RECQ4 selectively recognizes Holliday junctions

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Abstract

The RECQ4 protein belongs to the RecQ helicase family, which plays crucial roles in genome maintenance. Mutations in the *RECQ4* gene are associated with three insidious hereditary disorders: Rothmund–Thomson, Baller–Gerold, and RAPADILINO syndromes. These syndromes are characterized by growth deficiency, radial ray defects, red rashes, and higher predisposition to malignancy, especially osteosarcomas. Within the RecQ family, RECQ4 is the least characterized, and its role in DNA replication and repair remains unknown. We have identified several DNA binding sites within RECQ4. Two are located at the N-terminus and one is located within the conserved helicase domain. N-terminal domains probably cooperate with one another and promote the strong annealing activity of RECQ4. Surprisingly, the region spanning 322–400 aa shows a very high affinity for branched DNA substrates, especially Holliday junctions. This study demonstrates biochemical activities of RECQ4 that could be involved in genome maintenance and suggest its possible role in processing replication and recombination intermediates.

1. Introduction

The RecQ family is a highly evolutionarily conserved group of DNA helicases which are considered to be multiple genome "protectors" that maintain chromosomal stability and suppress tumorigenesis [1, 2]. In human cells, five RecQ helicases have been identified: RECQ1 [3, 4], BLM (RECQ2) [5], WRN (RECQ3) [6], RECQ4 and RECQ5 [7]. Mutations in three of the five human RecQ helicases, BLM, WRN and RECQ4, can lead to hereditary Bloom (BS), Werner (WS) and Rothmund–Thomson (RTS) syndromes, respectively. All syndromes are causally linked with clinical symptoms including chromosomal and developmental abnormalities, cancer predisposition and premature aging [5, 6, 8].

Mutations in RECQ4 are associated not only with RTS but also with two additional insidious diseases, the RAPADILINO and Baller–Gerold (BGS) syndromes [8-11]. BGS and RAPADILINO have clinical features overlapping with those of RTS, manifesting not only as hyper- and hypopigmentation (poikiloderma), radial ray defects, skeletal abnormalities, and slow growth, but also premature aging and a higher predisposition to cancer, especially osteosarcoma [12]. In addition, cells derived from patients with RTS show a high frequency of aneuploidy, trisomy and chromosomal rearrangements [13].

The *RECQ4* gene encodes a protein 1208 amino acids long (133 kDa) and containing a highly conserved 3´ to 5´ helicase domain in its center [7, 14]. The RECQ4 protein lacks homology to another two conserved RecQ motifs, namely RQC and HRDC, which are present in the BLM and WRN helicases. Although the RECQ4 protein's role in DNA metabolism remains unknown, it has been shown to possess ATPase activity and single-strand annealing activity while manifesting only limited helicase activity [15-19]. Due to its interactions with several replication initiation factors, such as MCM10 and TOPBP1, and its importance for the proper assembly of CDC45-MCM2-7-GINS complex at the replication fork, it has been suggested that RECQ4 plays a role in replisome assembly [20, 21]. This view is supported also by the presence of both nuclear targeting sequences and a region with similarity to the yeast Sld2 protein that is important for initiating DNA replication [22-24] at the N-terminus of RECQ4. RECQ4 is also implicated in DNA repair, as it interacts and co-localizes with key proteins for base excision repair [25] and also facilitates nucleotide excision repair of UV-induced DNA damage [26]. In addition, RECQ4 has been shown to function in telomere maintenance [27] and co-localize with RAD51 foci and regions of single-stranded DNA after induction of double-strand breaks (DSBs) [28]. While these findings further point to a role for

RECQ4 in the repair of DSBs by homologous recombination, additional studies are required to decipher the RECQ4 protein's role and molecular mechanism of action.

Most of the mutations identified in patients with RTS are located in the conserved helicase domain of *RECQ4*, and these can lead to incompletely functional protein truncation [29, 30]. Whereas mice carrying mutations within the helicase domain survive and show RTS phenotype [31, 32], disruption of the N-terminus of RECQ4 leads to early embryonic lethality. This has been confirmed, too, in the DT40 system, where the N-terminus of human RECQ4 (spanning region 1–496 aa) was able fully to rescue from lethality cells for which RECQ4 had been disabled [33]. This clearly indicates that the N-terminus of RECQ4 is essential for cell viability, and it also explains why mutations in this region are very rare.

In this work, we investigated the DNA binding properties of RECQ4. We identified several DNA-binding domains within RECQ4. Two domains were located within the N-terminus and a third was part of the helicase domain. Both domains within the N-terminus are required for annealing complementary strands. Particularly noteworthy is that the DNA-binding domain located in region 322–400 aa shows a very strong preference for Holliday junctions, thus implying a role of RECQ4 in the metabolism of replication and recombination intermediates.

2. Results

2.1. N-terminus of RECQ4 has high affinity for Holliday junctions

Since other RECQ helicases contain several DNA-binding domains, we tested for their presence in the RECQ4 protein. To this end, we generated several RECQ4 truncations (Fig. 1A) as MBP-fusion at N-terminus and 9-histidine at C-terminus, respectively, to improve solubility and protein yield. The purification of each RECQ4 fragment was carried out using a defined protocol (Supplementary Fig. 1A) and resulted in proteins with near homogeneity (Supplementary Fig. 1B).

To map the DNA-binding domains in RECQ4, we first tested fragments that split the RECQ4 protein into N-terminal (RECQ4[1–400], RECQ4[1–492]) and C-terminal/helicase (RECQ4[455–1208]) domains. As shown in Fig. 1B, RECQ4(1–400) and RECQ4(1–492) were able to bind 50% of dsDNA substrate at 100 nM, while C-terminal fragment required 200 nM protein for 30% of dsDNA to be bound. To exclude potential effects of MBP-tag on DNA

binding activity, we removed it from fragment RECQ4(1–400). However, no effect on DNA binding of this RECQ4 fragment was observed (Supplementary Fig. 2).

Next, we decided to test DNA binding preferences of RECQ4(1–400) for different DNA structures representing various recombination or replication intermediates, including ssDNA, dsDNA, 3' flap, bubble and Holliday junction (HJ) structures. To our surprise, we observed a very strong affinity for HJs and other branched DNA substrates (Fig. 2). Specifically, at 12 nM RECQ4(1–400) the affinity for HJs is about 9- or 3-fold higher in comparison to ss/dsDNA and 3' flap substrates, respectively (Fig. 2B), thus indicating a strong affinity for HJs. The DNA binding properties of the C-terminal part of the RECQ4 protein were also tested on the same DNA substrates. While RECQ4(455–1208) shows a significantly higher binding preference for HJ substrate (Supplementary Fig. 4A), its affinity for HJs compared to the RECQ4(1–400) fragment is about 6-fold lower (Fig. 2B and Supplementary Fig. 4B).

To confirm the strong preference of RECQ4 for HJs, we used two types of competition assays. In the first assay, increasing amounts of RECQ4(1–400) were incubated with an equimolar mixture of ssDNA and HJ. While RECQ4(1–400) is capable of binding around 70% of HJ substrate at 12 nM, the ssDNA remained unbound (Figs. 3A and B). In the second assay, RECQ4(1–400) was pre-incubated with ssDNA to form a complex and then challenged by addition of HJ substrate. Increasing HJ concentrations were able completely to outcompete ssDNA from its complex with RECQ4 (Figs. 3C and D). Taken together, these experiments clearly demonstrate that the N-terminus of RECQ4 has a very high and specific affinity for HJ substrate.

Next, we used atomic force microscopy to directly visualize RECQ4(1–400) protein binding to replication intermediates. We analyzed approximately 300 molecules of DNA isolated from *Xenopus laevis* egg extracts, amongst which we found nine clearly defined structures containing replication fork. All except one were bound by the RECQ4 protein (Figs. 4A, B and C), further demonstrating the high affinity of RECQ4 to these DNA structures. As a control, the same amount of DNA isolated from replication extracts was incubated without addition of RECQ4(1-400) excluding any potential protein contamination (Supplementary Fig. 5).

2.2. RECQ4 possesses several DNA-binding domains

To map the HJ-binding domain within the N-terminus of RECQ4, we constructed and purified additional truncations (Fig. 1A) and tested them using an electrophoretic mobility shift assay (EMSA). Truncations 1–269 already resulted in a 9-fold reduction of binding affinity for HJ compared to RECQ4(1–400) at 12 nM concentration (Fig. 5A). Therefore, we also tested an N-terminal fragment spanning the region 269–400 and observed a similar affinity to that of RECQ4(1–400) (Fig. 5A and Supplementary Fig. 3C). To further delineate the domain, we also generated truncation 1–322. While it was able to bind HJs, it had about one-sixth of the affinity as well as a different mode of binding compared to RECQ4(1–400) and RECQ4(269–400) (Fig. 5A and Supplementary Fig. 3C). Finally, fragment RECQ4(322-400) showed undistinguishable affinity for HJ as 1-400 and 269-400 (Fig. 5A and Supplementary Fig. 3C), thus suggesting that HJ interaction domain is located in the region 322–400.

Additional N-terminal truncations of the RECQ4 further reduced the specificity and binding affinity for HJ, although some DNA binding with fragment 1–189 was still observed. Almost no DNA binding was detected for fragment 1–90 (Supplementary Fig. 3C), indicating that the N-terminal region includes an additional DNA binding site within 90–189 aa. Furthermore, this DNA-binding domain seems not to be that specific, because its ability to bind HJ is about 8-times lower compared to RECQ4(1–400) (Fig. 5A and Supplementary Fig. 6A and B). Taken together, the N-terminus of RECQ4 contains two DNA-binding domains, one located at region 90–189 and the other, an HJ-specific domain, at region 322–400 (Fig. 5B).

2.3. N-terminus shows strong annealing activity

We have previously demonstrated the ability of RECQ4 to promote annealing of complementary single strands [15]. We therefore asked which region of RECQ4 is responsible for this activity. We tested two N-terminal fragments, RECQ4(1–492) and RECQ(1–400). Both were able strongly to promote annealing reaction, as 5 nM protein was able almost completely to generate annealing product (Fig. 6 and Supplementary Fig. 7A). On the other hand, the C-terminal RECQ4(455–1208) fragment showed only very weak annealing activity with only half of the annealed product observed at 40 nM concentration. Next we compared this activity to that of a known annealing protein, RAD52, and observed a lower but comparable ability to anneal complementary strands (Supplementary Fig. 7B and C).

To further narrow the domain responsible for the annealing activity, we also tested RECQ4(1–269) and RECQ4(269–400) containing two separate DNA-binding domains. We observed that the annealing ability of both fragments is significantly lower than that of RECQ4(1–400) with 4 times more RECQ4(1–269) and RECQ4(269–400) required to reach comparable annealing activity at 5 nM (Fig. 6B). Taken together, this indicates that the N-terminal region is responsible for the annealing activity of the RECQ4 protein, and both interaction domains within regions 90–189 and 322–400 are required for this activity.

2.4 The annealing does not counteract helicase activity

Seeing that the N-terminus of RECQ4 possesses annealing activity, we wished to determine if this activity counteracts the weak helicase activity observed for the full-length RECQ4 protein [17-19]. First, we assessed the ATPase activity of RECQ4(455–1208) fragment containing the helicase domain with the Walker A motif. ATPase activity was indistinguishable from that of RECQ4wt (Supplementary Fig. 8B), although it was very weak compared to that of Srs2, another DNA repair helicase (Supplementary Fig. 8A). Next, we tested the unwinding of this fragment and observed no difference compared to RECQ4wt (Supplementary Fig. 9A and B). This is in contrast to another RecQ helicase (BLM) that was able completely to unwind 3' overhang at 12.5 nM. In summary, our data indicate that the absence of the RECQ4 N-terminus with its annealing activity has no effect on ATPase and helicase activities.

3. Discussion

The RECQ4 protein plays multiple roles in the maintenance of genome stability. *In vivo* studies suggest that the N-terminus is essential for cell viability and embryogenesis [33], whereas the C-terminus (mainly the conserved helicase domain) is responsible for genome integrity and preventing cancer predisposition [31, 32]. Even though many biochemical experiments show interactions between RECQ4 and a wide range of proteins involved in DNA replication and repair, very little is known about the role of RECQ4.

Here, we wished to characterize the DNA binding properties of RECQ4. Our data demonstrate that RECQ4 contains at least three DNA-binding domains. One less specific DNA-binding domain is situated within the N-terminus of RECQ4 within 90–189 aa. Notably, MCM10, which is an integral component of the MCM replicative helicase complex

participating in DNA replication in humans, interacts with the N-terminus (1–200 aa) of RECQ4 [20, 21]. This interaction indicates a possible direct role of RECQ4 in initiating replication. It might be possible that the main function of the 90–189 aa DNA-binding domain is targeting of MCM10 or other components of replisome to the origin of replication. The overlapping protein and DNA interaction sites also suggest possible mutual interference, a point needing to be addressed in the future. Even though a DNA interaction motif within the first 54 amino acids has been observed previously [34], we were not able to detect significant DNA binding activity within the first 90 amino acids. Dramatically high concentrations of RECQ4 (32 μM) needed to be used in order to detect some DNA binding [34]. We cannot, however, exclude the possibility that an additional 40 amino acids might have affected the folding of an otherwise disordered region and thus affected the interaction.

The second DNA-binding domain was identified within the region 322–400 aa. To our surprise, this domain shows a very high affinity for HJ compared to ssDNA or other structures. In addition, the affinity of RECQ4(1-400) for HJ is undistinguishable from the wild-type RECQ4 (Supplementary Figs. 3A and B), indicating that this domain is responsible for HJ binding within the full-length protein. We cannot exclude that C-terminal domain could have an auxiliary effect to HJ binding of wild-type. However, the contribution of this domain for HJ binding is only minor, as 12 nM RECQ4(1-400) binds 90% of HJ compare to 10% bound by the same amount of RECQ(455-1208). This clearly indicates that RECQ4 might play a direct role in processing of HJ-containing structures. Even though RECQ4 does not contain the conserved HRDC domain of RecQ helicases, it might be possible that another DNA-binding domain has evolved in the N-terminal part to facilitate the molecular function of the RECQ4 protein. It is known that stalled replication forks can regress to form HJs in order to bypass DNA lesion and enable restart of replication forks [35-39]. Alternatively, the HJ, regressed replication forks or stalled replication fork can be cleaved by structure-selective endonucleases, thus leading to generation of DSBs which are then repaired by homologous recombination (HR) [40-42]. Among RecQ helicases, BLM already has been shown to dissolve double HJs and prevent sister chromatid exchanges and thus possibly to suppress the high incidence of cancer in BS patients [43, 44]. BLM also has been reported to co-localize within DNA bridges as well as ultra-fine anaphase bridges that are believed to represent un-replicated and difficult-to-replicate regions of genome and HR intermediates that re-formed

late in G2/M phase [45]. RECQ4 was recently identified as a BLM-interacting protein, although this interaction leads to inhibition of BLM-mediated unwinding of HJ by RECQ4 in contrast to other DNA substrates [46], indicating specific affinity of RECQ4 for this substrate. In addition, RECQ1 was shown to promote fork restoration and this activity is inhibited by PARP1 [47]. The ability of RECQ4 to bind both HJ and PARP1 (our as well as other data [48]) thus suggests a role in the regulation of fork restart. We therefore hypothesize that RECQ4 might recognize HJ structures and that even while it can stabilize and protect them against the activities of some proteins it might target others required for their processing.

Furthermore, the ability of RECQ4 to strongly bind HJs also can help to explain its role in mitochondrial genome and telomere maintenance [27, 49, 50]. Indeed, human heart mtDNA contains abundant HJs [51] and RECQ4 (alone or together with other factors) can be required to remove such intermediates and thus facilitate mitochondrial DNA segregation. In yeast mitochondria, a key component of the mtDNA recombination machinery is the HJ resolvase or cruciform cutting endonuclease, Cce1p, where it works in concert with Mhr1p to maintain mtDNA [52, 53]. While individual deletions have little effect on mtDNA stability, loss of both genes leads to frequent and rapid mtDNA depletion [53]. In humans, mitochondrial genetic diseases linked with nuclear genes affect replication of mtDNA either directly at the replication fork or by nucleotide supply [54]. In view of RECQ4's possible role during replication, it is not surprising that symptoms associated with deficiency in some of these genes (POLG, TWINKLE) show similarity to those of subsets of RTS patients, including mental retardation associated with atrophy of the brain [55-57]. A role of RECQ4 was recently suggested also in telomere maintenance, as knock-down and patient-derived cells exhibit elevated levels of fragile sites and breaks at telomeres, and it was shown to directly interact and promote D-loop unwinding together with the WRN helicase [27]. RECQ4 thus could help target WRN to the replication-stalled sites at telomeric regions and promote their unwinding, even though it blocks accessibility of HJs for BLM [58]. This might be similar to the action of TRF2, which has been shown to recognize and protect cleavage of HJs at telomeres [59, 60] and inhibits WRN activities at HJs with telomeric repeats or WRN-mediated formation of T-circles [61]. Accordingly, RECQ4 and TRF2 also have been shown functionally to interact [27] and could be responsible for recruitment and assembly of the shelterin complex after replication.

The third DNA-binding domain of RECQ4 resides within a conserved helicase motif, suggesting that this DNA-binding domain is likely to be crucial for the helicase and ATPase activity of RECQ4 [17-19]. Nevertheless, RECQ4's helicase activity is very low and is limited by substrate specificity [19]. Similarly, the ATPase activity of RECQ4 is very weak in comparison to that of yeast Srs2 helicase or other RecQ helicases [58]. Nevertheless, it cannot be excluded that post-translational modification or accessory factor can influence ATPase and helicase activities. Furthermore, it has been suggested that annealing activity of RECQ4 can mask helicase activity [18, 19, 62]. While we have mapped annealing activity to the N-terminal region, its separation from the helicase domain has no effect on RECQ4's ATPase or helicase activity. We therefore suggest that, in contrast to the other members of the RecQ family, the low level of ATPase and helicase activity does not point to a role for RECQ4 in direct unwinding of DNA intermediates during replication alongside MCM2-7 [63].

Taken together, the N-terminus of RECQ4 contains two DNA-binding domains which are responsible for the affinities of the full-length RECQ4 protein and facilitate single-strand annealing activity. In addition, the region of RECQ4 within 322–400 aa selectively recognizes HJs, and that could be essential for the function of RECQ4 in DNA replication and repair. This is in agreement with previous *in vivo* studies showing that the N-terminus of RECQ4 is indispensable and sufficient for cell viability [32, 33] and DNA repair [64]. We hypothesize that RECQ4 binds and targets other proteins to specific DNA substrates which are formed during DNA replication or recombination to ensure their timely and coordinated processing. Alternatively, it has been proposed that the role in strand annealing or annealing-mediated strand exchange is involved in template switching or fork regression. These activities might, however, be inhibited by the natural presence of another ssDNA binding protein, RPA [15, 20], and so this needs to be further evaluated *in vivo.* Despite links to defined genetic disorders, the role of RECQ4 remains mysterious. It is a point of great interest for further understanding the biochemical function of RECQL4 in maintenance of nuclear and mitochondrial genomes and telomeres. During the preparation of this manuscript a report appeared confirming identification of several DNA-binding domains with RECQ4 and high binding affinity to G4 structures [65].

4. Methods

4.1. Cloning of RECQ4 fragments

Individual *RECQ4* fragments were amplified by PCR from the template plasmid pET32a-*RECQ4* using corresponding primers (Table 1). The final PCR product included the coding sequence for a particular fragment and a C-terminal His-tag flanked by *Eco*RI restriction sites. PCR products were inserted into pCR-Blunt II-TOPO vector (Invitrogen) and verified by restriction analysis using *Eco*RI enzyme and sequencing. pCR-BluntII-TOPO vector containing corresponding *RECQ4* fragment and expression vector pMAL-c2x were digested with *Eco*RI endonuclease. Linearized expression vector was dephosphorylated by Antarctic phosphatase to prevent its rejoining, and this was followed by ligation with individual RECQ4 fragments.

4.2. Expression and purification of RECQ4 fragments

Bacterial strain *E. coli* BL21(DE3)pLysS was transformed with individual plasmids encoding various RECQ4 fragments. The cultures were grown overnight and protein expression was induced by addition of 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) at 16°C overnight. Twenty grams of cell pellet were sonicated in 80 ml of breakage buffer (50 mM Tris-HCl [pH 7.5], 10% sucrose, 2 mM ethylenediaminetetraacetic acid [EDTA], 200 mM KCl, 0.01% NP40, 1 mM β-mercaptoethanol) and a cocktail of protease inhibitors (aprotinin, chymostatin, leupeptin, pepstatin A and benzamidine hydrochloride at 5 mg/ml each). The lysate was clarified by ultracentrifugation (100,000 x g, 1 h, 4°C) and the supernatant was incubated with 1 ml of His-Select nickel affinity gel (Sigma) at 4°C overnight. The beads were washed with 15 ml of buffer K (20 mM K_2HPO_4, 10% glycerol, 0.5 mM EDTA, 150 mM KCl, 0.01% NP40, 1 mM β-mercaptoethanol) containing 10 mM imidazole. The bound protein was eluted by K buffer containing increasing concentrations of imidazole (150–1000 mM). Fractions containing RECQ4 fragments were then incubated with 0.5 ml of amylose beads (NEB) at 4°C for 1 h. The beads were washed with 15 ml of K buffer and proteins were eluted by K buffer containing 10 mM maltose. Pooled protein fractions were loaded onto a 0.5 ml MonoS/MonoQ column (GE Healthcare) and eluted using a 10 ml gradient of 150–1000 mM KCl in K buffer. Purified RECQ4 fragments were concentrated using a VivaSpin-2. RECQ4wt was expressed and purified as previously described [15].

4.3. DNA substrates

All oligonucleotides used in this study were supplied by VBC Biotech (Austria) and are summarized in Table 2. Synthetic DNA substrates used in the EMSA (Table 3) were prepared as described by Matulova et al. [66].

For atomic force microscopy, the replication intermediates isolated from *Xenopus laevis* egg extracts were kindly provided by Vincenzo Costanzo (IFOM, Italy). For further details on preparation, see Hashimoto et al. [67].

4.4. Electrophoretic mobility shift assay (EMSA)

The reaction mixture consisted of reaction buffer D (30 mM Tris [pH 7.5], 1 mM DTT, 100 mM KCl, 100 µg/ml BSA), 3 nM fluorescently labeled DNA substrate, and indicated concentrations of the RECQ4 protein. The reaction mixtures were incubated at 37°C for 20 min. After the addition of loading buffer (60% glycerol, 10 mM Tris [pH 7.5], 60 mM EDTA), the reaction mixtures were resolved in 7.5% native polyacrylamide gel in 0.5x TBE running buffer (45 mM Tris-ultrapure, 4.5 mM boric acid, 0.1 mM EDTA, pH 8). Gels were scanned by an Image Reader FLA-9000 Starion (Fujifilm) and quantified using MultiGauge V3.2 software (Fujifilm).

Two competition assays were used in this study. In the first, a mixture of 1.5 nM FITC-ssDNA and 1.5 nM FITC-Holliday junction (HJ) substrates was incubated with indicated concentrations of RECQ4 at 37°C for 20 min. After adding loading buffer, the reaction was separated on 7.5% native polyacrylamide gel in 0.5x TBE running buffer and analyzed as described above. In the second competition assay, RECQ4 was pre-incubated with 1.5 nM Cy5-ssDNA to form a protein–DNA complex at 37°C for 10 min. After pre-incubation, the increasing concentrations of FITC-HJs were added to the reaction followed by additional incubation at 37°C for 15 min. The reaction was resolved and analyzed as above.

4.5. Atomic Force Microscopy (AFM)

For AFM imaging, 10 µl reactions containing 1 ng/µl of MBP-RECQ4(1–400) and 0.5 ng/µl of replication intermediates from *Xenopus laevis* egg extracts were incubated for 20 min at 37°C in reaction buffer (30 mM HEPES-KOH [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 0.5

mM DTT). Upon incubation, the solution was deposited on freshly cleaved mica (V1 grade) (SPI Supplies), incubated for one minute, then washed with MilliQ water and air-dried.

Images were obtained on a JKP NanoWizard® 3a BioScience AFM (JPK Instruments) operating in AC (tapping) mode in air using standard AFM probe ACTA (APPNANO).

4.6. Single-strand annealing assay (SSA)

The reaction was set up essentially as described by Altmannova et al. [68]. Briefly, a mixture of 3 nM fluorescently labeled ssDNA (pR27*) and 7 nM non-labeled complementary ssDNA (pR28) was incubated with the indicated amount of RECQ4 in D buffer (30 mM Tris [pH 7.5], 1 mM DTT, 100 mM KCl, 5 mM $MgCl_2$, 100 µg/ml BSA) at 37°C for 20 min. The reaction was stopped by addition of 5% SDS and 0.5 mg/ml proteinase K and incubated at 37°C for 5 min. After adding loading buffer, the samples were resolved on a 10% native polyacrylamide gel in 1x TBE running buffer. Gels were scanned using an FLA-9000 Starion (Fujifilm) and quantified by MultiGauge software (Fujifilm).

In time-course experiments, the appropriate concentration of RECQ4 or RAD52 was mixed with the final reaction volume. The reaction was incubated at 37°C and the aliquots from reaction mixtures were taken at indicated time points (0, 2, 4, 6 and 8 min). To stop the reaction, 5% SDS and 0.5 mg/ml proteinase K were added to the collected samples. The reaction was resolved and analyzed as above.

4.7. ATPase assay

ATPase reaction was performed essentially as described elsewhere [69]. The reaction mixture included a purified protein, DNA cofactor (75 µM nucleotides, pR33), A buffer (30 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.5 mM DTT, 0.1 mg/ml BSA, 1 mM $MgCl_2$), and a mixture of unlabeled 10 mM ATP and 148 Bq/µl γ-^{32}P-ATP. The reaction was incubated at the chosen temperature and aliquots from reaction mixtures were taken at indicated time points (0, 5, 10, 20 and 30 min). To stop the reaction, 5% SDS was added to the collected samples. The reaction products were separated by thin-layer chromatography on cellulose plates. These were analyzed by phosphorimaging using an FLA-9000 Starion (Fujifilm) scanner and the amount of labeled phosphate released during ATP hydrolysis was quantified using MultiGauge software (Fujifilm).

4.8. Helicase assay

This assay has been described previously [69]. Briefly, fluorescently labeled 3' overhang substrate was incubated with appropriate concentrations of RECQ4/BLM protein in buffer H (30 mM Tris [pH 7.5], 1 mM DTT, 0.1 mg/ml BSA, 100 mM KCl, 20 mM creatine phosphate, 20 μg/ml creatine kinase, 2.4 mM $MgCl_2$, and 2 mM ATP) for 20 min at 37°C. The reaction was stopped by addition of 5% SDS and 0.5 mg/ml proteinase K and incubated for 5 min at 37°C. After adding loading buffer, the reaction products were separated on a 13% native polyacrylamide gel in 1x TBE running buffer. Gels were scanned by an Image Reader FLA-9000 Starion (Fujifilm) and quantified by MultiGauge V3.2 software (Fujifilm).

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FIGURE LEGENDS

Fig. 1. N-terminus of RECQ4 binds dsDNA more effectively than C-terminus. (A) Schematic illustration of RECQ4 and its fragments used in this study. (B) DNA binding of RECQ4. Increasing concentrations of RECQ4(1–400), RECQ4(1–492) and RECQ4(455–1208) (50, 100, 150 and 200 nM) were incubated with 3 nM FITC-labeled dsDNA for 20 min at 37°C. Quantification reported as mean ± SD based on three independent experiments.

Fig. 2. RECQ4(1–400) has high affinity for Holliday junction structures. (A) EMSA reactions containing increasing amounts of RECQ4(1–400) (6, 12, 25 and 50 nM, lanes 2–5) were incubated with 3 nM FITC-labeled ssDNA, dsDNA, 3' flap, bubble and HJ structures at 37°C for 20 min. (B) Quantification of the data in (A) reported as mean ± SD based on three independent experiments.

Fig. 3. DNA binding preference of RECQ4(1–400) using competition assays. (A) Increasing amounts of RECQ4(1–400) (6, 12, 25, 50 and 100 nM, lanes 2–6) were incubated with a mixture of FITC-labeled ssDNA and FITC-labeled HJ (3 nM each) in 1:1 ratio at 37°C for 20 min. (B) Quantification of the competition assay in (A) shown as mean ± SD based on three independent experiments. (C) RECQ4(1–400) was pre-incubated with Cy5-labeled ssDNA (green; 1.5 nM) to form a complex at 37°C for 15 min. After pre-incubation, increasing concentrations of FITC-labeled HJs (red; 10, 20, 30, 40 and 50 nM, lanes 2–7) were added. (D) The mean values for bound ssDNA from (C) shown ±SD from three independent experiments.

Fig. 4. Visualization of RECQ4(1–400) protein binding to replication forks by atomic force microscopy. (A) Overview of a larger area containing two DNA molecules. (B) Detail of replication fork with bound RECQ4(1–400) indicated by an arrow. (C) 3D profile demonstrating the difference in height between naked DNA and protein bound to DNA.

Fig. 5. RECQ4 contains several DNA-binding domains. (A) Quantification of various RECQ4 fragments and their affinities for HJ. Means ± SD shown are based on three independent

experiments. (B) Schematic representation of potential DNA-binding domains in the RECQ4 protein. Orange boxes represent the conserved helicase domain and the green box indicates the Sld2 domain. Violet and red boxes show mapped DNA binding sites in the RECQ4 protein.

Fig. 6. N-terminus of RECQ4 promotes annealing activity. (A) Reaction mixtures containing the indicated amounts (5, 10, 20 and 40 nM, lanes 2–5) of RECQ4(1–400), RECQ4(455–1208), RECQ4(1–269) and RECQ4(269–400) were incubated with 3 nM FITC-labeled ssDNA and non-labeled complementary ssDNA at 37°C for 20 min. (B) The plot presents mean values in (A) ±SD from three independent experiments.

Table 1

Primers used for cloning RECQ4 fragments.

Primer name	Primer usage	Sequence (5′-3′)	Length (nt)
pR401	RECQ4 fragment 500/600–1208 rev	AATGAATTCTTAGTGATGGTGATGGTGATGGTG ATGGTGCCC	42
pR405	RECQ4 fragment 1–500/600 for	AAGGAATTCATGGAGCGGCTGCGGGACGTGC	31
pR577	RECQ4 fragment 1–400 rev	ATTGAATTCTTAGTGGTGGTGATGATGATGGTG ATGGTGCTTGGTTGTGACTGTGGCACC	60
pR705	RECQ4 fragment 1–492 rev	CTTGAATTCTTAGTGGTGGTGATGATGATGGTG ATGGTGGATCCGCATGACTGCACGCTC	60
pR825	RECQ4 fragment 1–189 rev	GTTGAATTCTTAGTGGTGGTGATGATGATGGTG ATGGTGGCCAGGATCTAGGGAGCCCAG	57
pR826	RECQ4 fragment 1–269 rev	TTTGAATTCTTAGTGGTGGTGATGATGATGGTG ATGGTGCCAGGGCTCCTCGTTCCATCT	57
pR1011	RECQ4 fragment 1–322 rev	TTTGAATTCTTAGTGGTGGTGATGATGATGGTG ATGGTGGAGTCCGTGGTACCTGGGGTT	60
pR1012	RECQ4 fragment 1–90 rev	CCCGAATTCTTAGTGGTGGTGATGATGATGGTG ATGGTGTGGACTCTTGGTCGCAGCCC	59
pR1013	RECQ4 fragment 455–1208 for	TTGGAATTCCTGGGGCCCTCAGGGCAGTT	29
pR2001	RECQ4 fragment (269–400) for	AAGGAATTCTGGGAGAGCCCCGCACAGGTCCA	32

Table 2

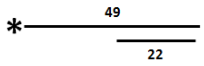
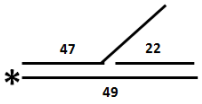
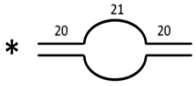
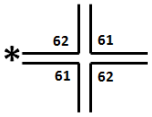
Primers used for preparing DNA substrates.

Primer name	Primer usage	Sequence (5‘-3’)	Length (nt)
pR26	3’ flap (with pR27* and pR29) 3’ overhang (with pR27*)	AATTCGTGCAGGCATGGTAGCT	22
pR27*	ssDNA, dsDNA (with pR28), 3’ overhang (with pR26) 3’ flap (with pR26 and pR29)	AGCTACCATGCCTGCACGAATTAAGCAATTC GTAATCATGGTCATAGCT	49
pR28	complementary to pR27*	AGCTATGACCATGATTACGAATTGCTTAATTC GTGCAGGCATGGTAGCT	49
pR29	3’ flap (with pR26 and pR27*)	AGCTATGACCATGATTACGAATTGCTTGGAA TCCTGACGAACTGTAG	47
pR33	83mer used for ATPase assay	TTTATATCCTTTACTTTATTTTCTATGTTTATTC ATTTACTTATTTTGTATTATCCTTATACTTATTT ACTTTATGTTCATTT	83
pR101*	bubble	GACGCTGCCGAATTCTACCAGTGCCTTGCTA GGACATCTTTGCCCACCTGCAGGTTCACCC	61
pR102		GGGTGAACCTGCAGGTGGGCGGCTGCTCATC GTAGGTTAGTTGGTAGAATTCGGCAGCGTC	61
pR139*	HJs	GACGCTGCCGAATTCTACCAGTGCCTTGCTA GGACATCTTTGCCCACCTGCAGGTTCACCC	61
pR140		TGGGTGAACCTGCAGGTGGGCAAAGATGTC CATCTGTTGTAATCGTCAAGCTTTATGCCGTT	62
pR141		GGGTGAACCTGCAGGTGGGCAAAAATGTCCT AGCAAGGCACTGGTAGAATTCGGCAGCGTC	61
pR142		GAACGGCATAAAGCTTGACGATTACAACAGA TGGACATTTTTGCCCACCTGCAGGTTCACCC	62
pR288**	ssDNA	AGCTACCATGCCTGCACGAATTAAGCAATTC GTAATCATGGTCATAGCT	49

*FITC-labeled primer, **Cy5-labeled primer.

Table 3

Structures of DNA substrates.

DNA substrate	Structure
3′ overhang	
3′ flap	
bubble	
HJ	

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