein binds to single- and double-stranded DNA and exhibits DNA-dependent ATPase activity. Using a topological assay, we demonstrate that hRad51 underwinds duplex DNA, in a reaction dependent upon the presence of ATP or its non-hydrolysable analogue ATPγS. Complexes formed with single- and double-stranded DNA have been observed by electron microscopy following negative staining. With nicked duplex DNA, hRad51 forms helical nucleoprotein filaments which exhibit the striated appearance characteristic of RecA or yeast Rad51 filaments. Contour length measurements indicate that the DNA is underwound and extended within the nucleoprotein complex. In contrast to yeast Rad51 protein, human Rad51 forms filaments with single-stranded DNA in the presence of ATP/ATPγS. These resemble the inactive form of the RecA filament which is observed in the absence of a nucleotide cofactor.
Key words: ATPase/DNA repair/genetic recombination/ nucleoprotein filaments

Introduction

Bacterial *recA* mutants are defective in genetic recombination and are sensitive to agents that promote DNA damage (Clark and Margulies, 1965). Biochemical studies show that the product of the *Escherichia coli recA* gene, RecA protein (M_r 37 842), plays a central role in recombination, since it catalyses homologous pairing and strand exchange reactions between two DNA molecules *in vitro* (reviewed in Cox and Lehman, 1987; Kowalczykowski, 1991; Radding, 1991; West, 1992). RecA is also an integral component of the cellular SOS response to DNA damage (Walker, 1985) and acts as an allosteric effector in the cleavage of LexA repressor (Smith *et al.*, 1991). RecA-mediated self-cleavage of LexA results in the expression of ~20 genes, most of which are directly involved in DNA repair and recombination. These properties make RecA protein one of the most intensively studied proteins in recent biology.

In addition to its catalytic function in genetic recombination, RecA provides the structural framework for homologous pairing (Howard-Flanders *et al.*, 1984). In the presence of ATP, RecA binds DNA (either single-stranded or gapped duplex) to form a right-handed helical nucleoprotein filament (West *et al.*, 1980; Stasiak *et al.*, 1981; Flory and Radding, 1982; Stasiak and DiCapua, 1982). Electron microscopic observations of filaments formed on duplex DNA, in the presence of ATP or a non-hydrolysable ATP analogue (ATPγS), show that the DNA is underwound as the pitch of the helix is extended to ~95 Å (Stasiak *et al.*, 1981). Thus, DNA is stretched from a 3.4 Å axial rise per base pair in B-form DNA to a 5.1 Å rise per base pair in the RecA–DNA complex. A similar stretching occurs with single-stranded DNA. This extended and regular filament structure, which contains 6.2 RecA monomers per turn of DNA (18.6 bp) (DiCapua *et al.*, 1982; Yu and Egelman, 1990), represents the active form of RecA with regard to ATPase, strand exchange activity and repressor cleavage. A second, non-extended, form of the RecA filament has been observed with single-stranded DNA in the absence of a nucleotide cofactor, though this structure is thought to be inactive (Yu and Egelman, 1992; Egelman and Stasiak, 1993; Ruigrok *et al.*, 1993). The crystal structure of the RecA helical polymer has been solved at a resolution of 2.3 Å, and resembles the inactive filament (Story and Steitz, 1992; Story *et al.*, 1992; Egelman and Stasiak, 1993).

The primary function of the RecA filament is to bring two DNA molecules into close proximity within a single filament, such that the DNA sequences are aligned ready for strand exchange and the formation of heteroduplex DNA (Howard-Flanders *et al.*, 1984; Stasiak *et al.*, 1984). This fundamental process may be highly conserved since: (i) all bacterial RecA proteins are highly related (Roca and Cox, 1990), (ii) a bacteriophage recombination protein (T4 UvsX protein) forms nucleoprotein filaments similar to those made by RecA (Yu and Egelman, 1993) and promotes homologous pairing and strand exchange reactions *in vitro* (Harris and Griffith, 1987; Kodadek *et al.*, 1988), and (iii) structural analogues of *recA* have been described in lower eukaryotes such as *Saccharomyces cerevisiae* (*RAD51*, *DMC1*) (Aboussekhra *et al.*, 1992; Bishop *et al.*, 1992; Shinohara *et al.*, 1992), *Schizosaccharomyces pombe* (*RAD51* = *rhp51*$^+$) (Muris *et al.*, 1993; Shinohara *et al.*, 1993) and *Neurospora crassa* (*mei-3*) (Cheng *et al.*, 1993).

The existence of structural *recA* analogues such as *RAD51*, *DMC1* and *mei-3*, is particularly interesting since mutations in these genes give rise to phenotypic properties that are consistent with a significant defect in DNA recombination and repair. For example, *S.cerevisiae rad51* mutants are sensitive to ionizing irradiation and are defective in DNA damage-induced mitotic recombination

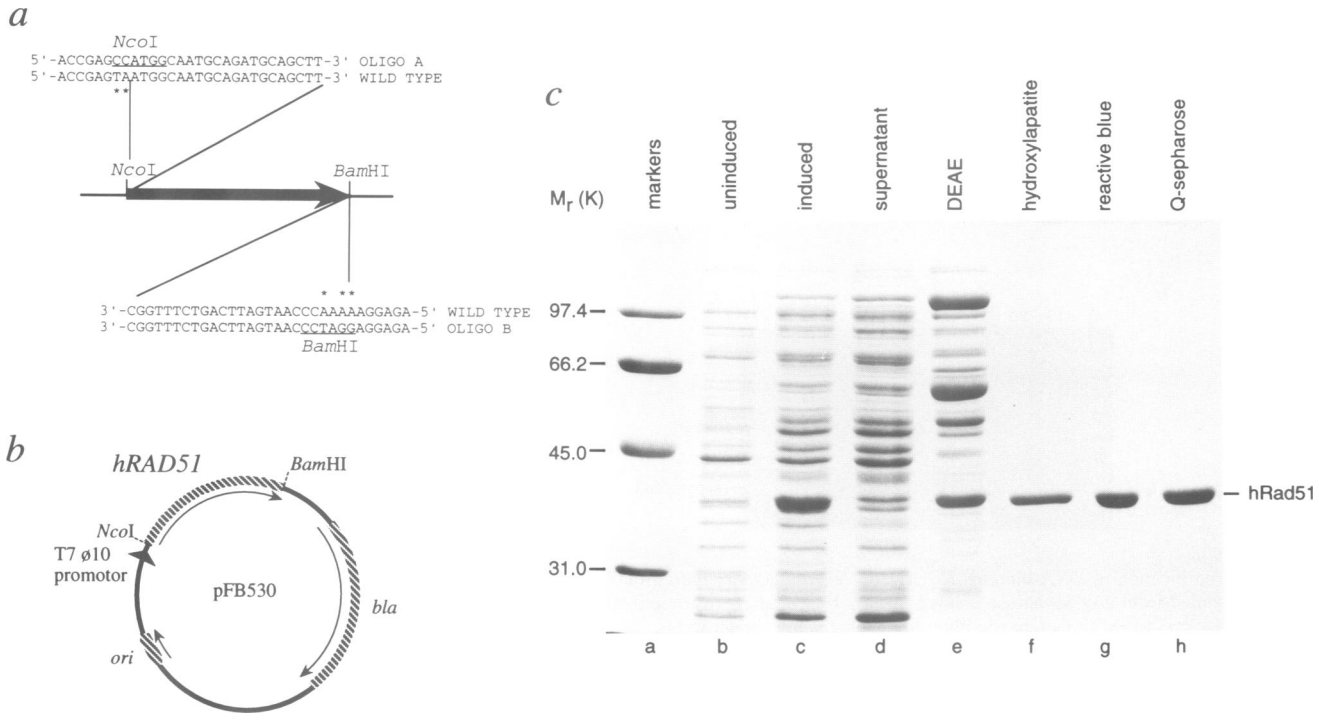

Fig. 1. Cloning of human *RAD51* gene and purification of hRad51 protein from *E.coli*. (**a**) The *RAD51* coding sequence was amplified by PCR from a human testis λgt11 cDNA library using oligos A (5′ of *RAD51*) and B (3′ of *RAD51*). These differ from the wild-type sequence as indicated by asterisks. (**b**) The PCR fragment was cloned into pET11d via the *Nco*I and *Bam*HI restriction sites to generate the plasmid pFB530. (**c**) SDS−PAGE showing the overexpression and purification of hRad51 protein from *E.coli*. Lane a, M_r standards; lanes b and c, cellular proteins before and after (4 h) induction with isopropyl-β-D-thiogalactoside (IPTG); lane d, soluble proteins after high-speed spin (fraction I); lanes e−h, fractions eluted from DEAE−Biogel (fraction II), hydroxylapatite (fraction III), reactive blue−agarose (fraction IV) and Q-Sepharose (fraction V), respectively; lane h contains 2 μg of purified hRad51 protein. The 10% polyacrylamide gel was stained with Coomassie blue.

(Game, 1983). During meiosis, *rad51* mutants accumulate double-strand breaks at recombination hotspots (Shinohara *et al.*, 1992). These observations point to an inability to promote the recombinational repair of broken chromosomal DNA. The *S.cerevisiae* Rad51 protein was recently shown to polymerize on duplex DNA to form a helical filament nearly identical in low resolution three-dimensional structure to that formed by RecA (Ogawa *et al.*, 1993). In this filament, the DNA was again elongated to form a structure with an axial rise of 5.1 Å per base pair and 18.6 bp per turn. However, unlike RecA protein, yeast Rad51 did not form filaments with single-stranded DNA (Ogawa *et al.*, 1993). In recent studies, the yeast Rad51 protein was shown to promote homologous pairing and strand exchange reactions *in vitro*, confirming that it is functionally analogous to RecA (Sung, 1994).

Remarkably, RecA structure may also be conserved in higher organisms since *RAD51*-like genes were identified recently in chicken (Bezzubova *et al.*, 1993), mouse (Morita *et al.*, 1993) and humans (Shinohara *et al.*, 1993; Yoshimura *et al.*, 1993). The human *RAD51* gene is transcribed at high levels in thymus, spleen, testis and ovary (Shinohara *et al.*, 1993) and encodes a putative 339 amino acid protein (M_r 36 966).

The experiments described here show that the human cDNA homologous to *recA/RAD51* produces a 37 kDa ATPase that binds single-stranded or duplex DNA to form nucleoprotein filaments. The filaments formed with nicked duplex DNA appear by electron microscopy to be almost indistinguishable from those formed by the *E.coli* RecA protein. In contrast, we observe that human Rad51 binds single-stranded DNA or superhelical duplex DNA in a manner different from that observed with either *E.coli* RecA or *S.cerevisiae* Rad51.

Results

Cloning of the human RAD51 gene from a testis cDNA library

The human *RAD51* gene was isolated from a testis cDNA library by the polymerase chain reaction (PCR) using flanking primers (Figure 1a) and cloned into the pET11d expression vector. The resulting plasmid, designated pFB530 (Figure 1b), contained *hRAD51* under control of the T7 ϕ10 promoter. Confirmation that pFB530 carried the wild-type *hRAD51* coding sequence was obtained by DNA sequencing.

Purification of hRad51 protein

To allow the overexpression of the *hRAD51* gene product, plasmid pFB530 was transformed into *E.coli* strain FB810, a *recA*$^-$ derivative of BL21(DE3). When FB810 pLysS carrying plasmid pFB530 was grown and treated with isopropyl-β-D-thiogalactoside (IPTG), the overexpression of a 37 kDa protein was observed by SDS−PAGE (Figure 1c, lane c). To purify this protein, 4 l of cells were induced with IPTG and, after 4 h, the cell paste was collected and the cells lysed by three cycles of freezing and thawing in the presence of 0.1% Triton X-100. Cell debris and insoluble materials were removed by high speed centrifu-

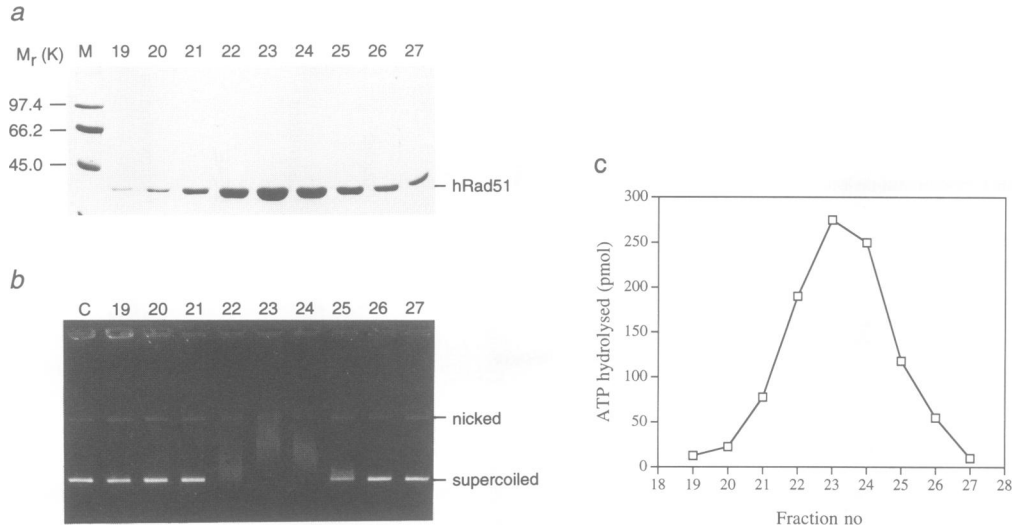

Fig. 2. Co-purification of DNA binding activity and ATPase activity with hRad51 protein. (**a**) 10% SDS−PAGE of peak hRad51-containing fractions 19–27, as eluted from a Q-Sepharose column using a KCl gradient (as described in Materials and methods). The 37 kDa hRad51 protein was visualized by staining with Coomassie blue. M_r standards are shown in lane M. (**b**) DNA binding activity of fractions eluted from Q-Sepharose. Lane C, control reaction to which no protein was added. Binding complexes were analysed by electrophoresis through a 1% agarose gel as described in Materials and methods. (**c**) ssDNA-dependent ATPase activity of Q-Sepharose fractions. Reactions were carried out as described in Materials and methods.

gation. The 37 kDa protein was then purified using DEAE−Biogel, hydroxylapatite, reactive blue−agarose and Q-Sepharose column chromatography (Figure 1c, lanes d−h). Although some of the overexpressed protein was lost through insolubility, we were able to purify 4 mg of homogeneous protein. The identity of the 37 kDa protein was confirmed as hRad51 by sequencing the first 21 N-terminal amino acid residues.

DNA binding and ATPase activity of hRad51 protein

During purification, we observed that hRad51 protein co-migrated with a DNA binding activity. To demonstrate this, samples from the final column (Q-Sepharose) were assayed for the ability to bind double-stranded circular DNA. We observed a single peak of DNA binding activity, as detected by a simple agarose gel band-shift assay (Figure 2b). DNA binding activity co-eluted precisely with the hRad51 protein peak, as detected by SDS−PAGE (Figure 2a). DNA binding was observed with supercoiled and relaxed circular ϕX174 duplex DNA. hRad51 protein also bound single-stranded circular ϕX174 DNA (ssDNA) (data not shown). The stable binding of hRad51 to ssDNA and dsDNA, as determined by this band-shift assay, preferred the presence of Mg^{2+} ions (≥1 mM), but did not depend upon a nucleotide cofactor since protein−DNA complexes were observed with ATP, ATPγS or without nucleotide cofactor (data not shown).

The peak fractions from Q-Sepharose were also assayed for ATPase activity. We observed a ssDNA-dependent ATPase activity which co-migrated with the peak of hRad51 protein (Figure 2c). Similar levels of ATPase activity were observed with superhelical duplex DNA (data not shown).

ATP-dependent underwinding of DNA by hRad51

When *E.coli* RecA protein binds duplex DNA in the presence of a nucleotide cofactor, it forms a nucleoprotein filament in which the DNA is extended and underwound (Stasiak and DiCapua, 1982). Since hRad51 binds duplex DNA *in vitro*, we next wished to determine whether, like RecA, it also promotes DNA underwinding. The method we used was based on a topological assay in which RecA-mediated DNA underwinding produces (+) superhelical turns which can be relaxed by eukaryotic topoisomerase I (Iwabuchi *et al.*, 1983). Upon deproteinization, the DNA is seen by agarose gel analysis as a highly negatively supercoiled product, defined as form X DNA (Figure 3a).

In control reactions, the binding of RecA to ^{32}P-labelled supercoiled DNA was carried out by pre-incubation at a low concentration of $MgCl_2$ followed by a shift up to 13 mM (Iwabuchi *et al.*, 1983). Addition of wheat germ topoisomerase I led to the formation of highly supercoiled form X DNA as expected (Figure 3b, lane c). The mobility of the form X DNA was greater than that of native supercoiled DNA (lane a), as shown by agarose gel electrophoresis following deproteinization. In the absence of RecA, the supercoiled DNA was relaxed by the topoisomerase to form a ladder of topoisomers (lane b).

Similar reactions were carried out with purified hRad51 protein, except that DNA binding could be carried out at 13 mM $MgCl_2$ rather than the two-step method needed for RecA. We observed that hRad51 also promoted the formation of form X DNA (Figure 3b, lane d). This reaction occurred in the presence of ATP (lane d) or the non-hydrolysable ATP analogue, ATPγS (lane f). Thus hRad51, like RecA, promotes ATP-dependent DNA underwinding.

In the absence of a nucleotide cofactor (Figure 3b, lane e), or in the presence of ADP (lane g), we did not observe the formation of highly supercoiled form X DNA. Under these conditions, the DNA ran at, or close to, the position of supercoiled DNA. Since binding studies indicate that hRad51 binds DNA in the absence of a nucleotide cofactor (data not shown), these results indicate that the protein protects the DNA from topoisomerase action or promotes

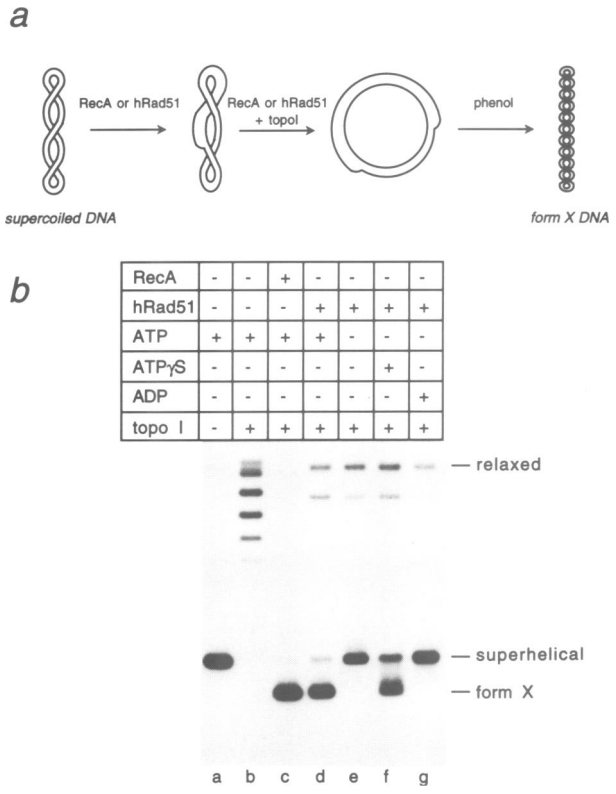

Fig. 3. Human Rad51 protein promotes ATP-dependent unwinding of DNA. Unwinding reactions were carried out as described in Materials and methods. hRad51 (8.25 μM), ATP (2 mM), ATPγS (0.5 mM) or ADP (0.5 mM) were included as indicated. Incubation was for 40 min at 37°C, then wheat germ topoisomerase I was added, as indicated, followed by a further incubation for 10 min at 37 °C. To permit RecA binding (lane c), it was necessary to modify the reaction such that RecA protein (30 μM) was pre-bound to the DNA in low Mg^{2+}. The $MgCl_2$ concentration was then increased to 13 mM and incubation continued for a further 10 min prior to topoisomerase addition. Following deproteinization, DNA products were analysed by electrophoresis through a 1% agarose gel.

partial DNA underwinding under these conditions. Further studies will be required to determine the nature of this DNA product.

To determine the stoichiometry of the hRad51−DNA interaction, the topological assay was performed using increasing amounts of hRad51 (at 2 mM ATP). In this experiment, we observed that a protein:DNA ratio of one hRad51 monomer per 2–3 bp of DNA was required to convert all the DNA into form X product (Figure 4, lanes h and i). The human Rad51 protein therefore binds DNA with a stoichiometry very similar to that shown by the *E.coli* RecA protein (DiCapua *et al.*, 1982).

hRad51 forms filaments with relaxed duplex DNA

When the human Rad51 protein was incubated with small (~1200 bp) nicked duplex DNA molecules in the presence of ATP, followed by stabilization with ATPγS, we observed the presence of nucleoprotein filaments by electron microscopy following negative staining (Figure 5, panel b). The hRad51−DNA complexes appeared very similar to those formed by *E.coli* RecA (panel a), since they exhibited the characteristic striated appearance of RecA−DNA complexes and because the double-stranded DNA was stretched to a similar extent in both cases.

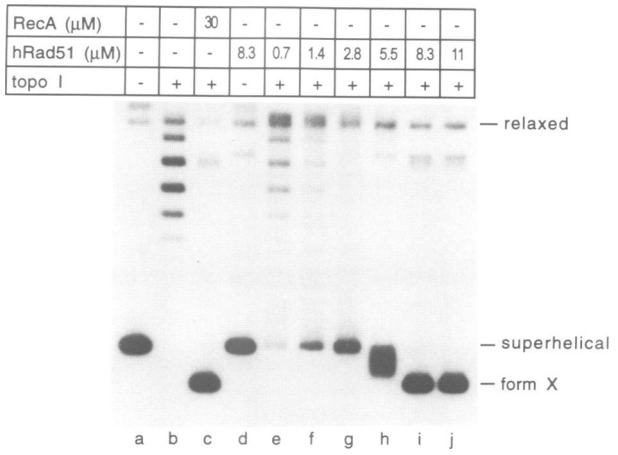

Fig. 4. Stoichiometry of the hRad51−DNA interaction. Standard unwinding reactions were carried out and analysed essentially as described in the legend to Figure 3 in the presence of 2 mM ATP, except that the initial incubation was for 10 min prior to topoisomerase treatment. The presence or absence of each protein, and the concentrations of hRad51 and RecA were as indicated. The DNA concentration was 30 μM (defined in nucleotides).

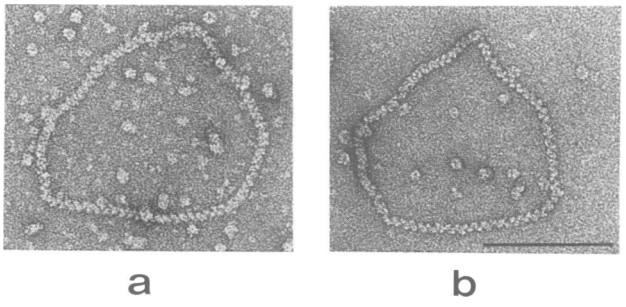

Fig. 5. Human Rad51 protein binds nicked circular DNA to produce nucleoprotein complexes similar to those formed by *E.coli* RecA protein. (**a**) Complexes formed by RecA. (**b**) Complexes formed by hRad51. Reaction mixtures (50 μl) containing 19 μM nicked circular duplex pM plasmid DNA (~1200 bp in length), 40 mM triethanolamine acetate pH 6.8, 2 mM $MgCl_2$, 2 mM ATP and RecA (6 μM) or hRad51 (6 μM) were incubated for 10 min at 37°C. ATPγS was then added to 1 mM and incubation was continued for a further 30 min. Complexes were fixed with glutaraldehyde and viewed after negative staining. The magnification bar denotes 100 nm.

The binding of RecA and hRad51 to nicked circular φX174 duplex DNA was also visualized, as shown in Figure 6 (panels a and b, respectively). The similarity of the two nucleoprotein complexes is quite striking. Preliminary measurements of the contour length of the DNA indicate that hRad51 stretched the DNA to ~2.55 μm, indicating an average axial rise of 4.7 Å (naked B-form DNA with an axial rise of 3.4 Å would be equivalent to a contour length of 1.8 μm). Under the same *in vitro* conditions, RecA protein produced a contour length of 2.66 μm (axial rise 5.0 Å). Even at saturating concentrations of hRad51, we found that many molecules were 5–10% shorter than similar complexes formed with RecA. Although this result may indicate that hRad51 extends the DNA to a lesser extent than RecA, it is likely that the observed extension by hRad51 is an underestimate since some regions of the hRad51−DNA filament showed a

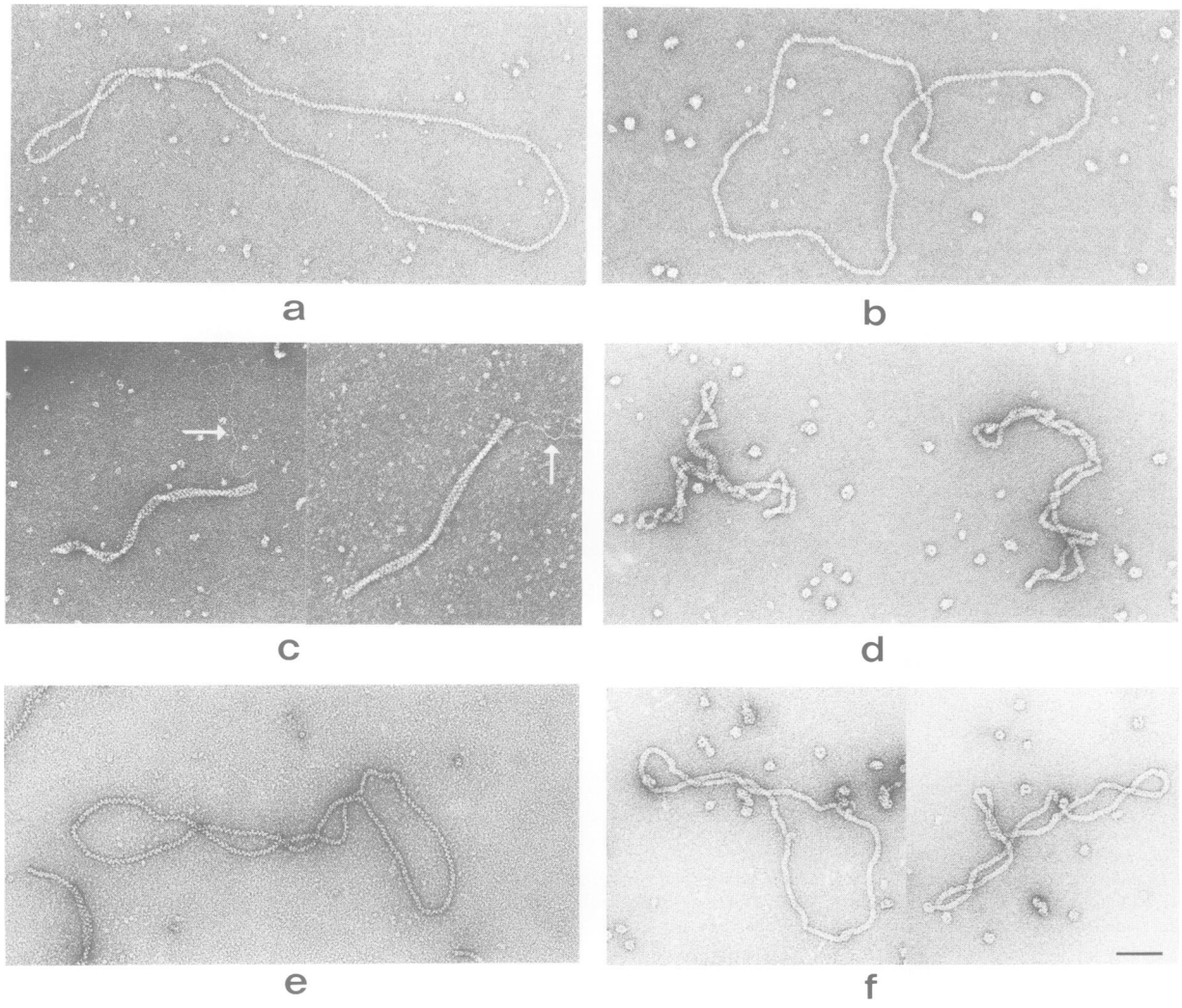

Fig. 6. Electron microscopic visualization of RecA and hRad51 filaments on double- and single-stranded ϕX174 DNA. The three panels on the left (**a**, **c** and **e**) show complexes made by RecA whereas those on the right (**b**, **d** and **f**) were made with human Rad51 protein. Complexes were formed on either nicked circular duplex (a and b), superhelical (c and d) or single-stranded (e and f) DNA. Reactions containing DNA (16 μM), RecA or hRad51 (6 μM for complexes with dsDNA and 12 μM for complexes with ssDNA) and 25 mM triethanolamine acetate pH 6.8, 1 mM magnesium acetate and 0.5 mM ATP were incubated for 5 min at 37°C and then stabilized by incubation with 2 mM ATPγS for 30 min at 37°C. Complexes with dsDNA were fixed with glutaraldehyde whereas those with ssDNA were not. Electron microscopy was carried out as described in the legend to Figure 5. In (c), the arrows indicate naked highly supercoiled DNA. The magnification bar denotes 100 nm.

less striated appearance than elsewhere, indicating that the DNA may be incompletely covered by protein.

Interaction of hRad51 with superhelical DNA

When hRad51 was reacted with negatively supercoiled DNA (Figure 6d), the complexes were quite different from those formed by RecA (Figure 6c). As expected, the binding of RecA to supercoiled DNA was limited by the torsional stress caused by extensive underwinding (Stasiak and DiCapua, 1982). As a consequence, the molecules adopted a folded, or side-by-side conformation, as two RecA-covered segments adhered laterally, with a region of naked DNA visible as a highly supercoiled tail (Figure 6c, arrows). In contrast, the Rad51 protein was able to cover the supercoiled DNA completely to form highly twisted molecules. These results indicate that the hRad51 protein was unable to fully underwind the supercoiled DNA due to the constraints imposed by torsional stress.

hRad51 forms filaments with single-stranded DNA

In contrast to the yeast Rad51 protein, human Rad51 formed filaments on single-stranded circular ϕX174 DNA in the presence of ATP/ATPγS (Figure 6f). However, these filaments were not as extended as similar filaments formed by RecA protein (panel e). The contour length of the hRad51−ssDNA filament was 1.47 ± 0.07 μm (n = 14) compared with 2.31 ± 0.12 μm for the RecA−ssDNA filaments. Indeed, the electron microscopic visualization of these complexes indicates that they resemble the inactive form of the RecA filament which are formed in the absence of a nucleotide cofactor.

Discussion

Similarity of human Rad51 to E.coli RecA and S.cerevisiae Rad51

The results presented here establish that the 37 kDa hRad51 protein, purified from a cloned human testis

cDNA, is analogous to the *E.coli* RecA protein. DNA binding studies show that hRad51 binds single- or double-stranded DNA, and that duplex DNA is underwound by hRad51 in a reaction that requires the presence of a nucleotide cofactor (either ATP or ATPγS). DNA binding by hRad51 appears to be cooperative and the DNA is saturated at one hRad51 monomer per 2–3 bp of duplex DNA. While these results point to a similarity between human Rad51 and *E.coli* RecA, the most compelling evidence for their remarkable similarity was provided by an electron microscopic visualization of hRad51–DNA complexes formed with nicked circular DNA. We found that hRad51 filaments were similar to those formed by RecA and exhibited the striated appearance characteristic of RecA filaments. Length measurements of hRad51 on nicked circular dsDNA molecules, formed in the presence of ATP or ATPγS, indicated that the DNA was underwound and extended, thus accounting for the formation of form X DNA in the topological assay.

DNA binding characteristics of hRad51 are different from E.coli RecA or yeast Rad51

Differences between hRad51 and RecA were observed when we visualized their binding to either supercoiled duplex or single-stranded DNA. We found that hRad51 was able to bind and fully cover the supercoiled DNA, whereas the binding of RecA was limited by the torsional stress caused by extensive underwinding (Stasiak and DiCapua, 1982). These results indicate that hRad51 is capable of exhibiting two modes of DNA binding to duplex DNA: in the absence of torsional stress, hRad51 binds to DNA and promotes extensive DNA unwinding, whereas binding under torsionally constrained conditions results in the formation of a non-extended form of the filament.

Differences were also observed between human Rad51 and yeast Rad51. Unlike the yeast Rad51 protein (Ogawa *et al.*, 1993), human Rad51 bound single-stranded DNA to form a nucleoprotein filament. However, the DNA within this filament was not extended to the same extent as that within the RecA–ssDNA complex. Our initial observations indicate that the hRad51–ssDNA filament more closely resembles the compact filament which RecA forms on ssDNA in the absence of a nucleotide cofactor. The reason why the yeast and human Rad51 proteins differ in their ability to bind ssDNA remains to be elucidated.

Interaction of hRad51 with ATP

DNA unwinding by hRad51 required the presence of ATP or the non-hydrolysable ATP analogue ATPγS. These results indicate that nucleotide binding rather than hydrolysis is necessary for the formation of the extended filament observed with dsDNA. At the present time however, little is known about the nucleotide binding characteristics of hRad51. The protein contains the conserved nucleotide binding domains A and B (Walker *et al.*, 1982) identical to those found in *S.cerevisiae* Rad51 and related to those in *E.coli* RecA (Yoshimura *et al.*, 1993). We have observed that hRad51 exhibits a DNA-dependent ATPase activity with either single- or double-stranded DNA. However, the rate of hydrolysis is lower than that observed with RecA, leading us to suggest that a reduced affinity for nucleotide cofactors may account for the differences observed by electron microscopy.

Conservation of filament structure: from bacteria to man

It is now becoming clear that the structure of RecA protein has been conserved from bacteria to man, indicating that biological mechanisms revealed during studies of RecA may also be applicable to higher eukaryotes. Overall, the human Rad51 sequence shows 56% homology (30% identity) with *E.coli* RecA. However, despite their similarity in size, the homology only extends from residues 33 to 240 in RecA, since hRad51 lacks the C-terminal region of RecA while containing 63 extra residues at the N-terminus of the protein (Shinohara *et al.*, 1993; Yoshimura *et al.*, 1993). C-terminal deletions in RecA are known to retain biochemical activity (strand exchange and repressor cleavage) (Rusche *et al.*, 1985; Benedict and Kowalczykowski, 1988; Tateishi *et al.*, 1992) indicating that the conserved regions are responsible for recombination, UV resistance and filament formation (Ogawa *et al.*, 1992, 1993).

Previous work showed that the bacteriophage T4 UvsX protein, the *E.coli* RecA protein and the *S.cerevisiae* Rad51 protein form nucleoprotein filaments with DNA. Moreover, all three proteins exhibited similar protein–DNA binding stoichiometries, cooperative DNA binding and ATP-dependent extension of DNA (Ogawa *et al.*, 1993; Yu and Egelman, 1993). Most importantly, all three proteins have now been shown to promote homologous pairing and strand exchange reactions *in vitro* (Kowalczykowski and Eggleston, 1994; Sung, 1994). The human Rad51 protein exhibits very similar physical and biochemical characteristics and can now be grouped with these prokaryotic and lower eukaryotic recombination proteins.

Although the human Rad51 protein is analogous to UvsX, RecA and yeast Rad51 protein, all of which are known to play important roles in genetic recombination, the precise cellular role of hRad51 remains to be elucidated. However, high level expression of mammalian *RAD51* mRNA has been observed in testis and ovary, reproductive organs that are active in meiotic recombination (Bezzubova *et al.*, 1993; Shinohara *et al.*, 1993; Yoshimura *et al.*, 1993). High level expression also occurs in thymus and spleen, tissues that contain dividing B and T lymphocytes, and it is possible that hRad51 plays a role in V(D)J rearrangements during lymphoid-specific recombination or antibody class switching. Future studies will focus on biochemical assays that will determine the function of hRad51.

In conclusion, we suggest that continued biochemical analyses of the human and yeast Rad51 proteins will yield important insights, not only into the generality of mechanisms of homologous pairing and strand exchange, but may also lead to significant advances in the use of homologous recombination as a means of gene targeting in mammalian cells.

Materials and methods

Proteins and DNA

Restriction enzymes and φX174 DNA were obtained from New England Biolabs. *E.coli* RecA protein was purified by modification of a published

procedure (Cox *et al.*, 1981). Protein M_r standards were obtained from Bio-Rad Labs. Wheat germ topoisomerase I and the 4.4 kb plasmid pDEA1, which was uniformly labelled with ^{32}P, were prepared as described (Adams *et al.*, 1994). The plasmid pM is a derivative of pAN13 (Sambrook *et al.*, 1989) and was provided by Dr B.Seed (Massachusetts General Hospital). For some experiments, pM and φX174 duplex DNA were nicked by treatment with DNase I. Amounts of DNA are expressed in moles of nucleotides.

Cloning and purification of human Rad51 protein

The *RAD51* coding sequence was amplified by PCR from a human testis λgt11 cDNA library (Clontech) using two synthetic oligonucleotides (Friedman *et al.*, 1988). Oligo A (5′ of *RAD51*) and oligo B (3′ of *RAD51*) differ from the wild-type sequence (Shinohara *et al.*, 1993; Yoshimura *et al.*, 1993) at several bases and are shown in Figure 1. Polymerase chain reactions were carried out using *Taq* polymerase (Boehringer Mannheim). The PCR fragment was cloned into pET11d (Studier *et al.*, 1990) via the *Nco*I and *Bam*HI restriction sites to generate the plasmid pFB530. Confirmation that pFB530 carried the wild-type *hRAD51* coding sequence was obtained by DNA sequencing.

Plasmid pFB530 was grown in *E.coli* strain FB810, a *recA*$^-$ derivative of BL21(DE3). It was constructed by introducing *recA269*::Tn*10* into BL21(DE3) by P1 transduction from N3072 (Lloyd *et al.*, 1987) with selection for tetracycline resistance.

The human Rad51 protein was purified as follows. Four litres of *E.coli* strain FB810 pLysS carrying pFB530 were grown in Luria broth containing 100 μg/ml carbenicillin and 25 μg/ml chloramphenicol to an absorbance at 650 nm of 0.45, and induced by the addition of IPTG followed by a further 4 h incubation at 37°C. Bacteria were harvested by centrifugation and resuspended in 100 ml of 0.1 M Tris−HCl pH 8.0, 2 mM EDTA, 5% glycerol, and frozen rapidly in a dry ice/ethanol bath. Cells were lysed by three cycles of freeze−thaw in the presence of 1 mM dithiothreitol (DTT) and 0.1% Triton X-100, and insoluble material was removed by centrifugation at 40 000 r.p.m. for 1 h in a Beckman 45 Ti rotor. A significant amount of the hRad51 protein was lost at this step due to insolubility. The soluble protein fraction (Fraction I; ~1 g in 70 ml) was dialysed overnight against 2×2 l of R buffer (20 mM Tris−HCl, pH 8.0, 1 mM EDTA, 0.5 mM DTT, 10% glycerol) containing 0.05 M KCl, and loaded onto an 80 ml DEAE−Biogel A (Bio-Rad) column equilibrated with the same buffer (flow rate 40 ml/h). Bound proteins were eluted with an 800 ml linear gradient of KCl (0.05–0.6 M) in R buffer. Fractions containing the 37 kDa hRad51 protein, which eluted at 0.2–0.3 M KCl were identified by SDS−PAGE. Pooled fractions (fraction II) were dialysed for 2×2 h against P buffer (0.1 M potassium phosphate pH 6.8, 10% glycerol, 0.5 mM DTT). The protein (~100 mg) was then loaded onto a 20 ml hydroxylapatite (Bio-Rad) column (flow rate 30 ml/h), equilibrated with P buffer. Bound proteins were eluted with a 200 ml linear gradient of 0.1–0.8 M potassium phosphate pH 6.8, containing 10% glycerol, 0.5 mM DTT, at a flow rate of 12 ml/h. Fractions containing hRad51, which eluted at ~0.4 M phosphate were identified by SDS−PAGE, pooled, diluted 1:1 with R buffer containing 0.1 M KCl and dialysed against 2–2 l of the same. Fraction III (~10 mg) was then applied to a 5 ml reactive blue 4−agarose (Sigma) column at a flow rate of 30 ml/h and bound proteins were eluted with a 50 ml linear gradient of 0.1–1.0 M KCl in R buffer. hRad51 eluted as a broad peak between 0.5 and 0.8 M KCl and was pooled and dialysed against 2×2 l of R-buffer containing 0.05 M KCl. Fraction IV (~6 mg) was then applied to a 3 ml Q-Sepharose (Pharmacia) column equilibrated in the same buffer, and bound proteins eluted with a 30 ml linear gradient of 0.05–0.6 M KCl in R buffer. Fractions containing hRad51 were identified by SDS−PAGE, pooled, dialysed against storage buffer (20 mM Tris−HCl pH 8.0, 2 mM EDTA, 50% glycerol, 0.5 mM EDTA) and stored at −70°C (Fraction V). The final yield of hRad51 was ~4.2 mg (2 ml of 2.1 mg/ml), determined using bovine serum albumin (BSA) as standard (Bio-Rad protein assay kit).

DNA binding assay

Binding reactions (30 μl) containing double-stranded circular φX174 DNA (20 μM) and 2 μl of each fraction to be analysed, were incubated for 10 min at 37°C in 30 mM Tris−HCl pH 7.5, 2 mM DTT, 2 mM ATP, 10 mM $MgCl_2$ and 100 μg/ml BSA. To each sample, 5 μl of sample loading buffer (40 mM Tris−acetate, pH 8.0, 1 mM EDTA, 50% glycerol and a trace of bromophenol blue) was added, and binding complexes were analysed by electrophoresis through a 1% agarose gel in Tris−acetate/EDTA buffer. DNA and DNA−protein complexes were visualized by ethidium bromide staining.

ATPase assay

Reactions (25 μl) contained single-stranded φX174 DNA (120 μM), 20 mM Tris−HCl pH 7.5, 2 mM DTT, 0.1 mM ATP, 2 mM $MgCl_2$, 100 μg/ml BSA and 2.5 μl of each fraction from Q-Sepharose. Each reaction contained 6.6 nCi [α-^{32}P]ATP. Incubation was for 30 min at 37°C, then 1 μl was spotted directly onto CEL 300 PEI/UV_{254} (Polygram) thin layer chromatography plates. The plates were developed in 1 M formic acid/0.5 M LiCl. The amount of ATP hydrolysed was determined from dried plates using a Molecular Dynamics Model 425E PhosphorImager. Quantitation was carried out using ImageQuant software.

DNA unwinding assay

Standard unwinding reactions (20 μl) contained supercoiled ^{32}P-labelled pDEA1 DNA (35 μM) in 30 mM Tris−HCl pH 7.5, 13 mM $MgCl_2$, 2 mM DTT and 100 μg/ml BSA. hRad51 (8.25 μM), ATP (2 mM), ATPγS (0.5 mM) or ADP (0.5 mM) were included as indicated in the figure legends. Reactions containing ATP were supplemented with an ATP regeneration system (20 mM phosphocreatine and 10 U/ml phosphocreatine kinase). Incubation was for 40 min at 37°C, then 20 U of wheat germ topoisomerase I were added followed by a further incubation for 10 min at 37°C. This amount of topoisomerase I is sufficient to relax protein-free DNA within 30 s. To permit RecA binding, it was necessary to modify the reaction such that RecA protein (30 μM) was pre-bound to the DNA in low Mg^{2+} (30 mM Tris−HCl pH 7.5, 1 mM $MgCl_2$, 2 mM DTT and 100 μg/ml BSA) for 30 min at 37°C. The $MgCl_2$ concentration was then increased to 13 mM and incubation continued for a further 10 min prior to topoisomerase addition. Reactions were stopped by addition of 5 μl of phenol stop mixture (45% phenol, 65 mM EDTA, 2% sarcosyl), vortexed briefly, and 5 μl of sample loading buffer (40 mM Tris−acetate, pH 8.0, 1 mM EDTA, 50% glycerol, and a trace of bromophenol blue) was added. Samples were subjected to electrophoresis through a 1% agarose gel (in 80 mM Tris−acetate buffer pH 7.5, containing 5 mM sodium acetate, 1 mM EDTA, 0.03% SDS) at 2 V/cm for 21 h. ^{32}P-labelled DNA was detected by autoradiography.

Electron microscopy

Protein−DNA complexes were fixed by addition of 0.2% glutaraldehyde followed by incubation for 15 min at 37°C. Samples were then diluted in 2 mM magnesium acetate and adsorbed onto freshly glow-discharged carbon coated grids and stained with 2% uranyl acetate.

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