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AtRECQ2, a RecQ helicase homologue from *Arabidopsis thaliana*, is able to disrupt various recombinogenic DNA structures *in vitro*

Daniela Kobbe, Sandra Blanck, Katharina Demand, Manfred Focke and Holger Puchta*

Botanik II, Universität Karlsruhe (TH), 76128 Karlsruhe, Germany

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*For correspondence (fax +49 721 608 4874; e-mail holger.puchta@bio.uka.de).

Summary

RecQ helicases play an important role in the maintenance of genomic stability in pro- and eukaryotes. This is highlighted by the human genetic diseases Werner, Bloom's and Rothmund–Thomson syndrome, caused by respective mutations in three of the five human *RECQ* genes. The highest numbers of *RECQ* homologous genes are found in plants, e.g. seven in *Arabidopsis thaliana*. However, only limited information is available on the functions of plant RecQ helicases, and no biochemical characterization has been performed. Here, we demonstrate that AtRECQ2 is a (d)NTP-dependent 3′→5′ DNA helicase. We further characterized its basal properties and its action on various partial DNA duplexes. Importantly, we demonstrate that AtRECQ2 is able to disrupt recombinogenic structures: by disrupting various D-loop structures, AtRECQ2 may prevent non-productive recombination events on the one hand, and may channel repair processes into non-recombinogenic pathways on the other hand, thus facilitating genomic stability. We show that a synthetic partially mobile Holliday junction is processed towards splayed-arm products, possibly indicating a branch migration function for AtRECQ2. The biochemical properties defined in this work support the hypothesis that AtRECQ2 might be functionally orthologous to the helicase part of the human RecQ homologue HsWRN.

Keywords: DNA repair, DNA recombination, RecQ, Holliday junction, D-loop, WRN.

Introduction

Members of the RecQ helicase family have attracted wide interest over the last two decades, as mutations in various RecQ genes have been linked to a higher incidence of cancer. RecQ helicases contribute to genome integrity. A considerable amount of data has been collected in recent years, and the functions of RecQ helicases have started to emerge (Bachrati and Hickson, 2003; Hanada and Hickson, 2007; Hickson, 2003; Opresko *et al.*, 2004; Sharma *et al.*, 2006).

RecQ homologues can be found in all kingdoms of life. However, organisms differ in the number of homologues, the presence or absence of certain domains, and the length and sequence of the non-conserved regions (Hartung and Puchta, 2006). *Escherichia coli* and *Saccahromyces cerevisiae* are two model organisms with a single homologue each, EcRECQ and ScSGS1. Humans have five homologues, and the plant model organism *Arabidopsis thaliana* has seven homologues (Hartung and Puchta, 2006; Hartung *et al.*, 2000; Figure 1).

Several observations imply specialization of function within the RecQ homologues of one organism, such as the varying modular domain composition of the proteins as well as various symptoms of the genetic diseases or mutant phenotypes. The various RecQ homologues of plants are especially interesting. Their analysis may reveal both unique plant-specific specializations and interesting evolutionary relationships for RecQ proteins.

Although *Arabidopsis thaliana* does not possess a RecQ homologue with the same modular composition as HsWRN, it possesses a protein that is homologous to the exonuclease domain of HsWRN called AtWRNexo (encoded by At4g13870) and three candidates with the same residual domain structure: AtRECQ2, AtRECQ4A and AtRECQ4B (Figure 1). AtWRNexo is able to interact with AtRECQ2 (encoded by At1g31360) in a yeast two-hybrid assay (Hartung *et al.*, 2000) and was analyzed biochemically (Li *et al.*, 2005; Plchova *et al.*, 2003). We decided to analyze

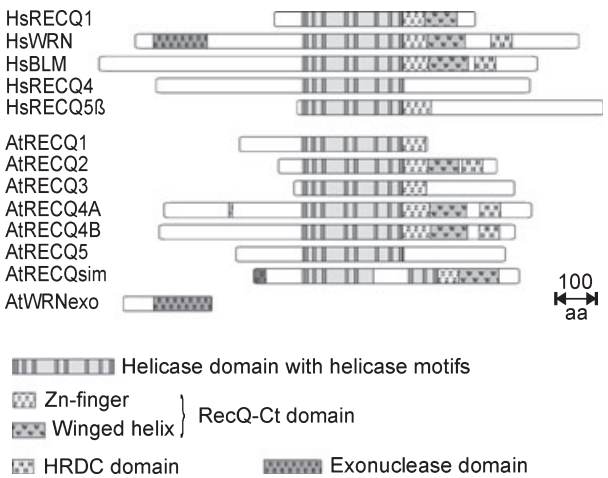

Figure 1. Modular structure of the RecQ proteins from *Homo sapiens* and *Arabidopsis thaliana*.
The domains shown were identified either by the ProDom entries PD209099 or PD215317, by Pfam entries or manually. The most important domains are defined; in addition, an EF-hand motif is present at the N-terminus of AtRECQ4A and a ubiquitin-associated domain is present at the N-terminus of AtRECQsim.

the biochemical functions of AtRECQ2 in order to classify it as either orthologous to the HsWRN helicase or as a protein with its own set of functions.

We expressed AtRECQ2 in *E. coli* and successfully purified it. Except for HsRECQ4 (Macris *et al.*, 2006), all characterized RECQ homologues are ATP-dependent 3′→5′ DNA helicases, as is AtRECQ2. One primary function of RecQ helicases is the processing of DNA structures resulting from stalled replication forks or recombination intermediates. In this respect, notable differences have been reported for various homologues, mirroring the specialization of the various members of the RecQ helicase family. In addition to characterization of the basal properties of AtRECQ2, we show that AtRECQ2 disrupts D-loop structures and a Holliday junction in the same way as HsWRN does.

Results

Purification of AtRECQ2 and AtRECQ2-K117M

The *Arabidopsis thaliana* RECQ2 protein (NCBI accession number CAC14866), which is deduced to have 705 amino acids and a molecular weight of 79.4 kDa, contains conserved helicase, RecQ-Ct (C-terminal) and HRDC (Helicase and RNaseD C-terminal) domains. The invariant lysine in the Walker A motif was determined to be K117 (counted without tags), and is equivalent to HsWRN-K577. As HsWRN-K577M did not show ATPase or helicase activity (Brosh *et al.*, 1999), we used the corresponding AtRECQ2 mutant as a negative control. After cloning both ORFs in respective vectors, we purified AtRECQ2 and AtRECQ2-K117M (total calculated mass including tags 85.5 kDa) from *E. coli* as described in Experimental procedures, making use of the N-terminal calmodulin-binding peptide (CBP) tag and the C-terminal histidine (His) tag. The purified proteins migrated as single bands of the expected size on a colloidal Coomassie-stained SDS–PAGE gel (Figure 2a). Using antibodies directed against the N-terminal FLAG tag and the C-terminal His tag, it was demonstrated that the proteins were full length (data not shown).

The ATPase activity of AtRECQ2 is dependent on DNA

The ATPase activity of AtRECQ2 was measured in the absence of DNA as well as using an 80-mer dT oligonucleotide and M13mp18 ssDNA. Significant activity is observed for the native protein but not with the K117M Walker A motif variant, and only in the presence of DNA (Figure 2b). Both the 80 nt and 7249 nt single-stranded DNA promote ATPase activity to the same order of magnitude.

AtRECQ2 has helicase activity

AtRECQ2 does not only contain helicase motifs but is able to displace an oligonucleotide with 17 bp of complementarity to M13mp18 ssDNA and 10 nt 3′ and 5′ overhangs. This reaction is dependent on ATP (Figure 2c). Additionally, comparison of AtRECQ2-K117M with the AtRECQ2 protein shows that the mutant enzyme preparation is not only devoid of significant contaminating ATPase activities (Figure 2b) but also helicase activities, and the AtRECQ2 preparation is thus suitable for usage in these studies.

Basic properties of AtRECQ2

Using the M13-based substrate described above, we defined the optimal pH and salt concentration for the strand-unwinding activity of AtRECQ2. The experiments (Figure 2d,e) revealed a broad range of both pH and potassium acetate concentration supporting unwinding activity. We thus carried out all further experiments at pH 8 in the presence of 50 mM potassium acetate. DNA unwinding by AtRECQ2 is also dependent on the presence of divalent cations (Figure 2f): without divalent cations, no unwinding was observed. For unwinding activity of AtRECQ2, the standard cation Mg^{2+} can be replaced by Mn^{2+}, and, to a lesser extent, also by Ca^{2+}. However, Zn^{2+} does not support strand unwinding. (d)NTPs must also be present for significant DNA unwinding by AtRECQ2 (Figure 2g): all (d)NTPs tested support strand unwinding. However, the extent of strand unwinding by AtRECQ2 is greatest with ATP and dATP.

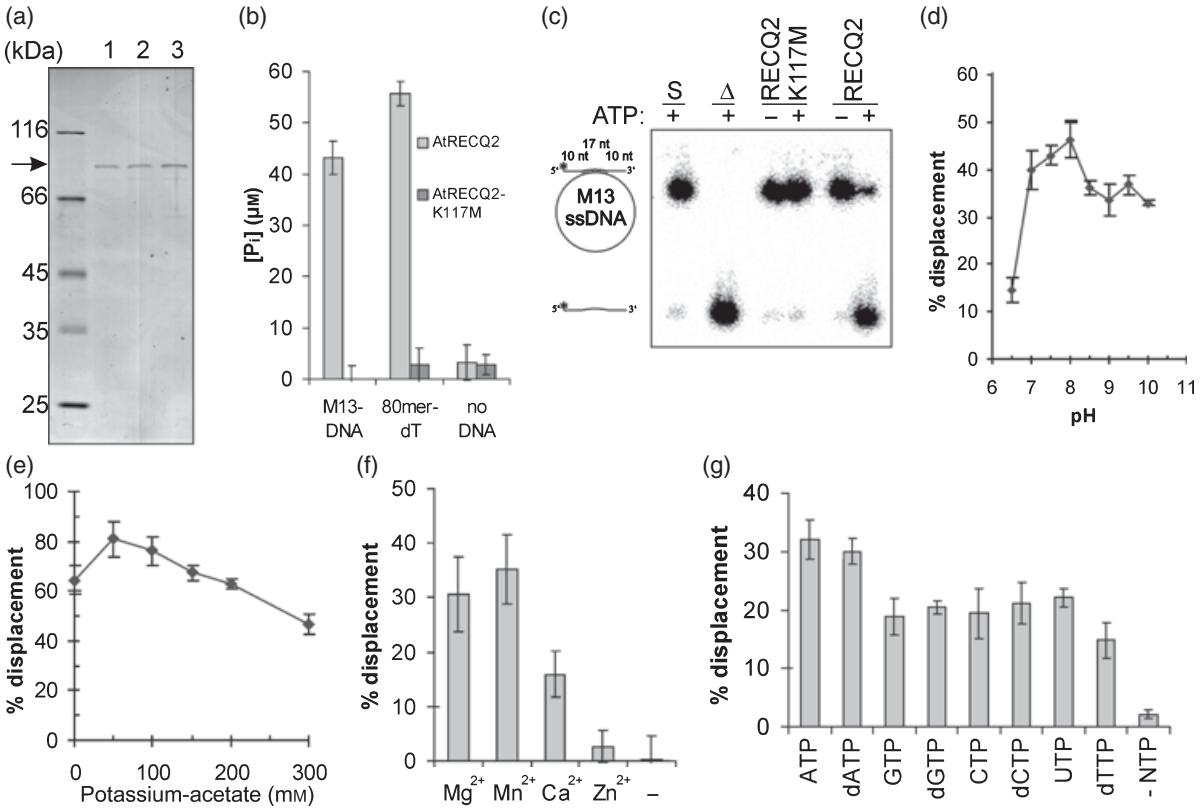

Figure 2. Purification, ATPase and DNA helicase activities of recombinant AtRECQ2.
(a) 10% SDS–PAGE analysis of two representative purifications of AtRECQ2 (lanes 1 and 2) and one of AtRECQ2-K117M (lane 3). The proteins were overexpressed in *Escherichia coli* and purified by Ni-immobilized metal ion affinity chromatography (IMAC) and CaM affinity chromatography. Approximately 40 ng of protein were electrophoresed and stained with colloidal Coomassie blue. The arrow indicates the proteins of interest, with a predicted molecular weight of 85.5 kDa.
(b) ATPase activity (as μM P_i released) of 8 nM AtRECQ2 and AtRECQ2-K117M in the presence and absence of DNA co-factors. M13-DNA, M13mp18 ssDNA; 80mer-dT, a DNA oligonucleotide comprising 80 thymidines.
(c) Dependence of helicase activity on the presence of ATP. The substrate and product are shown on the left. The asterisk marks the ^{32}P label. S, substrate, Δ, heat-denatured substrate. The reactions were incubated with 10 nM enzyme for 20 min.
(d–g) Reactions were performed with 5 nM AtRECQ2 for 20 min, using the M13-based substrate as shown in (c) and changing specific parameters. The displaced labeled strand was quantified. Helicase data are the means of three experiments, with SD indicated by error bars.
(d) Activity of AtRECQ2 at various pH values (all Tris-acetate buffer) without additional salt.
(e) Activity of AtRECQ2 with various concentrations of potassium acetate at pH 8.
(f) Cationic requirements of AtRECQ2 for strand unwinding. $MgCl_2$ was substituted by the same concentrations of $MnCl_2$, $CaCl_2$, $ZnCl_2$ and water, respectively. DTT was omitted from the reactions.
(g) Requirement for (d)NTPs for strand unwinding by AtRECQ2. ATP was substituted by dATP, GTP, dGTP, CTP, dCTP, UTP, dTTP and water.

AtRECQ2 is a 3′→5′ DNA helicase

The directionality of AtRECQ2 was determined using two oligonucleotide-based substrates: one providing a 23 nt 3′ tail and the other providing a 23 nt 5′ tail. AtRECQ2 unwinds the 3′-tailed partial duplex but not the 5′-tailed one in a concentration-dependent manner (Figure 3a). AtRECQ2 is therefore classified as a 3′→5′ helicase.

The extent of AtRECQ2-mediated unwinding is influenced by the length of the duplex

Under our standard conditions, the 23 bp directionality substrate with the 3′ tail was significantly unwound (Figure 3a), but the partial duplex was not fully unwound even at the highest AtRECQ2 concentration. We therefore wished to define the influence of the length of the double-stranded region on unwinding. Within the same sequence context, we used substrates with shorter complementary regions: 21, 19, 17 and 15 bp. We were able to demonstrate that, under our standard conditions, reduction in the length of the duplex region leads to a significant increase of strand displacement by AtRECQ2 (Figure 3b).

AtRECQ2 requires at least 5 nt of 3′ overhang to unwind a partial duplex DNA substrate

Within the same sequence context as above, we tested for the minimal length requirement of the 3′ overhang for AtRECQ2-dependent unwinding of a 15 bp duplex (at 9 mM

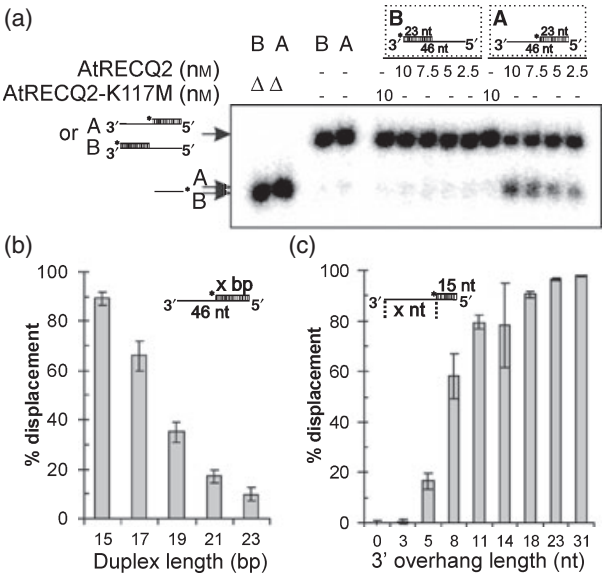

Figure 3. Directionality of AtRECQ2 and action on partial duplex DNA.
(a) Polarity of DNA unwinding by AtRECQ2. The partial duplexes used to demonstrate the directionality (A and B) are shown on the left; the asterisk indicates the ^{32}P label. The arrows indicate the positions of migration of the substrates (top) or the 23 nt unwinding products of substrates A and B (bottom). Incubation took place for 20 min in the presence of the indicated amounts of protein (nM). Δ, heat-denatured substrate. Helicase reaction products were resolved by 12% native TBE–PAGE and visualized by autoradiography.
(b, c) Quantification of the relative concentrations of displaced DNA from partial duplex DNA (means of three experiments with SD indicated by the error bars). AtRECQ2 (5 nM) was incubated with the various partial duplex DNA substrates for 20 min.
(b) Partial duplex with various duplex lengths, as shown.
(c) Partial duplex of 15 bp and various lengths of 3′ overhang as shown. The reaction was performed in 9 mM $MgCl_2$.

$MgCl_2$). Blunt-ended DNA and a duplex with 3 nt overhang were not processed by AtRECQ2. Duplexes with 3′ tails from 5–18 nt were unwound by AtRECQ2, with better unwinding observed as tail length was increased. However, no further enhancement was observed for a 23 or 31 nt 3′ tail (Figure 3c).

AtRECQ2 is able to process various D-loops

Previous studies have demonstrated that RecQ helicases are able to melt D-loops. We prepared an artificial DNA bubble structure with 21 unpaired nucleotides in the center and 20 complementary base pairs in both flanking sequences. In addition, D-loops composed of the same bubble and invading oligonucleotides providing either a 20 nt 3′ tail or a 20 nt 5′ tail or no tail were prepared. Melting of these structures was analyzed as a function of the AtRECQ2 concentration (Figure 4). Regardless of whether the invading strand had a 3′, 5′ or no tail, it was displaced from the bubble with approximately the same efficiency. This tendency was observed over the whole range of AtRECQ2 concentrations

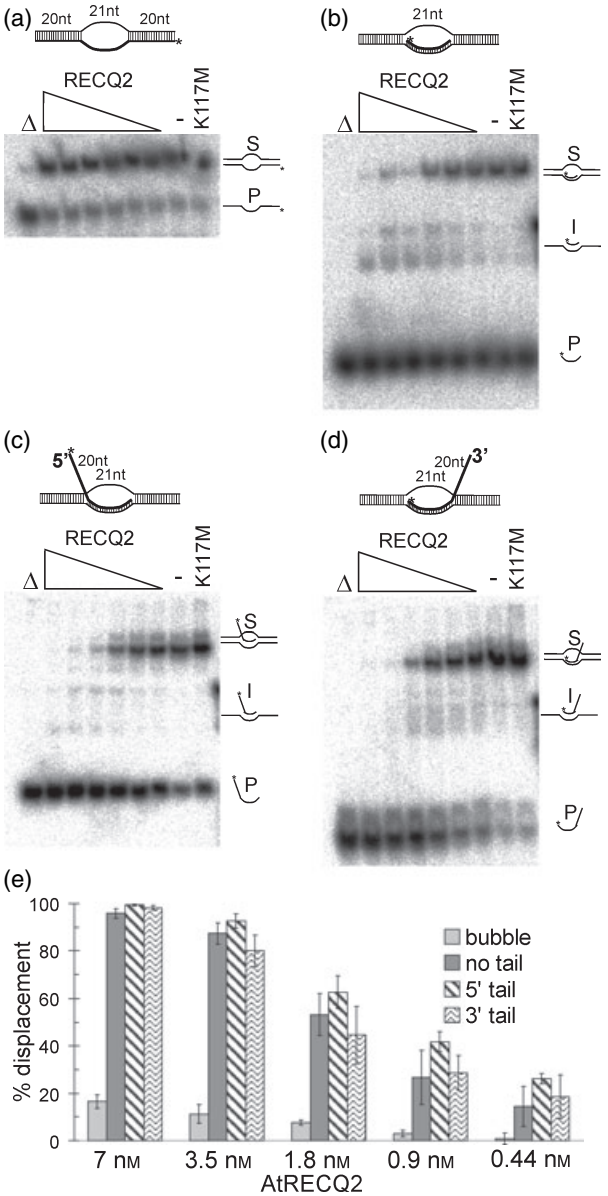

Figure 4. Melting of synthetic bubble and D-loops by AtRECQ2 helicase.
(a–d) Decreasing concentrations of AtRECQ2 (7, 5.3, 3.5, 1.8, 0.9 and 0.44 mM) and 10 nM of AtRECQ2-K117M (lanes labeled K117M) were incubated for 30 min under the conditions described in Experimental procedures. Helicase reaction products were analyzed by 10% native TBE–PAGE. On the right of each panel, the substrate (S), the respective intermediate forked structure (I) and the released invading strand product (P) are shown. The asterisk indicates the ^{32}P label. Δ, heat-denatured substrate control, –, no enzyme.
(e) Quantification of data obtained from repetitions of experiments shown in (a)–(d) with selected AtRECQ2 concentrations. Helicase data represent the mean of three experiments, with SD indicated by error bars.

tested. AtRECQ2 also disrupted the bubble structure, but much less efficiently. Similar experiments for which one oligonucleotide of the bubble was labeled (as in Figure 4a) confirmed these results, and revealed that the presence of an invading strand has no influence on unwinding of the bubble structure (data not shown).

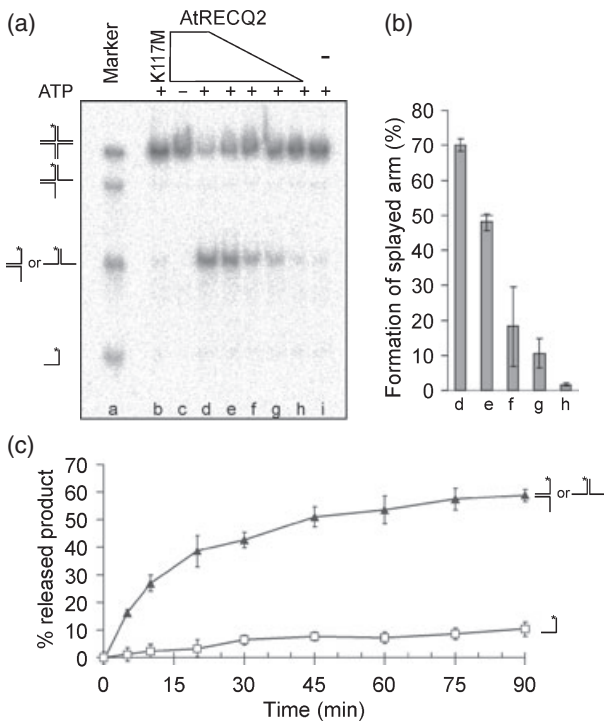

Figure 5. Branch migration of Holliday junctions by AtRECQ2.
(a, b) Various concentrations of AtRECQ2 (lanes c and d, 7 nM; lane e, 3.5 nM; lane f, 1.8 nM; lane g, 0.9 nM; lane h, 0.44 nM) and 10 nM of AtRECQ2-K117M (lane b) were incubated for 1 h with a partially mobile X12 Holliday junction under the conditions described in Experimental procedures, except for lane c, for which ATP was omitted.
(a) Autoradiography of the reaction products migrated on 10% TBE–PAGE. Lane a contains markers comprising (from top to bottom) an intact Holliday junction, a three-way junction, a splayed-arm product (the product of branch migration) and ssDNA.
(b) Relative concentrations of the splayed-arm product for various AtRECQ2 concentrations as a percentage of total DNA. Error bars indicate SD of the mean of three experiments.
(c) Kinetic experiments were performed with 7 nM of AtRECQ2 enzyme and the X12 Holliday junction as described in Experimental procedures. The splayed-arm (▲) and ssDNA (□)reaction products were quantified. Error bars indicate SD of the mean of six experiments.

AtRECQ2 can mediate branch migration of Holliday junctions

Holliday junctions are important intermediate structures formed during recombination and, under certain circumstances, during replication. When analyzing the reaction of AtRECQ2 with a synthetic Holliday junction containing a homologous core of 12 bp flanked by arms of 19 bp, we observed concentration-dependent and ATP-dependent branch migration, resulting in splayed-arm products (Figure 5a,b). Relatively small amounts of three-way junctions and single strands were formed. AtRECQ2-K117M did not process the Holliday junction. Time-course experiments (Figure 5c) showed an increase in splayed-arm products over 90 min. The amount of single-stranded product formed was less than 10%.

Discussion

An increasing amount of scientific literature places human RecQ helicases at the center of interest in molecular medicine. Plants are special in that they possess the highest numbers of RecQ homologues, e.g. seven in *Arabidopsis thaliana*. In this work, we analyzed the biochemical properties of AtRECQ2. This protein possesses all domains typical for RecQ helicases (Figure 1).

First of all, we were able to demonstrate that AtRECQ2 has strand-unwinding activity. This cannot be taken for granted, as strand unwinding by HsRECQ4 (the gene mutated in Rothmund–Thomson syndrome patients) could not be detected for a multitude of DNA substrates (Macris *et al.*, 2006). Optimal activity of AtRECQ2 occurred at pH 8 and in the presence of 50 mM potassium acetate, which are within the range of conditions published for RecQ helicases. AtRECQ2 also shows a typical RecQ-like behavior towards divalent cations: unwinding is displayed with Mg^{2+}, Mn^{2+} and Ca^{2+} but not with Zn^{2+}. Likewise, the unwinding reaction of ScSGS1 is significantly inhibited by the addition of Zn^{2+}, but not by Mn^{2+} or Ca^{2+} (Bennett *et al.*, 1998). For EcRECQ (Umezu *et al.*, 1990) and the large isoform of *Drosophila melanogaster* RECQ5 (Kawasaki *et al.*, 2002), Mg^{2+} can be substituted by Mn^{2+} and also very well by Ca^{2+}, but not by Zn^{2+} when ATP hydrolysis is analyzed.

However, AtRECQ2 is remarkable in its ability to efficiently use various (d)NTPs to promote strand unwinding of an M13-based substrate. AtRECQ2 is able to use all (d)NTPs to efficiently catalyze strand displacement, with a slightly higher efficiency if ATP or dATP are used. These properties are similar to those of HsWRN helicase, for which ATP and dATP are the best (d)NTPs, followed by dCTP and CTP. Strand unwinding by HsWRN also occurs if with GTP, dGTP, UTP and dTTP are used, but it is not efficient (Shen *et al.*, 1998). All other RecQ homologues analyzed for the usage of various (d)NTPs are more restricted, e.g. DmRECQ5 (Özsoy *et al.*, 2001), HsRECQ5β (Garcia *et al.*, 2004), ScSGS1 (Bennett *et al.*, 1998) and EcRECQ (Umezu *et al.*, 1990).

Consistent with all characterized RecQ helicases (with the exception of HsRECQ4), AtRECQ2 is a 3′→5′ DNA helicase. We were able to demonstrate this using the same substrate that had been used for HsWRN (Shen *et al.*, 1998). We further analyzed the properties and requirements of AtRECQ2 for unwinding of similar partial duplex DNA substrates. Under our standard conditions, we showed that the efficiency of unwinding increases with decreasing lengths of the duplex regions. Many RecQ helicases cannot displace very long stretches of double-stranded DNA *in vitro*, but their limits can be increased by the addition of cognate single-strand binding protein [SSB/replication protein A (RPA)].

In a second set of experiments, we showed that AtRECQ2 is not able to act on blunt-ended DNA but requires approx-

imately 5 nt of overhang to promote unwinding. Except for EcRECQ, which can initiate blunt DNA unwinding, the necessity for a 3′ overhang is common to all RecQ helicases tested.

Of special interest is the action of RecQ helicases on DNA substrates that mimic intermediates of replication and recombination. In order to provide proper comparison, we chose substrates that have been studied previously by others during biochemical characterization of RecQ helicases. Most importantly, we tested the activity of AtRECQ2 on D-loops and on a Holliday junction.

D-loops are formed in an early step of homologous recombination, when an ssDNA invades the homologous dsDNA and pairs with the complementary strand of the duplex. The process of homologous recombination is not only important in meiosis but also in DNA repair. If double-strand breaks are repaired by homologous recombination, repair according to the synthesis-dependent strand annealing model will lead to gene conversion and therefore contribute to overall genomic stability. According to this model, D-loops form and DNA synthesis takes place, but then the elongated strand is displaced to re-anneal with the original, complementary strand (Puchta, 1998, 2005). D-loops also occur to re-start stalled replication forks by homologous repair (Haber, 1999; Kowalczykowski, 2000). AtRECQ2 can displace invading strands of D-loops, regardless of whether there is protruding ssDNA or not, and irrespectively of the directionality of the protruding ssDNA. The analyzed bubble was partly unwound. The fact that displacement of the invading strand is more effective than disruption of the bubble is not necessarily of biological significance. It can be explained by the fact that, for unwinding of the bubble, 40 bp must be broken, but only 21 bp are involved in displacing the invading strand of the D-loops. As D-loops are formed at an early stage of homologous recombination, melting can be considered as anti-recombinogenic, thus enhancing genomic stability, a function generally attributed to RecQ helicases. The anti-recombinogenic function applies to D-loops in which the 3′ end of ssDNA is invading, because only these can be extended by DNA polymerases. However, with RAD51, the enzyme involved in the formation of D-loops in nature, the 5′ end of ssDNA is more invasive (at least for RAD51 from yeast and humans; Mazin *et al.*, 2000). Therefore, unwinding of non-productive D-loops with 5′ invasions and 3′ protruding tails by RecQ helicases may also be important. This has already been suggested for HsBLM one of the human RecQ homologues (Bachrati *et al.*, 2006; van Brabant *et al.*, 2000).

The same D-loops employed in this study were tested with HsRECQ1 (Sharma *et al.*, 2005) and HsBLM (van Brabant *et al.*, 2000). Both HsRECQ1 and HsBLM preferred to unwind the invading strand of D-loops in the order: 3′ tailed, 5′ tailed, no tail. The properties of HsWRN, analyzed using D-loops with different sequences (Orren *et al.* 2002), are similar to those of AtRECQ2: the invading strands of all three D-loop substrates are similarly well displaced. It was suggested that HsWRN helicase is directed to junctions between ssDNA and dsDNA (Brosh *et al.*, 2002, Orren *et al.* 2002).

During processes associated with homologous recombination, D-loops may evolve into Holliday junctions. Various studies on the processing of Holliday junctions by RecQ helicases have been performed. Branch migration leads to the appearance of splayed-arm products. Alternatively, ssDNA could be produced either directly or from the splayed-arm products. In this study, we used the partially mobile X12 Holliday junction (Mohaghegh *et al.*, 2001), and showed the appearance of splayed arms, the branch migration product, for AtRECQ2. This reaction was dependent on ATP and positively correlated with the enzyme concentration.

Splayed-arm products are the main product of RECQ helicases: HsWRN produced mainly splayed-arm products with the X12 Holliday junction substrate (Cheng *et al.*, 2006; Mohaghegh *et al.*, 2001). For HsRECQ1, the X12 Holliday junction substrate (Sharma *et al.*, 2005), and for HsRECQ5β, the X26 Holliday junction substrate (Garcia *et al.*, 2004), are processed towards splayed-arm products, and this branch migration can be inhibited by RuvA. More complex is the X12 Holliday junction conversion for HsBLM, which produced ssDNA, probably from a splayed-arm product formed first (Bachrati *et al.*, 2006; Janscak *et al.*, 2003; Mohaghegh *et al.*, 2001). Plasmid-based Holliday junctions, so called α-structures, are subject to branch migration by both HsBLM (Karow *et al.*, 2000) and HsWRN (Constantinou *et al.*, 2000). Thus probably, HsBLM also disrupts oligonucleotide-based Holliday junctions by branch migration (Mohaghegh *et al.*, 2001). Additionally, it has been shown that double Holliday junctions can be dissolved by HsBLM, together with HsTOPOIIIα, in a non-recombinogenic pathway (Wu and Hickson, 2003).

If a stalled replication fork has to be repaired, possible pathways may involve the formation of Holliday junctions: a lesion in the leading strand template may impede the polymerase, and uncouple the synthesis of the two daughter strands. The damage may be bypassed by a process called template switching. The replication fork is regressed to form a so-called chicken-foot structure – a Holliday junction in which the two newly synthesized strands pair together (Cox, 2002). The leading daughter strand can then be extended using the newly synthesized lagging daughter strand as the template. Then, the replication fork could be restored by reverse branch migration. RecQ helicases have been suggested to function in response to replication blockages (Bachrati and Hickson, 2003).

When comparing the RecQ homologues of *Homo sapiens* and *A. thaliana*, designation of specific orthologues is not possible based on sequence comparison. AtRECQ2, AtRECQ4A and AtRECQ4B in *A. thaliana*, and HsWRN and HsBLM in humans, possess the complete set of typical domains, comprising the helicase domain, the RecQ-Ct domain and the HRDC domain. Recent genetic analyses have suggested functional homology for AtRECQ4A and HsBLM for most aspects (Hartung *et al.*, 2006, 2007). Interestingly, despite homology of 70%, AtRECQ4B behaves differently from AtRECQ4A. AtRECQ4A suppresses homologous recombination (Bagherieh-Najjar *et al.*, 2005; Hartung *et al.*, 2007), but AtRECQ4B promotes recombination. It has been postulated that AtRECQ4B has taken over a recombinogenic function of a common 4A/4B ancestor that was functionally homologous to BLM (Hartung *et al.*, 2007). This phenomenon clearly demonstrates that sequence homology cannot be taken as a single criterion for functional homology.

In addition to the domains mentioned above, a functional exonuclease domain is present at the N-terminus of HsWRN. A homologue of this domain can be identified in *A. thaliana*, AtWRNexo, which shows conserved biochemical properties (Li *et al.*, 2005; Plchova *et al.*, 2003). Yeast two-hybrid assays revealed that AtRECQ2 interacts with AtWRNexo (Hartung *et al.*, 2000). In this work, we addressed the question as to whether AtRECQ2 and the helicase portion of HsWRN also possess similar biochemical properties. We conclude that the common behavior of AtRECQ2 and HsWRN helicases with respect to usage of various (d)NTPs and their non-preferential melting of various D-loops supports the hypothesis that these two proteins are functionally homologous. As mutations in the *HsWRN* gene lead to the severe genetic disorder Werner syndrome, analysis of the Arabidopsis *AtRECQ2* knockout phenotype will be of particular interest.

Experimental procedures

Plasmids for overexpression of AtRECQ2 and AtRECQ-K117M

The vector pCAL-n-FLAG (Stratagene, http://www.stratagene.com/) (providing an N-terminal calmodulin-binding peptide (CBP) and a thrombin recognition site as well as a FLAG epitope and enterokinase target) was modified by insertion of a linker composed of the two oligonucleotides 5′-TTGTTCCTCGTGGATCCCATCACCATCACCATCACTAAC-3′ and 5′-CATGGTTAGTGATGGTGATGGTGATGGGATCCACGAGGAACAA-3′ into the *Sma*I and *Nco*I sites to allow C-terminal attachment of a thrombin recognition site and a six-histidine tag (His-tag; LVPRGSHHHHHH) to the protein. Using a ligation-independent cloning strategy, the ORF of At*RECQ2* was inserted using the following primer pair: 5′-GACGACGACAAGATGGAAAGTGAAGCAATTCAA-3′ and 5′-CCACGAGGAACAAGAGCTTCTTCTTCTCCGCT-3′. The construct bearing the point mutation K117M in the Walker A motif of the conserved ATPase domain, as in Brosh *et al.* (1999) for example, was cloned by overlap extension (Sambrook and Russell, 2001) into the same vector using the primers and strategy mentioned above and 5′-GTGGTGGGATGAGTCTTTG-3′ and 5′-CAAAGACTCATCCCACCAC-3′ (codon for methionine 117 underlined).

Expression and purification of AtRECQ2

The protein was overexpressed in the *E. coli* strain BL21-CodonPlus®(DE3)-RIPL (Stratagene) by 0.2 mM isopropyl-β-D-thiogalactopyranoside induction for approximately 20 h at 16°C. All purification steps were performed at 4°C. The frozen cells were resuspended (approximately 0.1 g ml^{-1}) in buffer A [10 mM Tris/HCl pH 7.5, 200 mM NaCl, 20 mM imidazole, 5% glycerol, 10 mM 3-mercapto-1,2-propanediol (thioglycerol)] and disrupted by lysozyme (100 μg ml^{-1}) and sonication. The supernatant, after centrifugation at 40 000 ***g*** for 30 min, was filtered and loaded onto a 1 ml Ni^{2+}-charged HiTrap Chelating HP column (GE Healthcare, http://www.gehealthcare.com) at a flow rate of 1 ml min^{-1} using the low-pressure liquid chromatography system BioLogic LP (Bio-Rad, http://www.bio-rad.com/). The column was first washed with 45 ml of buffer A plus 0.5% Triton X-100, followed by 15 ml of buffer A. Contaminant proteins were removed for 20 min at 31% of buffer B (buffer A with 400 mM imidazole), and AtRECQ2 was eluted with 90% buffer B. The buffer was exchanged with buffer C (50 mM Tris/HCl pH 7.5, 500 mM NaCl, 2 mM $CaCl_2$, 1 mM $Mg(CH_3COO)_2$, 1 mM imidazole, 10 mM thioglycerol) on a PD-10 column according to the instructions of the manufacturer (GE Healthcare). The volume of the eluate was adjusted to 10 ml with buffer C and applied to 1 ml of equilibrated calmodulin (CaM) affinity resin (Stratagene). The column was washed with 10 ml of buffer C plus 0.5% Triton X-100 and 10 ml of buffer C, and the protein was eluted with buffer D (50 mM Tris/HCl pH 7.5, 500 mM NaCl, 2 mM EGTA, 10 mM thioglycerol). The buffer for the protein-containing fractions was exchanged with 50 mM Tris/HCl pH 7.5, 300 mM NaCl, 10% glycerol, 10 mM thioglycerol on a PD-10 column. The eluate was concentrated by dialysis against sucrose, and then mixed with the same volume of glycerol. Aliquots of the purified protein were stored at −80°C. AtRECQ2-K117M was purified by an identical method. The proteins were quantified on colloidal Coomassie-stained SDS–PAGE gels using BSA (Bio-Rad) as the standard.

DNA substrates

Appropriate oligonucleotides were labeled with [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$; GE Healthcare) and T4 polynucleotide kinase (NEB, http://www.neb.com). Preparation of the DNA substrates was similar to that described previously (Bachrati and Hickson, 2006; Brosh *et al.*, 2006). M13mp18 ssDNA was extracted from infected JM110 cells essentially as described by Sambrook and Russell (2001) but including an additional RNase A digest. The central region of 5′-AAAAAAAAAAGTCGACTCTAGAGGATCAAAAAAAA-AA-3′ is complementary to nucleotides 6252-6268 of M13mp18. This oligonucleotide was annealed to M13 DNA (molar ratio 2:1) and purified by gel filtration on Sephacryl™ S-400 material (GE Healthcare). The sequence of the oligonucleotides used to determine directionality was taken from Shen *et al.* (1998) (46-mer and both 23-mers). Further substrates were constructed based on these sequences with shorter oligonucleotides to yield the substrates shown in Figure 3(b,c). The X12 Holliday junction was first described in the context of RecQ helicases by Mohaghegh *et al.* (2001); the D-loop structures used were as described by van Brabant *et al.* (2000) (DL-1 to DL-5).

Helicase assay

The reactions were performed at 37°C with 150 pM of DNA substrate (100 pM for the replication fork) in 40 mM Tris acetate (pH 8.0), 50 mM potassium acetate, 6 mM DTT, 50 μg ml^{-1} BSA (NEB), 1.8 mM ATP and 1.8 mM $MgCl_2$ (unless otherwise indicated), and started (in general) by mixing the enzyme with the other reaction components. The reactions were terminated using one-third of the volume of stop solution (50 mM EDTA, 0.6% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue, 40% or 20% glycerol). For time-course experiments, aliquots were removed at the indicated time points. To determine background values, parallel reactions were performed without any enzyme. After running native Tris Borate EDTA (TBE)–PAGE gels at 4°C, the radioactive DNA was visualized by autoradiography using either a BIO-Imaging analyser BAS-1500 (Fujifilm, http://www.fujifilm.com) or Instant Imager (Canberra Packard, http://www.canberra.com) and quantified using Packard Imager for Windows software (version 2.05) from Canberra Packard. The fraction of DNA unwound was calculated as described previously (Mohaghegh *et al.*, 2001).

ATPase assay

Reactions (50 μl) were incubated under unwinding conditions, except that 0.54 mM ATP, 0.54 mM $MgCl_2$ and 25 μM of M13mp18 ssDNA or 80-mer dT were used and the reaction was allowed to run for 45 min. The release of free inorganic phosphate (P_i) was measured as described previously (Lanzetta *et al.*, 1979) but with the use of malachite green oxalate, 0.1% Triton X-100 instead of Sterox, and reading of the absorbance at 670 nm after 1 h. Reactions without enzyme, but otherwise identical, were performed to determine the zero value.

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