Interactions of Human Rad54 Protein with Branched DNA Molecules*

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The Rad54 protein plays an important role during homologous recombination in eukaryotes. The protein belongs to the Swi2/Snf2 family of ATP-dependent DNA translocases. We previously showed that yeast and human Rad54 (hRad54) specifically bind to Holliday junctions and promote branch migration. Here we examined the minimal DNA structural requirements for optimal hRad54 ATPase and branch migration activity. Although a 12-bp double-stranded DNA region of branched DNA is sufficient to induce ATPase activity, the minimal substrate that gave rise to optimal stimulation of the ATP hydrolysis rate consisted of two short double-stranded DNA arms, 15 bp each, combined with a 45-nucleotide single-stranded DNA branch. We showed that hRad54 binds preferentially to the open and not to the stacked conformation of branched DNA. Stoichiometric titration of hRad54 revealed formation of two types of hRad54 complexes with branched DNA substrates. The first of them, a dimer, is responsible for the ATPase activity of the protein. However, branch migration activity requires a significantly higher stoichiometry of hRad54, ~10 ± 2 protein monomers/DNA molecule. This pleomorphism of hRad54 in formation of oligomeric complexes with DNA may correspond to multiple functions of the protein in homologous recombination.

Rad54 is one of the key proteins of homologous recombination in eukaryotes (1, 2). Mutations in the RAD54 gene cause severe deficiency in homologous recombination and DNA repair (3, 4). Structurally, Rad54 protein belongs to the Swi2/Snf2 family of DNA-dependent ATPases, whose members are best known for their chromatin remodeling activity (5). Biochemical studies have demonstrated a remarkable spectrum of Rad54 activities in vitro, indicating its multiple functions at various stages of homologous recombination (6). Yeast and human Rad54 protein physically interacts with its cognate Rad51 protein (7–9) and stimulates the DNA pairing activity of human Rad54 protein physically interacts with its cognate Rad51 (10, 11). Rad51 to ssDNA2 and stabilizes the Rad51-ssDNA filament in an ATP-independent manner (25). hRad54 showed the strongest binding preference for the partial X-junction (PX-junction) in which one of the four arms contained ssDNA (Table 1). A lesser preference was seen for other branched DNA substrates, including forked DNA and the X-junction. Given the importance of the novel branch migration activity of Rad54, we set out to examine the basis for the observed binding specificity of hRad54 and to further characterize the mechanism of DNA binding.

EXPERIMENTAL PROCEDURES

Proteins—GST- and His6-tagged versions of hRad54 protein were expressed in Sf21 insect cells (11). GST-hRad54 was purified as described previously (11). His6-hRad54 purification was performed at 4 °C. Cells (10 g) stored at −80 °C were thawed and lysed by incubation in 10 volumes of ice-cold buffer A (50 mM Tris–HCl, pH 7.5, 200 mM KCl, 2 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol, 0.5% Nonidet P-40) supplemented with EDTA-free protease inhibitors mixture (Roche Applied Science) for 30 min with constant stirring. The crude extract

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S4.

2 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; hRad54, human Rad54; BMH, bis-maleimidohexane; nt, nucleotide(s); PX-junction and -structure, partial X-junction and -structure, respectively.

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was clarified by centrifugation (100,000 \times g for 60 min) (Fraction I) and loaded on a Q-Sepharose column (50 ml) equilibrated with T20 buffer (20 mM Tris-Cl, pH 7.5, 1 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol), containing 200 mM KCl. The flow-through fraction containing hRad54 (Fraction II) was diluted with K20 buffer (20 mM KH2PO4 (pH 7.5), 0.5 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol) to 150 mM KCl and applied onto a 25-ml heparin column, which was developed with a 180-ml gradient from 150 to 1000 mM KCl in K20 buffer without EDTA. The hRad54 fractions (Fraction III; 35 ml; ~300 mM KCl) were supplemented with 10 mM imidazole and loaded on a 1 ml of Histag HP column (GE Healthcare). The column was washed sequentially with K20 buffer without EDTA containing 500 mM KCl and 10, 20, and 30 mM imidazole. hRad54 protein was eluted with K20 buffer containing 500 mM KCl, 200 mM imidazole, and no EDTA. The eluate (Fraction IV; 2 ml), containing the bulk of the Rad54 protein, was further fractionated in a Superdex-200 column (58 ml), equilibrated with buffer K20 containing 500 mM KCl. hRad54 protein eluted from the S-200 column in a volume expected for a monomeric protein. The hRad54 eluate (Fraction V) was diluted with 4 volumes of K20 buffer containing 100 mM KCl, and eluted with a 20-ml gradient of KCl (100–600 mM) in K20 buffer. The fractions containing hRad54 protein were analyzed for nuclease contamination, pooled (Fraction VI), and stored in small aliquots at –80 °C. The protein appeared nearly homogeneous in a Coo-massie-stained SDS-polyacrylamide gel.

**DNA**—All oligonucleotides used in this study (supplemental Table S1) were purchased from Integrated DNA Technologies, Inc., in desalted form and further purified by electrophoresis in 6–10% polyacrylamide gels containing 50% urea. The concentrations of the purified oligonucleotides were determined spectrophotometrically using extinction coefficients provided by the manufacturer. To prepare ssDNA or dsDNA with protruding ssDNA tails, complementary ssDNA oligonucleotides were annealed as described (26) and stored at –20 °C. Oligonucleotides were labeled using [γ-32P]ATP and T4 polynucleotide kinase, as described previously (12). dsDNA fragments longer than a 135-mer were prepared by digestion of pUC19 plasmid DNA using the following restriction endonucleases (New England Biolabs): HaeIII to produce a 257-bp fragment; AluI to produce 521- and 679-bp fragments; Scal and AvIII to produce a mixture of two fragments of 1373 and 1313 bp (designated as 1300 bp); and SmaI to produce a 2686-bp fragment (linear pUC19 dsDNA). 257, 521, and 679 dsDNA fragments were separated from the smaller fragments by a 6% nondenaturing PAGE and recovered as described (26). 1300- and 2686-bp DNA fragments were prepared by phenol extraction followed by ethanol precipitation.

**ATPase Assay**—The hydrolysis of ATP by Rad54 protein was monitored spectrophotometrically as described previously (27). The oxidation of NADH, coupled to ADP phosphorylation, resulted in a decrease in absorbance at 340 nm, which was continuously monitored by a Hewlett-Packard 8453 diode array spectrophotometer using UV-visible ChemStation software. The rate of ATP hydrolysis was calculated from the rate of change in absorbance using the following formula: rate of \( A_{340} \) decrease (s\(^{-1}\)) \times 9880 = rate of ATP hydrolysis (µM/min).

**Kinetic Parameters**

The reactions in standard buffer containing 25 mM Tris acetate, pH 7.5, 10 mM magnesium acetate (unless indicated otherwise), 1 mM dithiothreitol, 2 mM ATP, 3 mM phosphoenolpyruvate, pyruvate kinase (20 units/ml), lactate dehydrogenase (20 units/ml), NADH (200 µg/ml), and the indicated concentrations of Rad54 protein and DNA were carried out at 30 °C. In preliminary experiments, it was determined that 2 mM was a saturating concentration of ATP for all DNA substrates used in this study. Kinetic parameters of these reactions were determined using GraphPad Prism 4.03 software. Concentrations of free Mg\(^{2+}\) ion were calculated using WebMaxC2.10 (available on the World Wide Web) applying the following parameters: temperature, 30 °C; pH 7.5; and ionic strength, 0.05 (28).

**Branch Migration Assay**—The hRad54 protein (100 nM, unless indicated otherwise) was incubated with 32P-labeled synthetic PX-junction (number 71/169/170/171) (33 nM, molecules), 32P-labeled synthetic X-junction (number 71/170/234/235) (33 nM, molecules), or 32P-labeled synthetic PX-junction (number 265/266/269/270) (20 or 30 nM, molecules) in a 90-µl branch migration buffer containing 25 mM Tris acetate, pH 7.5, 2 mM ATP, 1 mM dithiothreitol, 100 µg/ml bovine serum albumin, the ATP-regenerating system (10 units/ml creatine phosphokinase and 15 mM creatine phosphate), and the indicated concentrations of magnesium acetate. The reactions were carried out at 30 °C or in the case of PX-junction (number 265/266/269/270) at 20 °C. Aliquots (10 µl) were withdrawn, and DNA substrates were deproteinized by treatment with stop buffer (1.4% SDS, 960 µg/ml proteinase K, 7.5% glycerol, 0.015% bromphenol blue) for 5 min at 22 °C. Samples were analyzed by electrophoresis in 8% polyacrylamide gels (29:1) in 1X TBE buffer (90 mM Tris borate, pH 8.3, and 1 mM EDTA) at 22 and at 4 °C for PX- and X-structure, respectively. The gels were dried on DE81 chromatography paper (Whatman) and quantified using a Storm 840 PhosphorImager (Amersham Biosciences).

In hRad54 stoichiometric titrations, the initial rates of branch migration were determined using branch migration buffer containing 3 mM magnesium acetate. The reactions were carried out for 5 min at 20 °C. Time points were taken at 1, 3, and 5 min for each protein concentration tested. The initial rate was determined using the linear part of the kinetic curve during the first 3 min of the reaction. In some experiments, the ATPase regeneration system containing 3 mM phosphoenolpyruvate, pyruvate kinase (20 units/ml), lactate dehydrogenase (20 units/ml), and NADH (200 µg/ml) was used instead of the ATP regeneration system containing creatine phosphokinase and
DNA Binding by hRad54

![Graph showing the effect of DNA substrates of different structures on the ATPase activity of hRad54.](image)

FIGURE 1. Effect of DNA substrates of different structures on the ATPase activity of hRad54. ATPase assays were carried out in the presence of hRad54 protein (60 nM) and various DNA substrates (see Table 1) in the indicated concentrations (μM, nucleotides), which take into account only double-stranded regions of the substrates. The data are the mean of at least three measurements, and the error bars represent the S.E.

creatinine phosphate, with no apparent effect on the initial rate measurements.

RESULTS

hRad54 Shows Binding Preferences for Branched DNA Substrates with ssDNA Arms—Previously, we found that hRad54 shows DNA binding preference for branched DNA substrates (25). hRad54 possesses a dsDNA-dependent ATPase activity, which is essential for DNA branch migration and DNA transcription. Here, using the ATPase activity as a readout, we further characterized hRad54 DNA substrate specificity for a broad range of DNA substrates. We measured the initial rate of ATP hydrolysis of hRad54 protein in the presence of various branched oligonucleotide-derived DNA substrates over a range of DNA concentrations (Fig. 1). The results demonstrate that branched DNA structures (Table 1) with one single-stranded arm (PX-junction, 3′-flap, 5′-flap) stimulate the velocity ($V_{max}$) of hRad54 ATP hydrolysis $\sim 1.5$–$2.0$-fold greater than branched DNA substrates with fully dsDNA arms (X-junction, replication fork) or with two ssDNA arms (Kappa and forked DNA). The 3′-flap and 5′-flap structures containing two dsDNA arms and one ssDNA arm were nearly as efficient in stimulating the ATPase activity of hRad54 protein as the PX-junction, which was the best substrate among the previously examined structures (25). Overall, our results show the following order of preference for DNA substrates according to their efficiency in inducing hRad54 ATPase: PX-structure $\geq$ 3′-flap $\geq$ 5′-flap $\gg$ Kappa $\geq$ X-structure $=$ replication fork $>$ forked DNA $>$ dsDNA. Previously, it was shown that ssDNA supports the ATPase activity of hRad54 poorly (29).

Structural Requirements for Branched DNA Molecules That Serve as Substrates for hRad54—To gain a better understanding of how Rad54 protein interacts with branched DNA substrates, we determined the minimal length of ssDNA and dsDNA regions of branched DNA required for efficient hRad54 binding and stimulation of its ATPase activity. Starting from linear dsDNA, we gradually increased the complexity of DNA substrates by constructing additional DNA branches and varying the length of the branches.

To determine the minimal length of linear dsDNA that supports the ATPase activity of Rad54, we measured the initial rate of ATP hydrolysis by hRad54 as a function of dsDNA concentration for each of a set of dsDNA fragments with lengths varying from 17 to 2686 bp (see examples in supplemental Fig. S1). From the obtained data, we determined the $K_M$ for each dsDNA fragment ($K_M^{DNA}$) (Fig. 2A and Table 2). The $K_M$ values decrease sharply from 61.6 to 0.62 μM with an increase of dsDNA length from 17 to 63 bp. The $K_M$ decreases slightly to 0.44 μM for 90 bp dsDNA fragment and levels out within a 0.3–0.4 μM range for dsDNA fragments up to 2686 bp long. At the same time, the velocity of ATP hydrolysis increases sharply with an increase of dsDNA length from 17 to 63–90 bp and then less steeply until it plateaus at 1300 bp (Fig. 2B). It was observed previously for T4 gp41 helicase that a sharp decrease in the $K_M^{DNA}$ and concomitant increase in the ATPase $V_{max}$ occurs when the size of DNA approaches the DNA binding site size of the protein (30). By this estimate, the apparent size of the hRad54 DNA binding site required for basal ATPase activity is between 63 and 90 bp. Based on the model of Peter von Hippel and co-workers (30), the second phase of increase in the velocity of ATP hydrolysis for dsDNA fragments from 63–90 bp to 1300 bp at a constant $K_M^{DNA}$ value was consistent with translocation of hRad54 protein along the dsDNA (11, 30). The $V_{max}$ data for these DNA fragments have been fit to an equation hyperbolic in DNA length (Fig. 2B). The DNA length at half-plateau height, 280 ± 30 bp, indicates the average translocation distances for hRad54 under tested conditions.

To examine whether an ssDNA tail attached to a short dsDNA fragment can enhance the ATPase activity of Rad54 protein, we constructed a set of tailed DNA molecules containing an invariable 32-bp dsDNA region with ssDNA oligo(dT) tail of various lengths. An oligo(dT) sequence was chosen, because it does not support the ATPase activity of Rad54; in

| DNA substrate | Structure (annealed oligonucleotides)$^a$ |
|---------------|------------------------------------------|
| X-junction    | 175 $\rightarrow$ 174 $\rightarrow$ 177 |
| PX-junction   | 175 $\rightarrow$ 176 $\rightarrow$ 181 |
| 3′-flap       | 175 $\rightarrow$ 176 $\rightarrow$ 180 |
| 5′-flap       | 175 $\rightarrow$ 176 $\rightarrow$ 181 |
| Kappa         | 174 $\rightarrow$ 175 $\rightarrow$ 176 |
| Forked DNA    | 175 $\rightarrow$ 176 $\rightarrow$ 179 |
| dsDNA         | 175 $\rightarrow$ 179 $\rightarrow$ 179 |

$^a$ Numbers correspond to the oligonucleotides in supplemental Table S1.
FIGURE 2. Effect of DNA length on the apparent $K_{M}^{DNA}$ (A) and the $k_{cat}$ (B) of the hRad54 ATPase. For 17, 25, 32, 48, 63, 90, 135, 257, 521, 679, 1300, and 2686-bp dsDNA fragments, the rate of ATP hydrolysis was determined as a function of dsDNA concentration (see supplemental Fig. S1). DNA concentrations were in a range 0.1–32 μM (nucleotides). The dashed hyperbolic curve (inset B) is presented as a reference to emphasize a more rapid rise in ATP hydrolysis for short dsDNA fragments in a range 0.1–32 μM (nt). The apparent $K_{M}^{DNA}$ and $k_{cat}$ values were calculated by fitting the data (like the data shown in supplemental Fig. S1) to the Michaelis-Menten equation. The curve in A is an interpolation curve. The data in B were fit to a hyperbolic curve according to Ref. 30. The curve in inset B for short dsDNA fragments is an interpolation curve. The data are the mean of at least three measurements; the error bars represent the S.E.

TABLE 2
The $K_{M}^{DNA}$ of the ATPase activity of hRad54 as a function of the length of dsDNA substrates

| DNA length (bp) | $K_{M}^{DNA}$ (nt) | Oligonucleotides$^a$ |
|-----------------|---------------------|----------------------|
| 17              | 62 ± 29             | 17/18                |
| 25              | 10 ± 1.4             | 112/113              |
| 32              | 4.3 ± 0.6            | 5/6                  |
| 48              | 3.4 ± 0.4            | 25/26                |
| 63              | 62 ± 0.4             | 1/2                  |
| 90              | 0.44 ± 0.3           | 90/91                |
| 135             | 0.40 ± 0.2           | 114/115              |
| 1300            | 0.38 ± 0.1           | pUC19 fragment       |
| 2686            | 0.42 ± 0.1           | Linear pUC19         |

$^a$ Numbers correspond to the oligonucleotides in supplemental Table S1.

increase was dependent on the length of ssDNA tails, reaching a maximum, ~4-fold stimulation, with an ssDNA tail of 45–60 nt (dT45 and dT60) (Table 3). The polarity of ssDNA tails (3’ to 5’ or 5’ to 3’) relative to the duplex DNA had no effect on the ATPase activity of hRad54 (Table 3). The presence of ssDNA in the DNA substrate appeared to be important for stimulation of the hRad54 ATPase activity, because in trans dT45 ssDNA inhibited dsDNA-dependent ATPase activity of hRad54 (data not shown). Previously, it was shown that ssDNA inhibits the ATPase activity of yeast Rad54. In contrast to a 32-bp dsDNA fragment, the addition of 32- or 45-nt oligo(dT) ssDNA tails to longer dsDNA fragments (62 or 90 bp) did not lead to a noticeable stimulation of ATP hydrolysis (Fig. 3). We suggested that ssDNA tails stimulate the ATPase activity of hRad54 mainly by increasing the overall length of short DNA substrates up to the DNA binding site size of hRad54. The obtained value for this site, 80–90 nt/bp, agrees with the estimate reported above for dsDNA fragments.

We further increased the complexity of DNA substrates by adding a second ssDNA arm to dT45-tailed DNA, creating forked DNA (Table 3). We investigated the effect of the length of this ssDNA arm on the ATPase activity of hRad54. We found that forked DNA stimulated the ATPase activity of hRad54 stronger than tailed DNA (Table 3). The highest stimulation, ~2.5-fold over tailed DNA, was observed when the length of the second ssDNA arm was between 30 and 45 nt.

Next, using the optimal forked DNA substrate, containing two dT arms of 45 and 30 nt, we determined the minimal length of the dsDNA branch required for the ATPase activity of hRad54 protein. The results showed that the dsDNA branch can be decreased to 12 bp without significant loss of the ATPase activity (Table 3). However, shortening of the dsDNA region to 8 bp decreased the velocity of ATP hydrolysis dramatically, ~4-fold. The calculated melting temperature of the 8-bp duplex was 35.1 °C; therefore, it might become unstable at the reaction temperature (30 °C). However, even at 20 °C, forked DNA with a 12-bp dsDNA branch was a far superior substrate for Rad54 ATPase activity than that with an 8-bp dsDNA branch (data not shown). Thus, a 12-bp dsDNA branch in forked DNA substrate was sufficient for stimulation of ATP hydrolysis by hRad54. Shortening of the dsDNA region to 2 bp decreases the hRad54 ATPase activity to a very low level (7-fold lower than for an 8-bp dsDNA region), typically observed in the presence of ssDNA.

We then constructed a set of 3’-flap DNA molecules with a 45-nt ssDNA arm (dT45 or mixed base composition) and two dsDNA arms (mixed base composition) of variable length (Table 3). When the length of both dsDNA arms was 15 bp, the ATPase activity ($V_{max}$) was ~1.6-fold higher than for the best forked DNA substrate (Table 3). The increase in length of dsDNA arms to 25, 37, or 45 bp did not increase the ATPase activity of hRad54 protein further or even slightly decreased it when the dsDNA arms were 45 and 32 bp. The sequence of the ssDNA arm in the flapped DNA was not apparently essential for the ATPase activity, since replacement of the dT45 arm with the ssDNA arm of mixed base composition did not significantly

contrast, ssDNA sequences of mixed base composition support some small level of ATP hydrolysis (29). We found that the velocity of ATP hydrolysis by hRad54 protein was increased by the addition of ssDNA tails to a 32-bp dsDNA fragment. The

$^3$ O. M. Mazina and A. V. Mazin, unpublished data.

$^4$ S. Kowalczykowski and C. Bornarth, personal communication.
affect the rate of ATP hydrolysis. The decrease in length of ssDNA arm to 30 and 15 nt caused a decrease in the rate of ATP hydrolysis by hRad54 (data not shown).

Finally, we constructed the PX-junction containing three dsDNA branches of 15 bp each and an ssDNA arm of 45 nt. The rate of ATP hydrolysis with this substrate appeared to be approximately the same as with the flap DNA described above (Table 3).

Thus, in the course of analysis of a series of DNA substrates of ascending complexity, the flap DNA containing two 15-bp dsDNA arms and a 45-nt ssDNA arm, emerged as the minimal substrate that supports optimal Rad54 ATPase activity, about 15-fold better than 32-bp linear dsDNA. Henceforth we refer it as the “minimal flap DNA substrate.”

### Table 3

ATPase activity of hRad54 with various DNA substrates of ascending complexity

| DNA substrate       | Structure (annealed oligonucleotides) | Apparent $k_{cat}^a$, min$^{-1}$ | Oligos$^b$ # |
|---------------------|---------------------------------------|---------------------------------|--------------|
| 32 bp blunt duplex  | 32 bp                                 | 133 ± 24                        | 240/222      |
| 3'-ssDNA tail       | 32 bp dT$_{15}$                       | 198 ± 11                        | 240/223      |
|                     | 32 bp dT$_{30}$                       | 308 ± 30                        | 240/153      |
|                     | 32 bp dT$_{45}$                       | 545 ± 51                        | 240/224      |
|                     | 32 bp dT$_{60}$                       | 573 ± 54                        | 240/225      |
|                     | 32 bp dT$_{75}$                       | 545 ± 50                        | 240/226      |
| 5'-ssDNA tail       | 32 bp dT$_{45}$                       | 523 ± 48                        | 148/222      |
| Forked DNA          | 32 bp dT$_{15}$                       | 765 ± 84                        | 148/223      |
|                     | dT$_{45}$                             |                                |              |
|                     | dT$_{30}$                             | 1210 ± 84                       | 148/153      |
|                     | dT$_{45}$                             | 1240 ± 145                      | 148/224      |
|                     | dT$_{45}$                             | 1180 ± 126                      | 148/225      |
|                     | dT$_{60}$                             | 1180 ± 87                       | 150/155      |
|                     | dT$_{30}$                             |                                |              |
The Stoichiometry of hRad54 Binding to the Minimal Flap DNA Substrate—Although on a gel filtration column, Rad54 protein elutes as a monomer (31) the stoichiometry of Rad54-DNA complexes is still unknown. Here we measured the stoichiometry of hRad54 binding to the minimal flap DNA substrate. For this purpose, we measured the rate of ATP hydrolysis as a function of DNA concentration for each DNA substrate.

### Table 3—continued

| DNA substrate       | Structure (annealed oligonucleotides) | Apparent $k_{cat}^a$, min$^{-1}$ | Oligos$^b$ # |
|---------------------|---------------------------------------|----------------------------------|--------------|
| Forked DNA          | $d_{45}$ 12 bp $d_{30}$               | 953 ± 80                        | 152/157      |
|                     | $d_{45}$ 8 bp $d_{30}$                | 230 ± 30                        | 158/159      |
|                     | $d_{45}$ 2 bp $d_{30}$                | 34 ± 4                          | 162/163      |
| 3'-flap DNA         | $77$ bp                                | 1080 ± 80                       | 227/224/119  |
|                     | 32 bp $d_{45}$ 45 bp $d_{30}$-3'      |                                 |              |
|                     | 62 bp                                  | 1420 ± 128                      | 251/175/180  |
|                     | 37 bp $d_{45}$ 25 bp $d_{30}$-3'      |                                 |              |
|                     | 62 bp                                  | 1620 ± 136                      | 243/175/180  |
|                     | 37 bp $d_{45}$ 25 bp $d_{30}$-3'      |                                 |              |
|                     | 40 bp mixed$^{45}$ -3'                | 1970 ± 253                      | 244/245/180  |
|                     | 15 bp mixed$^{45}$ -3'                |                                 |              |
|                     | 30 bp mixed$^{45}$ -3'                | 1950 ± 340                      | 244/249/250  |
|                     | 15 bp mixed$^{45}$ -3'                |                                 |              |
| PX-structure        | $30$ bp                                | 1880 ± 235                      | 244/249/364/124 |

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*For each DNA substrate, the rates of ATP hydrolysis were determined in a broad range of DNA concentrations up to saturation (see supplemental Fig. 1). hRad54 concentration was 60 nM. The $k_{cat}$ values were calculated by fitting the data to the Michaelis-Menten equation.

* Numbers correspond to the oligonucleotides in supplemental Table S1.*
hydrolysis as a function of (i) DNA concentration at fixed hRad54 protein concentration (60 nM) and (ii) hRad54 protein concentration at fixed DNA concentration (30 nM, molecules). Since the GST domain is known for its ability to dimerize in solution (32), we performed these experiments with both GST-Rad54 and His6-Rad54 proteins to exclude any possible effect of such dimerization. The results, however, appeared to be identical for both versions of hRad54. Titration of hRad54 protein with the minimal flap DNA substrate yielded the stoichiometry of two hRad54 molecules/DNA molecule (Fig. 4A). Similar, when hRad54 was used as a titrant, the apparent stoichiometry was two hRad54 molecules/DNA molecule (Fig. 4B).

We next investigated whether an hRad54 complex with the minimal flap DNA is presented by two independently bound hRad54 monomers or hRad54 binds as a dimer. To address this question, we used a protein cross-linking agent, BMH, specific for free sulfhydryl groups. Previously, BMH was used to determine an oligomeric status of yeast Rad54 in a complex with phage DNA (31). His6-hRad54 migrates in an SDS-polyacrylamide gel in accord with the predicted size of 88.7 kDa (Fig. 5, lanes 2 and 6). In the absence of DNA, treatment with BMH yielded a novel Rad54 doublet that migrates as a protein with an apparent size of about 110 kDa (denoted as M1 and M2 in Fig. 5, lanes 3 and 7), which was previously attributed to intramolecular cross-linking of Rad54 monomer (31). In the presence of the minimal flap DNA, we observed the formation of additional hRad54 species (Fig. 5, lanes 4 and 5). One of them has an apparent size of about 180 kDa, consistent with the predicted dimer size (177.4 kDa). Another species, denoted D1, has a size of about 235 kDa, which is smaller than the predicted trimer size (266.1 kDa). We suggest that it can be a dimer, in which one or both monomeric units contain intramolecular cross-links. Finally, consistent with a previous report (31), there were also very large oligomers. A 4-fold increase in BMH concentration (100 μM) did not significantly change the Rad54 modification pattern, indicating that nearly all Rad54 groups accessible for the reagent were modified (Fig. 5, lanes 7–10). Since BMH can cross-link sulphydryl groups separated by only 13 Å, cross-link-
When the PX-junction concentration was 30 nM, the maximal initial rate of branch migration on both the X- and PX-junctions (25). Here we measured the effect of hRad54 concentration on DNA branch migration using synthetic PX junctions (number 265/266/269/270) in the three-strand reaction (Fig. 6A, top). For each tested hRad54 protein concentration, we measured the initial rate of branch migration. When the PX concentration was 20 nm (molecules), the maximal initial rate of branch migration was observed at the hRad54 protein concentration above 200 nm, yielding the stoichiometry of ~10.5 ± 2 hRad54 monomers/PX-junction molecule (Fig. 6A, closed circles). When the PX-junction concentration was 30 nm, the maximal initial rate was observed at ~300 nm hRad54 concentrations, yielding the stoichiometry of ~9.5 ± 2 hRad54 monomers/PX-junction molecule (Fig. 6A, open circles). It is not entirely clear why the initial rate of branch migration with 30 nm DNA is ~3-fold faster than with 20 nm DNA concentration (Fig. 6A). We suggest that slower DNA binding of hRad54 at lower DNA concentration (20 nm) may contribute to the observed difference in the rates of branch migration. The hRad54 stoichiometries were approximately the same regardless of whether His6- or GST tag hRad54 was used (data not shown). The increase in the reaction temperature from 20 to 30 °C did not seem to have a significant effect on the hRad54 stoichiometry, although at high hRad54 concentrations, the initial rates of branch migration were too fast, complicating the accuracy of our measurements (data not shown). Also, the hRad54 stoichiometries were approximately the same for 3-strand and 4-strand branch migration (data not shown).

Whereas efficient DNA branch migration required the hRad54 stoichiometry of ~10 ± 2 protein monomers/PX-junction molecule, only two hRad54 protein monomers were required for the maximal ATP hydrolysis rate on the minimal flap DNA. To exclude the effect of DNA substrate lengths, we then determined hRad54 stoichiometry in the ATPase assay using the same DNA substrate, PX-junction (number 265/266/269/270) (30 nm, molecules) and reaction conditions identical to that of DNA branch migration, shown in Fig. 6A. We found that, as in the case with the minimal flap DNA, ~2 molecules of hRad54/DNA molecule were required for the maximal rate of ATP hydrolysis (Fig. 6B).

Thus, DNA branch migration and ATP hydrolysis show different requirements in respect to the Rad54 stoichiometry, with

FIGURE 6. The rate of branch migration (A) or ATP hydrolysis (B) by His6-tagged hRad54 as a function of protein concentration on a movable PX-junction.

The scheme of the reaction is shown at the top of A. Four-base pair heterologies (denoted by TGAC and ACTG) were introduced into the PX-junction (number 265/266/269/270) to block spontaneous branch migration. The double wavy line denotes the heterologous DNA terminal branches. The PX-junction was designed in such a way that branch migration can proceed only in one direction of movement, shown by curved arrows. The asterisk indicates 32P label at the DNA 5′-end. The numbers correspond to the oligonucleotides in supplemental Table S1. The PX-junction concentrations are 20 nm (molecules; closed circles) or 30 nm (molecules; open circles). The closed circle data set was placed on the left y axis, and the open circle data set was placed on the right y axis. In control reactions, hRad54 protein was substituted by storage buffer. The ATPase assays (B) were carried out using conditions identical to those in A using a 30 nm (molecules) concentration of the PX-junction (number 265/266/269/270). The dashed lines indicate the tangents drawn to the curves to determine the apparent DNA binding stoichiometries of hRad54. For A and B, the error bars represent the S.E.; the data are the mean of two measurements.
DNA Binding by hRad54

DNA branch migration activity requiring an ~5-fold higher hRad54 stoichiometry than the ATPase activity, 10 ± 2 molecules of hRad54/molecule of the PX-junction.

Effect of the Conformation of Branched DNA on Its Interaction with Rad54—The observed hRad54 binding preference for branched DNA substrates with an ssDNA arm (PX-junctions and flap DNA) over fully double-stranded junctions (X-junctions, replication fork) (Fig. 1; see also Ref. 25) could not be due to its preference for ssDNA per se, because the affinity of hRad54 for ssDNA was significantly lower than for any of the branched DNA substrates (25, 33). We suggested that the hRad54 binding preferences are determined by the conformation of DNA junctions. It is known that the X-junctions exist in two conformations, open and stacked (Fig. 7, top) (28). The transition between these conformations is determined by the concentration of bivalent cations (e.g. magnesium) and occurs between 100 and 300 μM of free magnesium ions. The PX-junction resembles a nicked three-way junction with an additional ssDNA arm (34). Although three-way junctions can form the stacked conformation at elevated magnesium concentrations, it is less stable than the one formed by the X-junction (35). The Kappa structure, a branched DNA molecule containing a duplex and two ssDNA arms (Table 1), seems unlikely to form the stacked conformation, because it requires intramolecular interactions between dsDNA branches.

To test the effect of DNA conformation on DNA binding of hRad54, we measured the ATPase activity of hRad54 at different magnesium concentrations in the presence of the X-, PX-, and Kappa structures (Table 1). The maximal rate of ATP hydrolysis was observed at 3 mM magnesium acetate for the PX-junction and the Kappa structure (~1.1 mM of free magnesium ion; see “ATPase Assay”) (Fig. 7A). However, for the X-junction, the rate of ATP hydrolysis reached its maximum at 1.2 mM magnesium acetate (~85 μM free magnesium ion) and then declined at higher magnesium concentrations. We suggested that formation of the stacked X-junction conformation occurring at free magnesium concentrations higher than 85 μM is inhibitory for hRad54 binding.

If hRad54 binds preferentially to the open X-junction conformation, one would expect that preincubation of the protein with the X-junction at low magnesium concentration would stimulate its ATPase activity by facilitating DNA binding. Indeed, we found that preincubation of hRad54 with the X-junction at 0.3 mM Mg²⁺ causes an ~3-fold stimulation of the ATP hydrolysis rate after Mg²⁺ concentration was raised to 3 mM (Fig. 7B); the shift to other Mg²⁺ concentrations caused smaller but significant increases in the ATP hydrolysis rate. The increase in the ATP hydrolysis rate occurred within the first minute after the shift, followed by its gradual decline to the levels specific for any magnesium concentration when the shift was not applied (Fig. 7A). With the PX-junctions, the shift in Mg²⁺ concentration caused only ~1.6-fold increase in the ATP hydrolysis rate (Fig. 7, A and B). Thus, with magnesium shift, the ATP hydrolysis rate in the presence of the X-junction almost reached the rate observed in the presence of the PX-junction (Fig. 7B). As expected, the magnesium shift had no effect on the hRad54 ATPase activity in the presence of the Kappa structure, consistent with its expected inability to form the stacked DNA conformation; also, it had no effect on the hRad54 ATPase activity in the presence of a 63-bp linear dsDNA fragment (supplemental Fig. S3).

These results show that the ATPase activity of hRad54 in the presence of the X-junction can be greatly enhanced by preincubation of the protein and DNA at low magnesium concentrations. We concluded that hRad54 binds preferentially to branched DNA molecules in the open conformation that is predominant under low magnesium concentrations.

Effect of DNA Conformation on DNA Branch Migration Activity of Rad54 Protein—Here we wanted to ascertain that the open DNA conformation is important for DNA branch migration activity of hRad54 protein. For this purpose, we measured...
the rate of DNA branch migration on PX- and X-junctions in the presence of various magnesium concentrations (Fig. 8, A and B). We found that although the maximal rate of DNA branch migration on the PX-junctions was at 3.0 mM Mg$^{2+}$, on the X-junctions the maximal rate of branch migration was observed at 1.2 mM Mg$^{2+}$ (Fig. 8C). Thus, similar to the ATPase activity, branch migration activity of hRad54 protein on the X-junction was stimulated at low Mg$^{2+}$ concentrations, which support the open X-junction conformation.

**DISCUSSION**

The Holliday junction (or X-junction), a key structure of homologous recombination, is formed as a result of ssDNA invasion and branch migration (36). Branch migration of the Holliday junctions represents a fundamentally important process that is thought to affect the amount of genetic information contributed by each parent during meiotic recombination, to cause the disruption of recombination intermediates, or to promote regression of the stalled replication fork (25, 37–39). Previously, we found that Rad54 protein binds to branched DNA molecules and efficiently promotes branch migration of Holliday junctions (25). Here we characterized structural features of branched DNA substrates required for efficient hRad54 protein binding, ATP hydrolysis, and DNA branch migration. We determined the minimal length of each of the DNA arms required for efficient induction of the ATPase activity of Rad54 protein, which is essential for DNA branch migration.

Rad54 protein possesses a motor domain (also called the ATPase domain), which is conserved among all proteins of the helicase superfamilies 1 and 2 (SF1 and SF2), including the Swi2/Snf2 proteins (5, 40). The motor domain consisting of two RecA-like subdomains is primarily responsible for translocation of the proteins along ssDNA or dsDNA. The available structures show that the cleft between two RecA-like subdomains forms the nucleotide-binding site (41). A groove above the cleft binds the DNA molecule, which stimulates ATP hydrolysis. Openings and closings of the cleft in response to nucleotide binding and hydrolysis cause the relative affinity for DNA to alternate between two RecA-like domains, allowing the DNA to slide over one or the other of the DNA binding sites and ultimately to translocate (42). Recent analysis of the protein structure and protein-DNA complexes of the *Sulfolobus solfa*
DNA Binding by hRad54

taricus Swi2/Snf2 core ATPase (SsoRad54cd) and the zebrafish core Rad54 indicated that the contact interface between the double-stranded DNA segment and the protein consists of around 12–15 bp (5, 40). Thus, our estimates of the minimal dsDNA arms of ~12 bp in branched DNA that is required for induction of ATP hydrolysis by hRad54 are in good agreement with the structural data. We therefore suggest that a short dsDNA region of the fork DNA or the flap DNA occupies the DNA binding site of the motor domain. However, linear dsDNA fragments of this length do not support the ATPase activity of hRad54, indicating that additional DNA-protein contacts are required for stable binding.

The proteins of helicase superfamilies have different functions in the cell and show different substrate specificities. These specificities are conferred by additional protein domains that may be either insertions or extensions to the highly conserved core of the RecA-like domains (41). Prokaryotic branch migration protein RecG is an example of such modular domain organization. The N-terminal domain of RecG interacts specifically with the Holliday junctions, whereas its motor domain binds to one of the dsDNA arms promoting protein translocation along DNA and causing migration of the Holliday junctions (43). In RuvAB, another prokaryotic branch migrating enzyme, RuvA protein binds specifically to the Holliday junctions, whereas RuvB, a member of the AAA+ helicase family, interacts with this complex and then binds to two flanking dsDNA arms to promote DNA translocation (37).

Recent structure analysis of the core (residues 91–738) of the zebrafish Rad54, which is highly homologous to hRad54, did not identify an additional structured domain outside the ATPase but revealed several extensions of the motor domain, including the N terminus, two α-helical extensions (HD1 and HD2), and the very C terminus (5). The N-terminal extension and the very C terminus that was also found to contain a zinc-coordinating motif are unique to the Rad54 family of DNA translocases. The helical segments HD1 and HD2 are general hallmarks of all SWI/SNF helicases (5), including ISWI, which was shown not to promote branch migration of PX junctions (25). The specific extensions of Rad54 protein may provide additional protein-DNA contacts for interaction with the Holliday junction or other branched DNA substrates. They may also provide additional contacts for linear dsDNA binding.

Both yeast and human Rad54 protein exist in a monomeric form in solution (31). However, binding to DNA causes oligomerization of Rad54, as it was first reported for the Saccharomyces cerevisiae Rad54; the cross-linking experiments indicated a dimer as the principal oligomeric species, whereas larger oligomers were also detected (31). Formation of Rad54 oligomers in complexes with DNA was also detected using atomic force microscopy for hRad54 (18) and electron microscopy for S. cerevisiae Rad54 (44). We previously found that the velocity of ATP hydrolysis by hRad54 shows saturation at the stoichiometry of ~1 protein monomer/30 bp of linear pUC19 plasmid DNA (11). Taken together with our current results, which show that the minimal size of linear/tailed dsDNA required for basal ATPase activity is 60–80 bp, these data are also consistent with the formation of hRad54 dimers with DNA during ATP hydrolysis. Moreover, stoichiometric titration of hRad54 with linear 63-bp dsDNA substrate yields a stoichiometry of ~2 hRad54 monomers/DNA molecule, also indicating possible hRad54 dimer formation (supplemental Fig. S4).

Although all previous studies used linear or circular dsDNA, hRad54 protein, as we recently found, has an ~200-fold higher affinity for branched DNA molecules (25). In addition, branched DNA structures, PX- and X-junctions, represent the substrate for DNA branch migration activity of hRad54 (25). Here we investigated the oligomeric status of hRad54 complex with branched DNA. We identified two types of hRad54-DNA complexes. The first of them is probably represented by a dimer. In the ATPase assay, hRad54 shows the binding stoichiometry of 2 monomers/DNA molecule; a dimer can be cross-linked and identified in an SDS gel even in the presence of an excess of free DNA.

The minimal flap DNA containing one ssDNA arm of 45 nt flanked by two dsDNA arms of 15 bp each was selected as a very proficient substrate to induce the ATPase activity of hRad54 protein. The central location of the branch point, where an ssDNA arm was attached to the duplex, appears to be important; the substrate in which a 45-nt ssDNA tail was attached to the terminus of a 32-bp dsDNA fragment was ~4-fold less efficient in inducing ATP hydrolysis. This arrangement of DNA branches suggests an interaction of hRad54 dimer with the flap DNA substrate, where the two centrally located specificity domains interact with the branch point, and two motor domains bind to the flanking dsDNA arms (Fig. 9A). We suggest that hRad54 also forms dimeric complexes of similar head-to-head configuration with the X- and the PX-structures or even with dsDNA (Fig. 9A). Such complexes with dsDNA would require DNA bending and formation of loops. The latter are consistent with known accumulation of positive and nega-
tive supercoils in covalently closed dsDNA during its interaction with hRad54 (16, 17, 45).

hRad54 binding to DNA molecules that leads to formation of dimeric hRad54 complexes is probably responsible for specific recognition of the branched DNA structure. These complexes are stable, as indicated by their persistence in the presence of a 10-fold DNA excess. Surprisingly, hRad54 at concentrations required for dimer formation showed relatively low branch migration activity on the PX junctions. Efficient branch migration activity requires a significantly higher hRad54 stoichiometry relative to branched DNA, 10 \( \pm \) 2 hRad54 monomers/DNA molecule, indicating the formation of large multiprotein complexes. The formation of large complexes of hRad54 with branched DNA is also consistent with the results of cross-linking experiments. This additional hRad54 binding to DNA junctions does not cause an increase in ATP hydrolysis, indicating that formation of these large complexes either involves different (ATPase-independent) DNA binding sites or is mediated by protein–protein interactions. By analogy with some other DNA-translocating proteins, we suggest that hRad54-DNA complexes may be presented by two hexameric complexes bound symmetrically to the opposite dsDNA arms flanking the Holliday junction (Fig. 9B).

An increase in the \( V_{\text{max}} \) of ATP hydrolysis with the length of dsDNA fragments at a constant \( K_{\text{M}} \) value was previously attributed to an ATPase-dependent translocation of proteins along DNA (11, 30). Applying this model for hRad54, we determined the average distance \( N \) translocated by this protein prior to stopping or dissociating from dsDNA. Under our experimental conditions, \( N \approx 280 \) bp. From these data, we can calculate the processivity of translocation \( (P) \), which is defined as the probability of advancing at each translocation step, relative to the probability of dissociating (46). Assuming a translocation step size of 1 bp, \( P \) is equal to \( (N - 1)/N \), yielding \( P = 0.996 \). This value is lower than the processivity of translocation by yeast Rad54 (0.99991), which was determined in single molecule experiments (20). It is possible that these values reflect intrinsic differences in translocation processivity of two Rad54 orthologs. Also, the measurements might be affected by different reaction conditions (e.g. magnesium concentration was 10 mM in our study versus 2 mM in Ref. 20). Finally, the \( P \) value obtained by the single molecule approach might reflect primarily the behavior of large multiprotein Rad54-DNA complexes, as was suggested by S. Kowalczykowski and co-workers (20), whereas the ATPase assay provides an average \( P \) value for the dimeric and multimeric hRad54 complexes.

The structure of the X-junction depends on the presence of divalent metal ions (e.g. magnesium). At low magnesium ion concentration, four helical arms of the X-structure are unstacked and extended with approximately square-planar symmetry (47). At 100–300 \( \mu \)M free magnesium ion, the helices undergo pairwise coaxial stacking into a right-handed antiparallel structure, termed the stacked X-structure (48, 49). The extended junction conformation greatly favors branch migration of the X-junction (49). Our data indicate that the maximal ATPase and DNA branch migration activity of Rad54 protein on the X-structure is observed at 1.2 mM magnesium ion, the concentration that in the presence of ATP corresponds to \( \approx 85 \mu \)M free magnesium ions. These data suggest that Rad54 binds preferably to the X-junction in the open conformation. The increase in magnesium concentration causes a gradual decrease in the rate of ATPase activity of Rad54, indicating an inhibitory effect of the stacked X-junction conformation on Rad54 binding. Consistent with this interpretation, the ATPase activity of hRad54 protein was significantly stimulated by preincubation with the X-structure at low magnesium concentration, reaching the rate of ATP hydrolysis equal to that observed in the presence of the PX-junction. Thus, the open conformation of the X-junction, which probably exists at low magnesium concentration, facilitates DNA binding of hRad54 and thereby stimulates its ATPase. Low magnesium concentrations also stimulate branch migration activity of hRad54 on X-junctions presumably in two ways: by facilitating hRad54 binding and by helping to maintain X-junctions in the open conformation that is required for branch migration.

The binding preference for the open conformation of the X-junction appears to be common for many proteins that bind to Holliday junctions. Electron microscopy studies showed that RuvA and RuvB complexes stabilize Holliday junctions in the open conformation (50, 51). There have been crystal structures of several different proteins bound to Holliday junctions, including RuvA (52–54), the Cre and Flp recombinases (55, 56), and RecG (43). In all of these cases, the conformation of the junction bound to protein has been square-planar rather than the stacked X-junction. However, some proteins, such as the hMSH4-hMSH5 complex, preferentially bind to Holliday junctions in the stacked conformation and act as a clamp for branch migration (57).

Under physiological magnesium ion concentration (0.5–1.0 mM free ions) (28), the PX-junctions (and flap DNA) are more efficient than the X-junctions in supporting the ATPase and branch migration activity of hRad54, consistent with a lower propensity of these structures to form the stacked DNA conformation. These data indicate that the PX-junction, which structurally resembles one end of the D-loop structure, a product of the initial step of homologous recombination, may represent a substrate for Rad54 protein in vivo. Other auxiliary proteins may also exist that help to induce or maintain the open conformation of the X-junctions, thereby stimulating binding and branch migration activity of hRad54 protein.

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